Kidins220 (kinase D-interacting substrate of 220 kDa) is a novel neurospecific protein recently cloned as the first substrate for the Ser/Thr kinase protein kinase D (PKD). Herein we report that Kidins220 is constitutively associated to lipid rafts in PC12 cells, rat primary cortical neurons, and brain synaptosomes. Immunocytochemistry and confocal microscopy together with sucrose gradient fractionation show co-localization of Kidins220 and lipid raft-associated proteins. In addition, cholesterol depletion of cell membranes with methyl-β-cyclodextrin dramatically alters Kidins220 localization and detergent solubility. By studying the putative involvement of lipid rafts in PKD activation and signaling we have found that active PKD partitions in lipid raft fractions after sucrose gradient centrifugation and that green fluorescent protein-PKD translocates to lipid raft microdomains at the plasma membrane after phorbol ester treatment. Strikingly, lipid rafts disruption by methyl-β-cyclodextrin delays green fluorescent protein-PKD translocation, as determined by live cell confocal microscopy, and activates PKD, increasing Kidins220 phosphorylation on Ser916 by a mechanism involving PKCε and the small soluble tyrosine kinase Src. Collectively, these results reveal the importance of lipid rafts on PKD activation, translocation, and downstream signaling to its substrate Kidins220.

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Kidins220 was cloned as the first physiological substrate for protein kinase D (PKD) (1). This kinase, also known as PKD1 or protein kinase Cμ (PKCμ) (6, 7), belongs to a novel family of diacylglycerol (DAG)-stimulated Ser/Thr kinases distantly related to the PKC family, characterized by unique enzymatic properties and domain architecture (for a recent review, see Refs. 8 and 9). PKD has multiple domains, including two cysteine-rich repeats that constitute a C1 domain, a pleckstrin-homology domain, and a C-terminal catalytic domain. The C1 domain binds the lipid second messenger DAG and phorbol esters with high affinity (6, 10). PKD can be activated in vivo by phorbol esters or by stimulation of plasma membrane receptors, such as growth factors, neuropeptides, or antigen receptors, through a PKC-dependent pathway (11–17). PKD via tyrosine phosphorylation of the pleckstrin homology domain mediated by the Src/Abl-signaling pathway (22–24). Activated PKD autophosphorylates at Ser916, and this phosphorylation event is frequently used to determine the activation state of this kinase (16, 25). Functional studies carried out with PKD implicates this isofrom in the control of the c-Jun NH2-terminal kinase and mitogen-activated protein kinase pathways (26–30) and in the regulation of cellular events such as Golgi apparatus organization and function (31–33), immune cell regulation (17, 34), and cell invasion, proliferation, apoptosis, or survival (23, 35–37).

Depending on the cell context and the stimulation conditions PKD can be targeted to different intracellular locations such as the cytosol, the plasma membrane, the Golgi apparatus, or the nucleus (for review, see Refs. 8 and 9). In many cell types where amino acid sequence contains several structural and functional domains and diverse motifs that may link this protein to membranes, cytoskeleton, and different signaling pathways. The N terminus bears 11 ankyrin repeats that are likely to be involved in protein-protein interactions specially with the cytoskeleton (3). Downstream, the sequence presents four transmembrane domains and a proline-rich region that may serve as a binding site for adaptor modules like SH3 domains. Kidins220 C-terminal half is very abundant in phosphorylatable residues, serine, threonine, and tyrosine, that could constitute docking sites for Ser/Thr binding domains of proteins or phospho-tyrosine binding modules such as SH2 domains. It also bears a sterile-α motif or SAM domain (4) and a potential PSD95/SAP90, DGL/ZO-1 (PDZ) binding motif at the very C terminus (5), both candidates for protein-protein interactions.

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PKD is mainly cytosolic, treatment with phorbol ester or receptor stimulation provokes a rapid recruitment of the enzyme to the plasma membrane (16, 38–41). Although this translocation process has been well documented for PKD, very little is known regarding its plasma membrane microdomain distribution. In eukaryotic cells, the plasma membrane structure and composition are not homogeneous and present dynamic structures termed lipid rafts that are rich in low density lipids, such as cholesterol and sphingolipids (42, 43). Caveolae represent a sub-population of lipid rafts characterized containing the scaffolding protein caveolin-1 (44–46). All lipid rafts including caveolae typically partition into low buoyant density detergent-resistant membranes that float during sucrose gradient centrifugation (47). The lipid rafts contribute to surface molecules on specific locations of the cell membrane, concentrating a number of signaling molecules, including transmembrane and glycosylphosphatidylinositol (GPI)-anchored receptors as well as intracellular signaling intermediates, while excluding many others (44–48). Rafts are believed to function as platforms specialized for signaling where, for example, kinases and their substrates could be in close proximity, facilitating signal transduction events to occur rapidly upon the appropriate signal (49).

Kidins220, which is predominantly expressed in neuroendocrine and neural tissues, is found concentrated in certain plasma membrane subdomains reminiscent of lipid rafts in PC12 cells (1). Here we report that Kidins220 is present in lipid rafts in brain and cells of nervous origin such as PC12 cells and primary neurons and that PKD translocates to these membrane microdomains after phorbol ester stimulation. Importantly, in cells where lipid rafts have been disrupted, PKD translocation is delayed, indicating that lipid microdomains are sites facilitating the recruitment of PKD to the plasma membrane. Furthermore, lipid raft integrity is important for keeping PKD activity at low levels since cholesterol depletion induces a significant increase on PKD autophosphorylation and Kidins220 phosphorylation by molecular mechanisms that involve PKCζ and the small tyrosine kinase Src. Collectively, this study integrates spatial and temporal data on PKD activation, translocation, and signaling to its substrate Kidins220 and defines the importance of lipid rafts in all these events.

**EXPERIMENTAL PROCEDURES**

**Materials**—Phorbol 12,13-dibutyrate (PDBu), methyl-β-cyclodextrin (MCDX), filipin, poly-L-lysine, and laminin were from Sigma. GFP (1 GF 109283X or bisindolylmaleimide D and PP2 were both purchased from Calbiochem. Nerve growth factor (NGF) was from Alexis Corp. (San Diego, CA). The polyclonal rabbit antisera against Kidins220 and phospho-Kidins220-Ser919 were purchased from AbCam (Cambridge, UK) and the mouse monoclonal antibodies anti-Thy-1 and anti-pho-Kidins220-Ser919 were prepared as described before (1). Anti-Kidins220 monoclonal antibody was purified from supernatants of a hybridoma that were harvested from mouse immune spleens of Dr. P. Storz (Harvard Medical School, Boston, MA). Texas Red- and Alexa 488-conjugated secondary antibodies as well as Texas Red and Alexa 488-conjugated anti-rabbit and anti-mouse secondary antibodies and ECL were from Amersham Biosciences. All other reagents were from standard suppliers or as indicated in the text.

**Cell Culture and Transfection**—Primary dissociated E19 rat cortical primary cultures were prepared from the cerebral cortices of 19-day-old fetal Wistar rats as described (50). Briefly, meninges were removed from the embryonic brains, and cortices were dissected. Tissue was resuspended in minimal essential medium (Invitrogen) complemented with 10% fetal calf serum, 10% horse serum, 0.1% penicillin, 16 μg/ml gentamicin, and 2 mM glutamine. Cells were counted and seeded on laminin (4 μg/ml)- and poly-lysine (10 μg/ml)-covered glasses in 35-mm dishes at a final concentration of 5 × 10^4 and incubated at 37 °C in an atmosphere of 5% CO₂. Cells were used after 2–8 days in culture, as indicated in the text. Under these conditions >95% of the cells in culture were neurons as assessed by immunostaining with polyclonal antibodies against the neuronal-specific enolase and the glial fibrillary acidic protein (not shown). 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 NGF (75 ng/ml) for 2 days; GFP (1.5 μM), or PP2 (50 μM) for 30 min, MCDX (15 μM) or filipin (10 μg/ml) for 1 h, or PDBu (200 nM) for different times as specified in the text. For transfection, cells were seeded at 50–60% confluence on poly-lysine (10 μg/ml)-coated glass coverslips. Cells were transfected in serum-free medium 24 h after plating by using 1 μg of DNA and 2.5 μl of LipofectAMINE2000 reagent (Invitrogen) per 35-mm dish according to the manufacturer specifications and, 24 h later cells were fixed and processed for immunofluorescence. The cDNA containing wild-type GFP-PKD and a translocation-deficient mutant GFP-PKD-P287G have been previously described (38).

**Preparation of Synaptosomes from Rat Brain**—Synaptosomal fractions were obtained from P15 Wistar rats by differential centrifugation of brain homogenates according to Gray and Whittaker (51) with slight modifications. Whole brains were placed in 40 volumes (w/v) of ice-cold phosphate buffer at pH 7.4 supplemented with sucrose (0.32 M) and homogenized in a Potter homogenizer in the presence of protease and phosphatase inhibitors (1 mM phenylmethylsulfon fluoride, 2 μg/ml aprotinin, 1 mM dithiotreitol, 10 μg/ml aprotinin, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, and 5 mM sodium orthovanadate) and then centrifuged at 5000 rpm in a SS34 rotor for 5 min at 4 °C. The resultant supernatant was further centrifuged at 11,000 rpm in a J2A20 rotor for 20 min at 4 °C. The crude synaptosomal pellet was resuspended in 0.32 M sucrose and loaded onto a Ficoll gradient (4 of 13%, 1 ml of 14%, and 4 ml of 50% Ficoll) prepared in 5 mM Hepes, pH 7.4, or 0.32 M sucrose) and centrifuged at 22,500 rpm in a SW41 rotor for 35 min at 4 °C. Bands at the interface between 5 and 9% Ficoll were isolated, diluted in sodium buffer (10 mM glucose, 5 mM KCl, 140 mM NaCl, 5 mM NaHCO₃, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, and 20 mM HEPES, pH 7.4), and centrifuged in a microcentrifuge at 4 °C and maximal speed for 12 h. The synaptosomal fraction was resuspended in the presence of protease inhibitors at a protein concentration of 1.5–2.0 mg/ml in sodium buffer.

**Isolation of Lipid Rafts**—Lipid rafts were isolated running sucrose gradients as described (52). Briefly, proliferating PC12 cells (seeded at 15 × 10⁶ cells/100-mm dish) or 10 × 10⁶ primary E19 rat cortical neurons cultured for 8 days were used. Cells were put in serum-free medium and pretreated or not for 1 h at 37 °C with 15 mM MCDX. Trypan blue staining showed that MCDX treatment did not affect cell viability. Synaptosomal preparations were resuspended in sodium buffer and treated or not for 1 h at 37 °C with 15 mM MCDX and centrifuged in a microcentrifuge at 4 °C and maximal speed for 30 min. The synaptosomal pellets were resuspended in 5 mM Hepes, pH 7.4, or PDBu (200 nM) for different times as specified in the text) solubilization buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 or 0.25% Triton X-100 (depending on the protein subject to study, as specified in the text) solubilization buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 or 0.25% TritonX-100) plus inhibitors and solubilized for 30 min at 4 °C. Sucrose concentration of the lysates was adjusted to 41% before they were over laid with 8.5 ml of 35% sucrose and 2.5 ml of 16% MCDX. Lysates were incubated at 4 °C for 30 min. The whole lysates were ultracentrifuged (35,000 rpm in a SW41 rotor, 18 h, 4 °C), and 10 fractions were collected from each gradient (from the top to the bottom, fractions 1–10), precipitated with 6.5% trichloroacetic acid in the presence of 0.05% sodium deoxycholate, washed with 80% cold acetone, dissolved, and boiled in 2× Laemmli sample buffer. Samples were separated by SDS-PAGE and visualized by Western blotting and autoradiography.

**Immunoprecipitation and Western Blot Analysis**—PC12 and 8-day rat primary cortical neurons were lysed in radiommune precipitation assay buffer (25 mM Tris-HCl, pH 7.6, 1% Triton X-100, 1% sodium deoxy-
cholate, 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. PKD was immunoprecipitated as described previously (1). For Western blot analysis, total cell lysates, immunoprecipitates, or different fractions of cells or synaptosomes were boiled for 5 min in 2× Laemmli sample buffer and analyzed by SDS-PAGE followed by transfer (200 mM glycine, 25 mM Tris, 10% CH3OH) to nitrocellulose (Schleicher & Schuell) at 100 V for 1 h at 4 °C. Membranes were blocked in TBST (20 mM Tris-HCl, pH 7.6, 137 mM NaCl 0.05% Tween 20) plus 5% lowfat 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 Vivo Cell Imaging, Immunofluorescence, and Confocal Microscopy—For live cell imaging cells were seeded on bottom glass 35-mm dishes coated with poly-lysine (MatTek Corp.) and placed inside a chamber at 37 °C with a 5% CO2 atmosphere on an inverted confocal microscope. For immunofluorescence cells grown on coverslips were fixed for 1 min in 4% paraformaldehyde in phosphate-buffered saline at 4 °C followed by methanol for 5 min at −20 °C. After blocking (1% bovine serum albumin for 15 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 antibodies. Before fixation and visualization some cells were preincubated with Texas Red-conjugated cholera-toxin B (50 μg/ml) for 30 min at 4 °C, which binds to the ganglioside type 1 (GM1) on the cell surface. All confocal images were acquired using a Leica TCS SP2 inverted confocal laser microscope with a 63× Plan-Apochromatic oil immersion objective and were normalized for each color separately. GFP fluorescence was excited with an argon laser emitting at 488 nm. Confocal images presented are single sections of a series or two-dimensional maximal projections of a z-series through the cell depth, as specified in the text and figure legends. Images were processed for presentation with Leica Confocal Software Lite version and Adobe Photoshop 6.0 (Adobe Systems Inc, CA).

RESULTS

Kidins220 Extensively Localizes at Lipid Rafts in Rat PC12 Cells and Primary Cultures of Cortical Neurons—Kidins220 is an integral membrane protein that was originally identified in PC12 cells, where it presents an intense discontinuous punctate staining at the plasma membrane and is concentrated at the tip of extending neurites in NGF-differentiated PC12 cells (1). Because Kidins220 is enriched in brain and in the cells of nervous origin (1, 2), we wanted to investigate the cellular distribution of Kidins220 in E19 rat primary cortical neurons in culture and compare it to the one first observed in PC12 cells. Using anti-Kidins220 antibodies we examined Kidins220 localization by immunofluorescence in both cell types. As shown in Fig. 1A, immunostaining of cortical primary neurons revealed the presence of Kidins220 in the cell body and in the neuronal extensions, where it concentrated at the tip and showed a very similar punctate pattern to the one observed in NGF-treated PC12 cells, brighter in certain subdomains of the plasma membrane (see the arrows in Fig. 1A).

Some plasma membrane components, including various GPI-anchored proteins, transmembrane proteins, and signaling molecules, together with cholesterol and other sphingolipids accumulate in microdomains of the plasma membrane that are resistant to non-ionic detergent solubilization, known as detergent-insoluble glycolipid-enriched domains or lipid rafts (44, 47, 53, 54). To investigate whether the Kidins220 uneven plasma membrane distribution could be due to its association with these specific detergent-resistant membrane subdomains both cultures were double-stained with antibodies against Kidins220 and Thy-1, a GPI-anchored protein targeted to lipid rafts (55). Confocal microscopy analysis showed that, although there are some membrane regions that present differential staining between these two proteins, an extensive co-localization of Kidins220 with Thy-1 could be observed in discrete regions of the plasma membrane in primary cortical neurons and NGF-differentiated PC12 cells (Fig. 1B). This result shows that a significant amount of Kidins220 co-localizes with the lipid raft marker Thy-1 in primary cortical neurons as well as in NGF-treated PC12 cells.

Because lipid rafts are enriched in low density lipids (such as glycosphingolipids and cholesterol) that are insoluble in 1% Triton X-100, rafts components are easily separated by floating after detergent solubilization and sucrose gradient centrifugation (48). We took advantage of this widely used method to study in detail the association of Kidins220 to lipid rafts. To this end, PC12 cells were solubilized in 1% Triton X-100, and extracts were subjected to sucrose gradient centrifugation. Western blot analysis of fractions collected from the gradients detected most Kidins220 in the top fractions, where the lipid
cholesterol in the maintenance of Kidins220 at lipid rafts we used MCDX, a drug that extracts cholesterol from the membranes (57). We first investigated how cholesterol depletion could affect Kidins220 membrane localization by confocal microscopy. Rat PC12 cells and primary cortical neurons (Fig. 2, A and B, respectively) showed a completely different immunostaining and hardly any co-localization of Kidins220 with Thy-1 when treated with MCDX compared with untreated cells, indicating the importance of cholesterol on Kidins220 membrane distribution.

To further analyze if the changes in Kidins220 localization followed by MCDX treatment were accompanied by alterations on Kidins220 detergent solubility, 1% Triton X-100 extracts were prepared from both cell types pretreated or not with MCDX before isolation of rafts by sucrose gradient centrifugation. Gradient fractionation and Western blot analysis of extracts obtained from untreated PC12 cells showed the majority of Kidins220 and Thy-1 floating in fractions 2 and 3 (Fig. 3A). In cortical neurons the bulk of Kidins220 is present in the same fractions; however, Thy-1 is predominantly present in fraction 1, being less abundant in fraction 2 (Fig. 3B). This result is in agreement with the lower degree of co-localization of Kidins220 and Thy-1 in cortical neurons when compared with PC12 cells presented in Fig. 1 and further indicates that Kidins220 and Thy-1 may co-exist in a smaller sub-population of membranes of low buoyancy in neurons than in PC12 cells. Preincubation with MCDX induced a consistent shift of Kidins220 and Thy-1 to higher buoyant density fractions, appearing in more soluble fractions of the gradient both in PC12 cells and cortical neurons (Fig. 3, A and B, respectively).

To demonstrate that the Kidins220 presence in detergent-insoluble glycosphingolipid domains was not an exclusive characteristic of cells in culture, we also examined Kidins220 solubility in brain membrane preparations. Extracts from postnatal rat brain synaptosomes were prepared with 1% Triton X-100 and loaded on sucrose gradients. After Western blot
analysis, Kidins220 and Thy-1 were mainly found in fraction 1 of the gradient. Pretreatment of brain synaptosomes with MCDX completely shifted the Kidins220 signal from fraction 1 to the bottom fractions of the gradient, mainly to fraction 10 (Fig. 3). However, the drug was much less effective in removing Thy-1 from the top fraction to more soluble fractions, indicating that distinct raft components in brain synaptosomes can display different sensitivities to MCDX and suggesting that Kidins220 and Thy-1 may be found together in a lipid raft sub-pool also in the brain.

**Active PKD Translocates to Detergent-insoluble Membranes in PC12 Cells Stimulated with Phorbol Esters**—The presence of Kidins220 in lipid microdomains prompted us to investigate whether PKD, the kinase that phosphorylates Kidins220 (1), could be associated to lipid rafts in neuronal cells. In other cell systems, it has been described that treatment with phorbol esters or receptor stimulation provokes the redistribution of PKD from the cytosolic compartment to the plasma membrane (16, 38–41), but no studies have been published on PKD translocation in cells of nervous origin. Additionally, a detailed analysis of PKD recruitment to differential plasma membrane microdomains has not been reported so far. To approach this issue, we transfected PC12 cells with GFP-PKD, stimulated them with PDBu, and visualized by confocal microscopy the distribution of GFP-PKD compared with the staining pattern of several lipid rafts markers. In resting PC12 cells GFP-PKD is mainly cytosolic (Fig. 4, A–C, upper panels). Stimulation with PDBu for 15 min provokes the recruitment of GFP-PKD to the plasma membrane where it highly co-localizes with Kidins220 as well as with Thy-1 (Fig. 4, A and B). Furthermore, in vivo binding of Texas Red-conjugated cholera toxin B to the lipid raft-enriched ganglioside GM1 at the surface of PC12 cells overlapped significantly with translocated GFP-PKD (Fig. 4C).

To ascertain the nature of PKD membrane association to lipid rafts we further examined its detergent solubility by biochemical fractionation of extracts subjected to sucrose gradient centrifugation. In the case of membrane-associated proteins, for example the members of the Ras family of small GTPases, the differential compartmentalization in membrane microdomains has been widely studied by solubility test assays performed with 0.25% Triton X-100. As shown in Fig. 5, after partitioning of 0.25% Triton X-100 extracts from unstimulated PC12 cells in sucrose gradients, the majority of endogenous PKD appeared in the most soluble material at the bottom of the gradient in fraction 10, although a slight signal of the kinase distributed along the gradient up to lipid raft fractions. After PDBu stimulation a significant amount of total PKD was shifted to fraction 2 and 3, where Kidins220 also floats. MCDX treatment efficiently eliminates active PKD from the raft compartment to the soluble fractions at the bottom of the gradient. Results are representative of three independent experiments.

**Fig. 5. Association of active PKD to detergent-insoluble membranes.** PC12 cells were untreated (−) or pretreated (+) for 1 h with MCDX (15 mM) and then left unstimulated (A) or stimulated with PDBu (200 nM) for 15 min (B). Cells were solubilized in 0.25% Triton X-100 and fractionated after ultracentrifugation in sucrose gradients as described under “Experimental Procedures.” The presence of Kidins220, total PKD (PKD) or active PKD phosphorylated at serine 916 (PKD-Ser916) was analyzed by Western blot with specific polyclonal antibodies. PDBu stimulation provokes a clear shift of active PKD to fractions 2 and 3 (see PKD and PKD-Ser916 panels), where Kidins220 also floats. MCDX treatment efficiently eliminates active PKD from the raft compartment to the soluble fractions at the bottom of the gradient. Results are representative of three independent experiments.
PKD Activation by Cholesterol Depletion

Fig. 6. PKD translocation is delayed after cholesterol depletion. PC12 cells transiently expressing GFP-PKD were imaged in real time before and after PDBu (A) or MCDX and PDBu (B) treatment. In resting cells GFP-PKD was distributed throughout the cytosol of untreated PC12 cells (panel A). After PDBu stimulation, membrane targeting of GFP-PKD started to be appreciable within 5–7 min and was maximal within 15–17 min (panels B–E). Image analysis of cells treated with MCDX up to 60 min showed no major changes on GFP-PKD distribution (panels F–K). After PDBu addition to these cells (panels L–S), the recruitment of GFP-PKD to the plasma membrane only started to be detectable at later time points (15–17 min; compare panel E with panel O), not reaching the maximum within 25 min were recorded. Similar results were obtained in the majority of PC12 cells observed (>95%). The results shown are representative of three independent experiments where each time 4–6 individual cells were imaged. Calibration bar, 4 μm.

at Ser916 (25). In unstimulated cells, Ser916 phosphorylation was not detectable (not shown). Immunoblotting of fractions obtained from PDBu-treated cells with the phospho-Ser916 antibody showed that fractions 2 and 3 were the ones containing the bulk of active PKD (Fig. 5B, PKD-pSer916), thereby demonstrating a prominent association of active PKD to detergent-insoluble membranes after PDBu stimulation.

We next investigated whether cholesterol plays a major role on PKD recruitment to these low buoyancy membrane domains. Incubation of control PC12 cells with MCDX and extraction with 0.25% Triton X-100 completely shifted total PKD to fraction 10, only partially affecting Kidins220 fraction localization (Fig. 5A). The lack of Kidins220 extraction from the raft fractions by pretreatment with MCDX in this case, when compared with previous Figs. 1 and 3, might be due to the use of 0.25% instead of 1% Triton X-100 buffer. In PDBu-stimulated cells, pretreatment with MCDX efficiently eliminated total (PKD) and active PKD (PKD-pSer916) from the raft compartment to the soluble fractions at the bottom of the gradient, where it remained phosphorylated at Ser916 (Fig. 5B, see the PKD-pSer916 signal in fraction 10). These results show the importance of cholesterol in maintaining active PKD associated to detergent-insoluble fractions and strongly indicate that PDBu-induced PKD activation is not blocked by lipid raft disruption.

Delayed Translocation of PKD after Cholesterol Depletion—The previous results obtained by sucrose gradient centrifugation showed that active PKD translocation could be affected by disruption of lipid rafts with MCDX. To further investigate this notion, we carried out confocal microscopy analysis of PC12 cells transiently expressing GFP-PKD. A comparison of the distribution of GFP-PKD in untreated and MCDX-pretreated cells and the ability of GFP-PKD to translocate to the plasma membrane by PDBu stimulation under both conditions was made by real time confocal imaging of live cells. As shown in Fig. 6A, at time 0 GFP-PKD was distributed throughout the cytosol of untreated PC12 cells. After PDBu stimulation, membrane targeting of GFP-PKD started to be appreciable within 5–7 min and was maximal within 15–17 min (Fig. 6A, panels B–E). Image analysis of cells treated with MCDX up to 60 min (Fig. 6B, panels F–K) showed no major changes on GFP-PKD distribution. After PDBu addition to these cells, the recruitment of GFP-PKD to the plasma membrane only started to be detectable at later time points (15–20 min) and did not reach the maximum within the 25 min recorded (Fig. 6B, with PDBu, panels L–S). The same results were obtained when cells were pretreated with filipin, another cholesterol-sequestering drug (not shown). These images clearly demonstrate that pretreatment with MCDX delays the starting point of GFP-PKD targeting to the plasma membrane after PDBu addition <2–3 times, clearly impairing the PKD translocation process.

MCDX Treatment Enhances PKD Activity and Kidins220 Phosphorylation without Predominantly Increasing Their Co-localization—Our results from in vivo time-lapse confocal microscopy show that in MCDX-treated cells GFP-PKD hardly starts to translocate after 15 min of PDBu stimulation (Fig. 6B, panel O). If PKD recruitment to plasma membrane microdomains is a prerequisite for enzymatic activation, the delay on PKD translocation suffered after lipid raft disruption may be accompanied by an impairment of its activation time course. To analyze in detail PKD activation and signaling after cholesterol depletion and to correlate these data with the state of PKD translocation observed by in vivo imaging, we measured PKD autophosphorylation at Ser916 and PKD-mediated phosphorylation of Kidins220 at Ser916. We have previously published that PDBu activation of PKD provokes Kidins220 phosphorylation at this residue (1). Cell extracts from PC12 cells pretreated...
with MCDX and stimulated with PDBu for 15 or 25 min, 2 time points in which we had detected a lack or partial translocation of PKD, were Western-blotted with antibodies specifically recognizing each phosphoserine residue. Unexpectedly, both PKD-Ser916 and Kidins220-Ser919 phosphorylation were consistently increased by MCDX pretreatment in unstimulated cells (Fig. 7A), indicating that disruption of lipid rafts by cholesterol depletion results in a significant increase of PKD activity, signaling, and the ability to autophosphorylate and transphosphorylate its downstream substrate. This effect on PKD activity was also observed when filipin was used (results not shown). Treatment with phorbol esters for 15 or 25 min induced PKD maximal activation, making differences among cells exposed or not to MCDX not appreciable (Fig. 7A). Together with the in vivo confocal images, these results indicate that PKD can be fully active before a complete translocation to the plasma membrane occurs.

Regarding Kidins220, in agreement with our previously published data (1), a 15-min incubation with PDBu raised Kidins220-Ser919 phosphorylation (Fig. 7A). Importantly, MCDX treatment further enhanced this phosphorylation. Maximal phosphorylation of Kidins220 at Ser919 was obtained after stimulation with PDBu for 25 min independently of MCDX pretreatment (Fig. 7A). The increase in Kidins220 phosphorylation could be just a consequence of PKD activation by MCDX treatment. Alternatively, a more complex situation would result from the combination of this fact together with the dramatic effects of cholesterol depletion on both Kidins220 cellular localization (shown in Fig. 2) and PKD translocation (detailed in Fig. 6) that could affect their co-localization pattern (presented in Fig. 4A). To test this last hypothesis PC12 cells were transfected with GFP-PKD, and we made a comparative analysis of Kidins220 immunostaining and GFP-PKD distribution in fixed cells by confocal microscopy, studying how their co-localization varied after MCDX treatment followed by 15 or 25 min of PDBu stimulation. When the cells were treated with MCDX, GFP-PKD, and Kidins220 co-localization was not significantly increased (Fig. 7B, + MCDX, see Merge and Zoom images). As observed in vivo (Fig. 6), MCDX treatment strongly blocked GFP-PKD translocation after 15 min of PDBu stimulation (Fig. 7B, + MCDX + PDBu, 15 min). Compare these images with Fig. 4, showing GFP-PKD translocation after 15 min of PDBu treatment. Here again, no major changes on their co-localization pattern were detected. Only after incubating the cells 25 min with PDBu, an increase of both proteins co-localizing at the plasma membrane was evident even though GFP-PKD was not fully translocated and considerable amounts of GFP-PKD were still in the cytosol (Fig. 7B, + MCDX + PDBu, 25 min). From all the data, one can speculate that cholesterol depletion, by delaying the time course of PKD translocation, favors a situation in which active PKD phosphorylates Kidins220 in a common intracellular compartment. Because PKD translocation is achieved after longer times of PDBu incubation, active PKD would be able to fully phosphorylate Kidins220 at the plasma membrane. A translocation-deficient mutant of PKD (GFP-PKD-P287G, Ref. 38) that can be equally

**Fig. 7.** MCDX treatment induces PKD activation and Kidins220 phosphorylation but no major changes in their co-localization pattern. **A,** PC12 cells were untreated (−) or pretreated (+) for 1 h with MCDX (15 mM) and then left unstimulated or stimulated with PDBu (200 nM) for 15 or 25 min. The presence of active PKD phosphorylated at Ser916 (PKD-pSer916), phosphorylated Kidins220 (Kidins220-pSer919), and total PKD and Kidins220 was analyzed by Western blot of total cell lysates. MCDX treatment enhances basal PKD autophosphorylation on Ser916 and provokes a significant increase in Kidins220-Ser919 phosphorylation. **B,** PC12 cells transiently transfected with wild type GFP-PKD and untreated (−) or treated (+) for 1 h with MCDX (15 mM) were unstimulated or stimulated for 15 or 25 min with PDBu (200 nM), fixed, and immunostained with Kidins220 antibodies. Kidins220 staining is shown in red and GFP-PKD is shown in green. Confocal microscopy images correspond to a single section. Merge and Zoom pictures show co-localization of GFP-PKD with Kidins220 in yellow. Results are representative of three independent experiments.
activated by PDBu as wild type PKD (38) is able to increase Kidins220 phosphorylation although not to the same extent as wild type PKD (data not shown). These results support the notion that PKD, once activated, can phosphorylate Kidins220 in an intracellular compartment and that it will further phosphorylate its substrate at the plasma membrane once translocation has occurred. Taken together, these results demonstrate that the integrity of lipid rafts is crucial for the correct localization and signaling of these two molecules.

**MCXD Treatment Increases PKD-Ser748 and Tyrosine Phosphorylation and Its Association to PKC and Src**—We next wanted to analyze the molecular mechanisms participating in PKD activation by MCXD treatment. Phosphorylation is a key event in the control of PKD activation. Two major pathways controlling PKD phosphorylation state and activity have been described. The first one implicates the phosphorylation of Ser744/Ser748 at the activation loop by the novel PKCs, PKCδ/H9280 and PKCζ/H9257 (18–21). The second is mediated by Src/Abl signaling and activates PKD through tyrosine phosphorylation of the activation loop by the novel PKCs, PKCδ/H9280 and PKCζ/H9257 (18–21). We finally investigated whether PKCδ and Src interact with PKD, participating in the catalytic activation of PKD triggered by MCXD treatment. Tyrosine phosphorylation was also examined in PKD immunoprecipitates from the same lysates were Western-blotted with PKD-pY463 and total PKD antibodies, showing that both tyrosine-phosphorylated bands (arrow heads) correspond to PKD and Src. Results shown are typical of three independent experiments.

**PKCδ and Src associate with PKD in MCXD-treated PC12 cells.** Total lysates or PKD immunoprecipitates were prepared from PC12 cells untreated (+) or pretreated (+) for 1 h with MCXD (15 mM). A, PKCδ interacts with PKD in PC12 cells, and MCXD treatment increases both PKCδ association and PKD-Ser748 phosphorylation. The levels of PKCδ and PKCζ in the total lysates or in PKD immunoprecipitates (IP) were determined by Western blot. PKD-Ser748 phosphorylation was also detected in PKD immunoprecipitates by Western blotting with a phospho-specific antibody (PKD-pSer748). B, PKD is tyrosine-phosphorylated and associates with Src in MCXD-treated PC12 cells. Tyrosine phosphorylation was determined by Western blotting total cell extracts or PKD immunoprecipitates using anti-phosphotyrosine antibodies (PY). Blots were stripped and re-probed with anti-PKD and anti-Src antibodies, showing that both tyrosine-phosphorylated bands (arrow heads) correspond to PKD and Src. Results shown are typical of three independent experiments.
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specifically by PKCe (21) and Src/Abl (23, 24); they are Ser744 at the activation loop and Tyr463 at the pleckstrin homology domain, respectively. Pretreatment of PC12 cells with GF I (3.5 μM for 30 min) reduced PKD autophosphorylation on Ser126 and transphosphorylation by PKCe on Ser744/Ser748, indicating that PKC activity was required for MCDX-induced PKD activation by cholesterol depletion was also shown after incubating the cells with the Src inhibitor PP2 (50 μM for 30 min), which resulted in a decrease on PKD-Ser391 phosphorylation after GF I or PP2 treatment (Fig. 9C). Altogether, these results demonstrate that both PKC and Src/Abl pathways participate in the observed activation of PKD after lipid rafts disruption.

**DISCUSSION**

In this study we show by confocal microscopy analysis and biochemical fractionation that Kidins220 is constitutively associated with membrane rafts in brain synaptosomes and in cells of nervous origin, primary cortical neurons and PC12 cells. More importantly, we demonstrate that active PKD is inducibly recruited to lipid rafts after PDBu stimulation and that cholesterol depletion delays the kinetics of translocation to the plasma membrane. Finally, MCDX treatment results in the activation of the endogenous PKD, evidenced by increased Ser744, Ser748/Ser742, and Tyr463 and the phosphorylation of the downstream substrate Kidins220 at Ser570 by mechanisms involving PKCe and Src/Abl signaling pathways.

Kidins220 associates constitutively to rafts, and one obvious question that remains to be answered deals with the mechanism by which this occurs. Kidins220 could directly target to or interact with the raft lipids or it could bind raft-associated proteins. A number of receptors and intracellular signaling molecules have been localized to lipid rafts in neurons (60, 61). Most membrane raft-associated proteins are constitutive residents because of GPI modification or through palmitoylation at membrane-proximal cysteine residues (43, 62). Kidins220 contains several cysteine residues in its very C-terminal tail susceptible to palmitoylation (Cys-1639, Cys-1649, Cys-1663)2 that could participate in the anchoring of Kidins220 to lipid rafts. An alternative mechanism may involve Kidins220 binding to other detergent-resistant membrane-associated proteins. The best candidates are ephrin and neurotrophin receptors, shown to interact with Kidins220 (2) and whose association with low density lipid rafts has been well documented (61, 63–71). Further studies will need to examine the structural features that target Kidins220 to lipid rafts, and mutagenesis experiments will determine the raft association signal/s within Kidins220 sequence.

Regarding PKD, many factors may contribute to its inducible recruitment to lipid rafts. PKD translocation to the plasma membrane depends on the C1 domain (16, 38). This segment is known to have a high affinity binding site for DAG and phorbol ester (6, 10) and plays a crucial role on PKD plasma membrane targeting (17, 38, 40). PKD-C1 domain would be strongly attracted toward lipid rafts since they cluster many G-protein-coupled and tyrosine kinase receptors and phospholipase C proteins, which convert these in hot spots for DAG production under physiological stimulation conditions. Additionally, protein-protein interactions could also play an important role in this process. Not only Kidins220 but several PKD-interacting proteins such as the B-cell receptor complex, Syk and phospholipase Cy1 (34), PKCe (19), Src (24), and Gα, (40) together with other lipids involved in PKD signal transduction, such as phosphatidylinositol-4,5-bisphosphate (72), have been shown to concentrate at rafts constitutively or after receptor stimulation (47, 73–75). Thus, PKD association to lipid rafts can be a multifactorial process where lipid and protein interactions may play a key role, and although not only the C1 domain but the different domains of the kinase may be involved.

MCDX treatment causes a major delay on PDBu-induced PKD translocation, indicating an essential role of lipid raft integrity in the plasma membrane recruitment of the kinase. After lipid rafts disruption, the enzyme may need more time to find the mislocalized interacting partners, lipids or proteins, at the plasma membrane. Although alternative explanations are possible, these findings could now further explain some unique physiological features of PKD translocation. One of them is the apparent irreversibility of PKD translocation to the plasma membrane, recently proposed to be mediated by its interaction with Gα (40). The association of PKD to lipid rafts could also contribute to the persistent nature of PKD translocation, which would be characterized by a very low dissociation constant due to the multivalent binding nature of both lipid raft resident proteins and lipids and PKD. An interesting issue to investigate would be whether the persistent translocation and association of PKD with Gα occurs in these detergent-insoluble membranes.

What might be the function of segregating Kidins220 and active PKD into lipid rafts? Compartmentalization of proteins is needed to organize and regulate cellular activities, and although lipid rafts are known to play a key role in polarized membrane trafficking and proteolytic processing, their best established role is signal transduction (43, 45, 47, 62). Many reports implicate neuronal rafts in the compartmentalization of the multitude of signals impinging on the cell surface into distinct signaling cascades by including or excluding key signaling molecules, like cell-surface receptors, intracellular signal-transduction molecules, and transmembrane ligands (60, 61, 66, 76), and regulating in this way cellular functions such as cell adhesion, axon guidance, or synaptic transmission (45, 62, 77). Whereas it is tempting to hypothesize a possible function for rafts in the polarized sorting of Kidins220 and PKD in the brain and in neurons, as appears to be the case for other neuronal proteins (78, 79), their raft association could serve primarily to cluster these two proteins with other signaling molecules.

In this study we show that the majority of Kidins220 partitions into detergent-resistant membrane fractions, and given the established role of lipid rafts and caveolae in signal transduction, this probably reflects a physiologically relevant pool of Kidins220 involved in signal transduction through these microdomains. The differential targeting of Kidins220 to various lipid raft subpopulations, observed by the different co-localization pattern and detergent solubility in PC12 cells, neurons, and brain, may segregate Kidins220 access to different signaling cascades emerging from these microdomains. In agreement to this, Kidins220 has so far been found downstream of two different signaling pathways. After Kidins220 identification as a PKD substrate, the same protein was cloned and named ARMS (ankyrin repeat membrane spanning) as a downstream effector of ephrin and neurotrophin tyrosine kinase receptors (2). Neurotrophins form a family of neurotrophic factors, including NGF, brain-derived neurotrophic factor, NT3, and NT4/5, whose signaling is mediated by two kinds of surface receptors, the Trk family of tyrosine kinase receptors and p75

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2 N. Cabrera-Poch, L. Sánchez-Ruiloba, M. Rodríguez-Martinez, and T. Iglesias, unpublished observations.
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Iglesias, T., Cabrera-Poch, N., Mitchell, M. P., Naven, T. J. P., Rozengurt, E., and Schiao, G. (2000) J. Biol. Chem. 275, 40048–40056

REFERENCES

1. Iglesias, T., Cabrera-Poch, N., Mitchell, M. P., Naven, T. J. P., Rozengurt, E., and Schiao, G. (2000) J. Biol. Chem. 275, 40048–40056
2. Kong, H., Boulter, J., Weber, J. L., Lai, C., and Chao, M. V. (2001) J. Neurosci. 21, 176–185
3. Sedgwick, S. G., and Smerdon, S. J. (1999) Trends Biol. Sci. 24, 311–316
4. Schultzt, J., Ponting, C. P., Hofmann, K., and Bork, P. (1997) Protein Sci. 6, 249–263
5. Craven, S. E., and Breder, D. S. (1998) Cell 93, 495–498
6. Valverde, A. M., Sinnet-Smith, J., Van Lint, J., and Rozengurt, E. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 8572–8576
7. Johannes, F. J., Prestle, J., Eus, S., Oberhageman, P., and Pfizenmaier, K. (1994) J. Biol. Chem. 269, 6140–6148
8. Van Lint, J., Ryka, A., Maeda, Y., Vantus, T., Sturany, S., V Malhotra, V, Malhotra, V., Van Lint, J., and van Meir, E. (1999) J. Biol. Chem. 274, 27627–27637
9. Ryka, A., De Kimpe, L., Mihalap, S., Vantus, T., Seufferlein, T., Vandenheede, J. R., and Van Lint, J. (2003) J. Biol. Chem. 278, 25313–25317
10. Lugaza, J. L., Sinnet-Smith, J., Van Lint, J., and Rozengurt, E. (1996) EMBO J. 15, 6220–6230
11. Lugaza, J. L., Waldron, R. T., Sinnet-Smith, J., and Rozengurt, E. (1997) J. Biol. Chem. 272, 23652–23660
12. Palucchi, L., and Rozengurt, E. (1999) Cancer Res. 59, 572–577
13. van Lint, J., and Rozengurt, E. (2001) FEBS Lett. 489, 101–106
14. Guba, S., Rey, O., and Rozengurt, E. (2002) Cancer Res. 62, 1632–1640
15. Matthews, S., Iglesias, T., Rozengurt, E., and Cantrell, D. (2000) EMBO J. 19, 2925–2934
16. Matthews, S., Rozengurt, E., and Cantrell, D. (2001) J. Exp. Med. 191, 2075–2082
17. van Lint, J., Ryka, A., Maeda, Y., Vantus, T., Sturany, S., V Malhotra, V., Malhotra, V., Van Lint, J., and van Meir, E. (1999) J. Biol. Chem. 274, 27627–27637
18. Hauser, A., Storz, P., Hubner, S., Brandl, I., Martinez-Moya, M., Link, G., and Johannes, F.-J. (2001) FEBS Lett. 492, 39–44
19. Brandl, I., Hubner, S., Kieser, M., Martinez-Moya, M., Horschinek, A., Hauser, A., Link, G., Rupp, S., Storz, P., Pfizenmaier, K., and Johannes, F.-J. (2002) J. Biol. Chem. 277, 6490–6496
20. Brandl, I., Einele, T., Salowsky, R., and Johannes, F.-J. (2002) J. Biol. Chem. 277, 45451–45457
21. Hurd, C., Waldron, R. T., and Rozengurt, E. (2002) Oncogene 21, 2154–2160
22. Prestle, J., Pfizenmaier, K., Brenner, J., and Johannes, F.-J. (1996) J. Cell Biol. 134, 1401–1410
23. Jamora, C., Yuanomez, N., Lint, J. V., Van Lint, J., and Malhotra, V. (1999) Cell 98, 59–68
PKD Activation by Cholesterol Depletion

33. Lijedahl, M., Mada, Y., Colanisi, A., Ayala, I., Van Lint, J., and Malhotra, V. (2001) Cell 104, 409–420
34. Sidorenko, S., Law, C., Klaus, S., Chandran, K., Takata, M., Kurosi, T., and Clark, E. (1996) Immunity 5, 353–363
35. Bowden, E. T., Barth, M., Thomas, D., Glazer, R. I., and Mueller, S. C. (1999) Oncogene 18, 4440–4449
36. Zhukova, E., Sinnett-Smith, J., and Rozengurt, E. (2001) J. Biol. Chem. 276, 40298–40305
37. Johannes, F. J., Horn, J., Link, G., Haas, E., Siemieniuk, K., Wajant, H., and Pfizenmaier, K. (1998) Eur. J. Biochem. 257, 47–54
38. Matthews, S., Iglesias, T., Cantrell, D., and Rozengurt, E. (1999) FEBS Lett. 457, 515–521
39. Rey, O., Young, S. H., Cantrell, D., and Rozengurt, E. (2001) J. Biol. Chem. 276, 104298–104305
40. van Deurs, B., Roepstorff, K., Hommelgaard, A. M., and Sandvig, K. (2003) Trends Cell Biol. 13, 92–100
41. Simons, K., and Tomare, D. (2000) Nat. Rev. Mol. Cell Biol. 1, 31–39
42. Brown, D. A., and London, E. (2000) J. Biol. Chem. 275, 17221–17224
43. Hunter, T. (2000) Cell 100, 113–127
44. Brewer, G., and Cotman, C. (1989) Brain Res. 494, 65–74
45. Gray, E., and Whittaker, V. (1962) J. Anat. 96, 79–88
46. Herreros, J., Ng, T., and Schiavo, G. (2001) Mol. Biol. Cell 12, 2947–2960
47. Brown, D., and London, E. (1998) Annu. Rev. Cell Dev. Biol. 14, 111–136
48. Jacobson, K., and Dietrich, C. (1999) Trends Cell Biol. 9, 87–91
49. Madore, N., Smith, K. L., Graham, C. H., Jen, A., Brady, K., Hall, S., and Clark, E. (1999) Cell 100, 113–127
50. Neufeld, E. B., Conney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21694–21613
51. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) Nat. Cell Biol. 3, 368–375
52. Herreros, J., Ng, T., and Schiavo, G. (2001) J. Anat. 200, 611–623
53. Brown, D., and London, E. (1998) J. Biol. Chem. 273, 3554–3559
54. Jacobson, K., and Dietrich, C. (1999) J. Biol. Chem. 274, 1150–1157
55. Neufeld, E. B., Conney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21694–21613
56. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) Nat. Cell Biol. 3, 368–375
57. Neufeld, E. B., Cooney, A. M., Pitha, J., Dawidowicz, E. A., Dwyer, N. K., Pentchev, P. G., and Blanchette-Mackie, E. J. (1996) J. Biol. Chem. 271, 21694–21613
58. Prior, I. A., Harding, A., Yan, J., Sluimer, J., Parton, R. G., and Hancock, J. F. (2001) Nat. Cell Biol. 3, 368–375
59. Kranegeb, O., Verlaan, I., and Moolenaar, W. H. (2001) Curr. Biol. 11, 1880–1884
60. Tansey, M. G., Baloh, R. H., Milbrandt, J., and Johnson, J., E. M. (2000) Neurosci. 25, 611–623
61. Wu, C., Butz, S., Ying, Y.-S., and Anderson, R. (1997) J. Biol. Chem. 272, 3554–3559
62. Brown, R. E. (1998) J. Cell Sci. 111, 1–9
63. Bilderback, T. R., Grigsby, R. J., and Dobrowsky, R. T. (1999) J. Biol. Chem. 274, 257–263
64. Bilderbach, T. R., Gazala V. R., Lisanti M. P., and Dobrowsky, R. T. (1999) J. Biol. Chem. 274, 257–263
65. Huang, C.-S., Zhou, J., Feng, A. K., Lynch, C. C., Klumperman, J., DeArmond, S. J., and Mobley, W. C. (1999) J. Biol. Chem. 274, 36707–36714
66. Bruckner, K., Labrador, J. P., Scheiffele, P., Herb, A., Seeburg, P. H., and Klein, R. (1999) Neuron 22, 511–524
67. Davy, A., Gale, N. W., Murray, E. W., Klinghoffer, R. A., Soriano, P., Feuerstein, C., and Robbins, S. M. (1999) Genes Dev. 13, 3125–3135
68. Peiro, S., Comella, J. X., Enrich, C., Martin-Zanca, D., and Rocamora, N. (2000) J. Biol. Chem. 275, 37846–37852
69. Tsu-Pierrchala, B. A., Encinas, M., Milbrandt, J., and Johnson, J., E. M. (2002) Trends Neurosci. 25, 412–417
70. Paratcha, G., and Baize, C. F. (2002) Curr. Opin. Neurobiol. 12, 542–549
71. Higuchi, H., Yamashita, T., Yoshikawa, H., and Tohyama, M. (2003) EMBO J. 22, 1780–1800
72. Hope, H. R., and Pike, L. J. (1996) Mol. Biol. Cell 7, 843–851
73. Cheng, P. C., Dykstra, M. L., Mitchell, R. N., and Pierce, S. K. (1999) J. Exp. Med. 189, 1549–1560
74. Lang, M. L., Chen, Y., Shen, L., Gao, H., Lang, G. A., Wade, T. K., and Wade, W. F. (2002) Biochem. J. 364, 517–525
75. Mukherjee, A., Arnauld, L., and Cooper, J. A. (2003) J. Biol. Chem. 278, 40806–40814
76. Galbiati, F., Ratani, B., and Lisanti, M. P. (2001) Cell 106, 403–411
77. Tooz, S. A., Martens, G. J. M., and Huttner, W. B. (2001) Trends Cell Biol. 11, 116–122
78. Ledesma, M. D., Simons, K., and Dotti, C. G. (1998) Trends Cell Biol. 8, 359–363
79. Colman, D. R. (1999) Neuron 23, 649–651
80. Masserini, M., Palestini, P., and Pito, M. (1999) J. Neurochem. 73, 1–11