Elevated expression of protein kinase Cδ induces cell scattering upon serum deprivation

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Summary

Tumor metastasis might be evoked in response to microenvironmental stress, such as a shortage of oxygen. Although the cellular response to hypoxia has been well established, we know little about how tumors adapt themselves to deprivation of growth factor. Protein kinase Cδ (PKCδ), a stress-sensitive protein kinase, has been implicated in tumor progression. In this study, we demonstrate that elevated expression of PKCδ in Madin-Darby canine kidney cells induces a scatter response upon serum starvation, a condition that mimics growth-factor deprivation. Serum starvation stimulates the catalytic activity and Y311 phosphorylation of PKCδ through reactive oxygen species (ROS) and the Src family kinases. Mutation of PKCδ at Y311 and Y322, both of which are phosphorylation sites for Src, impairs its activation and ability to promote cell scattering upon serum deprivation. Once activated by ROS, PKCδ itself activates ROS production at least partially through NADPH oxidase. In addition, the c-Jun N-terminal kinase is identified as a crucial downstream mediator of ROS and PKCδ for induction of cell scattering upon serum deprivation. We demonstrate that the C1B domain of PKCδ is essential not only for its localization at the Golgi complex, but also for its activation and ability to induce cell scattering upon serum deprivation. Finally, depletion of PKCδ in human bladder carcinoma T24 cells restores their cell–cell contacts, which thereby reverses a scattered growth pattern to an epithelial-like growth pattern. Collectively, our results suggest that elevated expression of PKCδ might facilitate the scattering of cells in order to escape stress induced by growth-factor deprivation.

Key words: PKCδ, ROS, Cell scattering, Serum deprivation

Introduction

It is known that tumors aggravated by microenvironmental stress might be evoked to metastasis (for a review, see Gupta and Massagué, 2006). For instance, if a tumor has outgrown its blood supply, the tumor cells at the front line become short of essential nutrients, growth factors, and oxygen. In tumors, a shortage of oxygen (hypoxia) is a strong selective pressure that promotes the outgrowth of malignant cells with a diminished susceptibility to undergo apoptosis. Several clinical studies have shown that the presence of hypoxic regions within tumors correlates with poor prognosis and an increased risk of metastasis (for a review, see Höckel and Vaupel, 2001). The cellular response to hypoxia involves the stabilization of a hypoxia-inducible factor, which is a master transcriptional factor capable of activating genes that promote angiogenesis, anaerobic metabolism, cell survival, and invasion (reviewed by Harris, 2002). It is apparent that lack of blood supply also leads to deprivation of nutrients (amino acids and glucose) and growth factors. However, adaptation of tumor cells to such deprivation is less clear. Culturing cells in serum-free medium (also known as serum starvation) has been used as a condition to mimic growth-factor deprivation, which causes increased levels of intracellular reactive oxygen species (ROS) (Rygiel et al., 2008).

ROS, including superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical, affect many cellular functions by damaging nucleic acids, oxidizing proteins, and causing lipid peroxidation. Such oxidative stress is associated with several human pathologies, such as cancer and cardiovascular disease (reviewed by Halliwell, 1997). Recent studies indicate that ROS act as second messengers in signal transduction (Irani, 2000; Lander, 1997; Veal et al., 2007), leading to cell apoptosis (Cai, 2005), proliferation (Geiszt and Leto, 2004), differentiation (Sauer et al., 2000), aging (Finkel and Holbrook, 2000), and migration (Ushio-Fukai, 2006). ROS have been shown to modulate protein function through oxidation of susceptible cysteine side-chain moieties (Rhee et al., 2000). A number of tyrosine kinases have been identified as being activated by direct cysteine oxidation (Giannoni et al., 2005; Kato et al., 2000). More significantly, ROS lead to the functional inactivation of protein tyrosine phosphatases through the formation of intramolecular disulfide bridges or sulfenyl-amide bonds, which in turn activates tyrosine kinases (Finkel, 1998). However, different ROS levels could have opposite effects in the same type of cells. For example, low ROS levels are mitogenic and anti-apoptosis, whereas high ROS levels induce growth arrest or apoptosis (Finkel and Holbrook, 2000).

Protein kinase Cδ (PKCδ) is the most thoroughly studied member of the novel PKC subfamily and has been implicated as participating in a wide variety of cell functions, including cell proliferation (Ashton et al., 1999; Kitamura et al., 2003), differentiation (Corbit et al., 1999; Pessino et al., 1995), apoptosis (Brodie and Blumberg, 2003; Kajimoto et al., 2004; Zhong et al., 2002), and tumor suppression (Lu et al., 1997; Reddig et al., 1999). Increasing evidence also indicates that PKCδ plays a positive role in cell motility (Chen et al., 2007; Gliki et al., 2002; Iwabu et al., 2004; Li et al., 2003) and the metastatic potential of tumor cells (Alonso-Escolano et al., 2006; Kharait et al., 2006; Kiley et al., 1999;

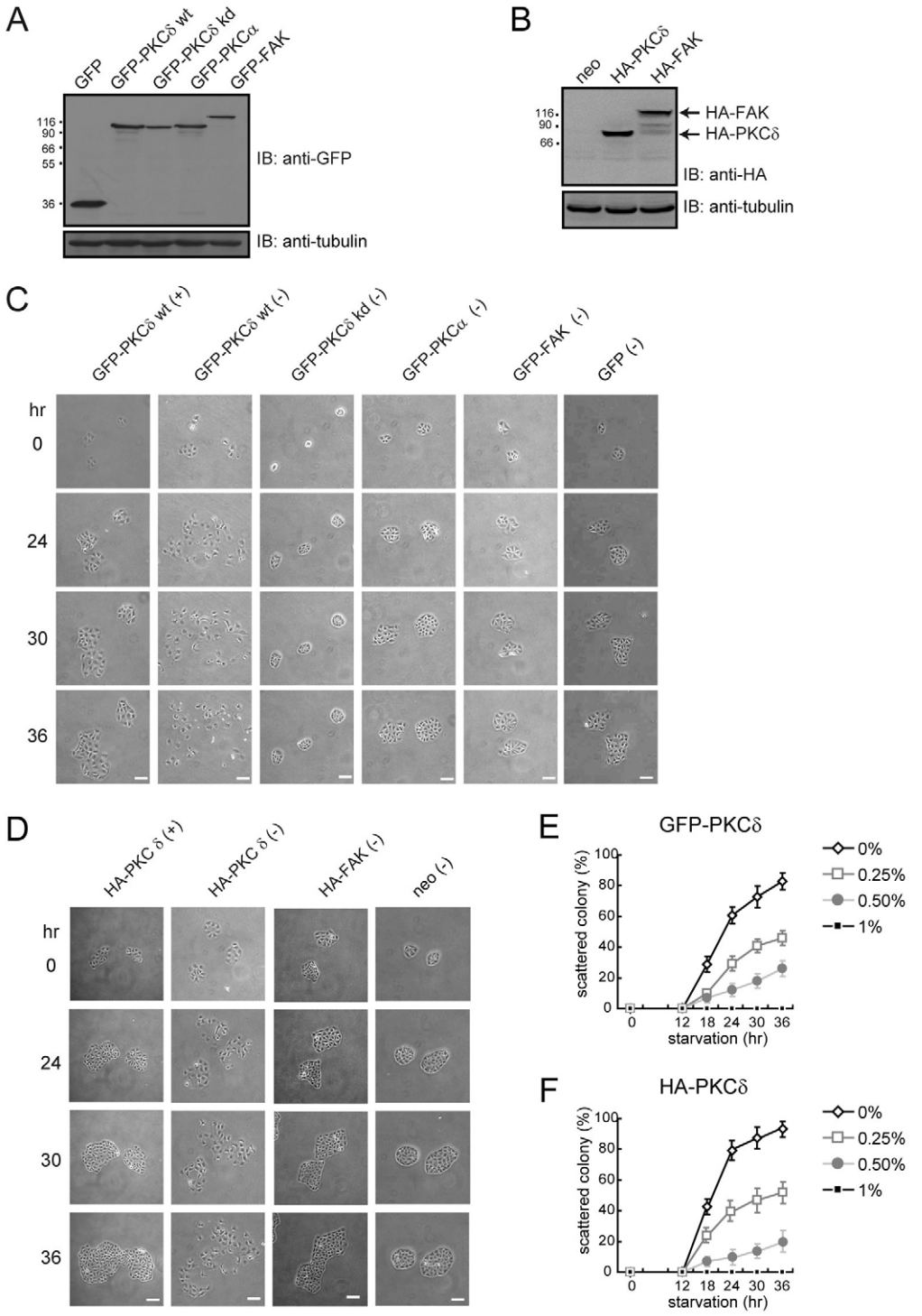

Fig. 1. Elevated expression of PKCδ induces scatter of MDCK cells in response to serum deprivation. (**A**) An equal amount of whole cell lysates from MDCK cells stably expressing GFP, GFP-PKCδ, GFP-PKCα, or GFP-FAK was analyzed by immunoblotting with anti-GFP. wt, wild-type; kd, kinase-deficient mutant. (**B**) An equal amount of whole cell lysate from neomycin-resistant control MDCK cells (neo) and those stably expressing HA-PKCδ or HA-FAK was analyzed by immunoblotting with anti-HA. (**C**) MDCK cells as described for A were allowed to grow as cell colonies in medium supplemented with 10% serum. Subsequently, they were maintained in medium with (+) or without (–) 10% serum and monitored by time-lapse video-microscopy. Representative micrographs at 0, 24, 30, and 36 hours are shown. Scale bars: 150 μm. (**D**) Cell colonies formed by MDCK cells stably expressing HA-PKCδ or HA-FAK were maintained in medium with (+) or without (–) 10% serum and monitored by time-lapse video-microscopy. Representative micrographs at 0, 24, 30, and 36 hours are shown. Scale bars: 150 μm. (**E**) MDCK cells stably expressing GFP-PKCδ were allowed to grow as colonies and then maintained in medium containing different amounts of serum. A cell colony was judged as a "scattered" one when one third of the cells in it had lost contact with their neighbors and exhibited a fibroblast-like phenotype. The percentage of scattered colonies in total counted colonies (n>200) was measured. Values (means ± s.d.) are from three independent experiments. (**F**) MDCK cells stably expressing HA-PKCδ were allowed to grow as colonies and then maintained in medium containing different amount of serum. The percentage of scattered colonies in total counted colonies (n>200) was measured. Values (means ± s.d.) are from three independent experiments.

Kruger and Reddy, 2003; Villar et al., 2007). Recently, our laboratory demonstrated that PKCδ is localized at cell–cell junctions and is capable of suppressing the function of E-cadherin (Chen and Chen, 2009). Notably, PKCδ has been identified as a stress-sensitive kinase that can be activated in response to a wide variety of stresses, including oxidative stress (Brodie and Blumberg, 2003; Steinberg, 2004). However, the activation mechanism and function of PKCδ can vary considerably, depending on the biological context such as the cell type, the nature of the extracellular stimuli, etc. (Brodie and Blumberg, 2003; Steinberg, 2004). In this study, we aim to examine whether PKCδ is involved in the cellular response to growth-factor deprivation.

Results

Elevated expression of PKCδ induces scatter of MDCK cells in response to serum deprivation

To examine whether elevated expression of PKCδ could render epithelial cells susceptible to adaptation upon growth-factor deprivation, GFP-PKCδ and HA-PKCδ were stably overexpressed in Madin-Darby canine kidney (MDCK) cells (Fig. 1A,B). We

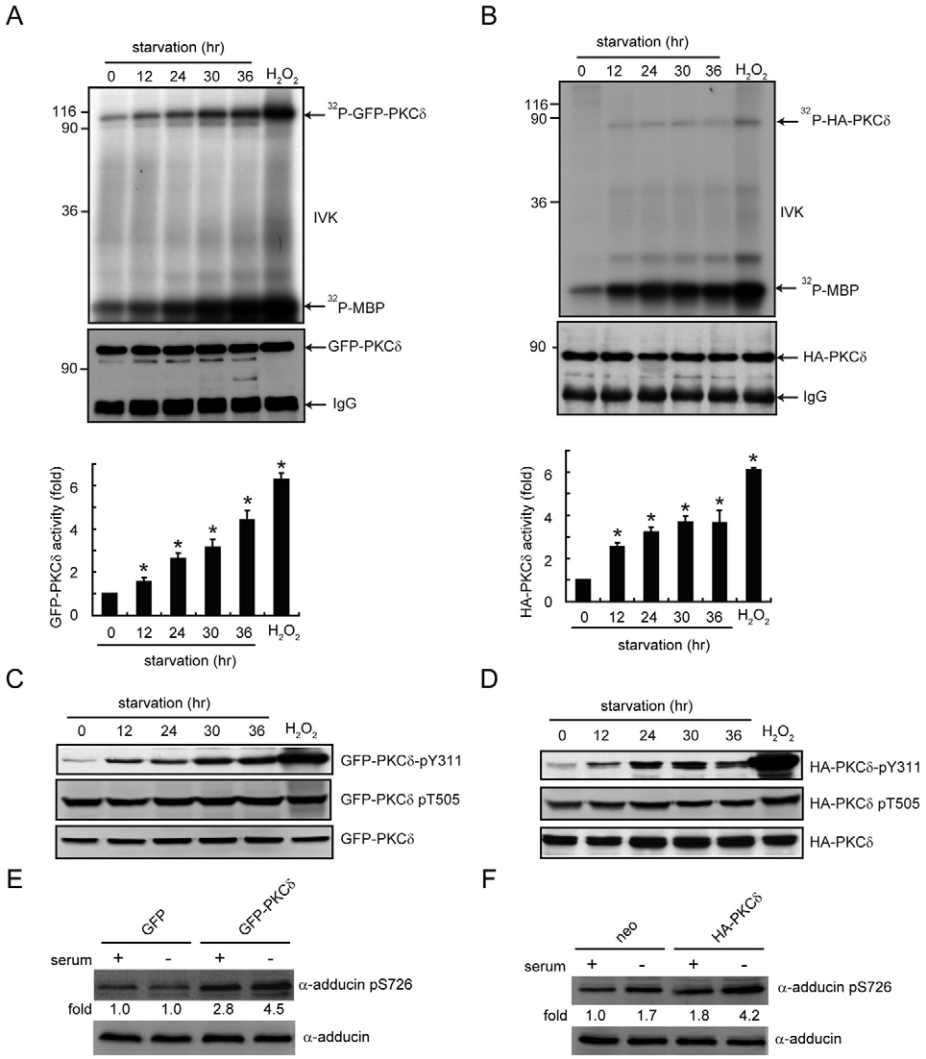

Fig. 2. Serum deprivation stimulates catalytic activity of PKCδ, accompanied by an increase in phosphorylation at Y311. (**A**) MDCK cells stably overexpressing GFP-PKCδ were treated with 5 mM H_2O_2 for 10 minutes or serum-starved for various times, as indicated. GFP-PKCδ was immunoprecipitated by anti-GFP and the washed immunocomplexes were subjected to an in vitro kinase assay using MBP as a substrate, or to immunoblotting with anti-GFP. The position of immunoglobulin G (IgG) is indicated. The ^{32}P-incorporation of myelin basic proteins was quantified and expressed as fold relative to the level of the control. Values (means ± s.d.) are from three independent experiments. *P<0.05. IVK, in vitro kinase assay. (**B**) MDCK cells stably overexpressing HA-PKCδ were treated with 5 mM H_2O_2 for 10 minutes or serum-starved for various times, as indicated. HA-PKCδ was immunoprecipitated by anti-HA and subjected to the in vitro kinase assay as described for A. The ^{32}P-incorporation of myelin basic proteins was quantified and expressed as fold relative to the level of the control. Values (means ± s.d.) are from three independent experiments. *P<0.05. (**C**) An equal amount of whole cell lysate from the cells as described for A was analyzed by immunoblotting with anti-phospho-PKCδ (Y311-*P*), anti-phospho-PKCδ (T505-*P*), or anti-GFP. (**D**) An equal amount of whole cell lysate from the cells as described for B was analyzed by immunoblotting with anti-phospho-PKCδ (Y311-*P*), anti-phospho-PKCδ (T505-*P*), or anti-HA. (**E**) An equal amount of whole cell lysate from the cells expressing GFP or GFP-PKCδ was analyzed by immunoblotting with anti-adducin or anti-phospho-adducin (S726-*P*). (**F**) An equal amount of whole cell lysate from neomycin-resistant control cells (neo) and those expressing HA-PKCδ was analyzed by immunoblotting with anti-adducin or anti-phospho-adducin (S726-*P*).

found that serum starvation, a condition mimicking growth-factor deprivation, induced scatter of MDCK cells stably overexpressing PKCδ, but not PKCα or focal adhesion kinase (FAK), a cytoplasmic tyrosine kinase known to promote cell migration and tumor progression (for a review, see McLean et al., 2005) (Fig. 1C,D). To quantify cell scattering, a cell colony was judged as a "scattered" one when one third of the cells in it had lost contact with their neighbors and exhibited a fibroblast-like phenotype. No cell colony was considered as scattered until after 12 hours of serum starvation (Fig. 1E,F). After 36 hours of serum starvation, more than 80% of cell colonies formed by the PKCδ-overexpressed cells exhibited a scattered phenotype (Fig. 1E,F). Meanwhile, the survival rate of the cells was more than 90% (data not shown), which allowed them to restore cell–cell contacts upon replenishment of serum to the medium (supplementary material Fig. S1). However, prolonged (>48 hours) serum starvation eventually led to cell death (data not shown). Induction of this PKCδ-promoted cell scattering was truly driven by serum deprivation, because as low as 1% serum in the medium was sufficient to inhibit this event (Fig. 1E,F). In addition, this scatter response was not through an autocrine mechanism, because the conditioned medium collected from the PKCδ-overexpressed cells did not trigger cell scattering (data not shown).

Serum deprivation stimulates the catalytic activity of PKCδ, which is dependent on ROS and Src

The effect of PKCδ on promotion of cell scattering upon serum deprivation was found to be completely suppressed by rottlerin, a selective inhibitor of PKCδ, at a concentration as low as 0.4 μM (supplementary material Fig. S2). Moreover, the expression of the kinase-deficient (kd) mutant of PKCδ in MDCK cells failed to induce cell scattering in response to serum deprivation (Fig. 1C). These results indicate that the catalytic activity of PKCδ is required for it to be able to induce cell scattering upon serum deprivation. To examine whether serum deprivation modulates the catalytic activity of PKCδ, an in vitro kinase assay for PKCδ was performed. Serum deprivation apparently stimulated the catalytic activity of PKCδ in a time-dependent manner, which reached the maximal activation (four- to fivefold) after 36 hours of serum starvation, comparable to that (about sixfold) stimulated by a short-term (10 minutes) treatment with hydrogen peroxide (H_2O_2) (Fig. 2A,B). The increased PKCδ activity was accompanied by an increase in the phosphorylation of PKCδ Y311 (Fig. 2C,D) and α-adducin S726 (Fig. 2E,F). α-Adducin has been identified as a physiological substrate of PKCδ (Chen et al., 2007). Phosphorylation of PKCδ on T505 in the activating loop has been reported to be important

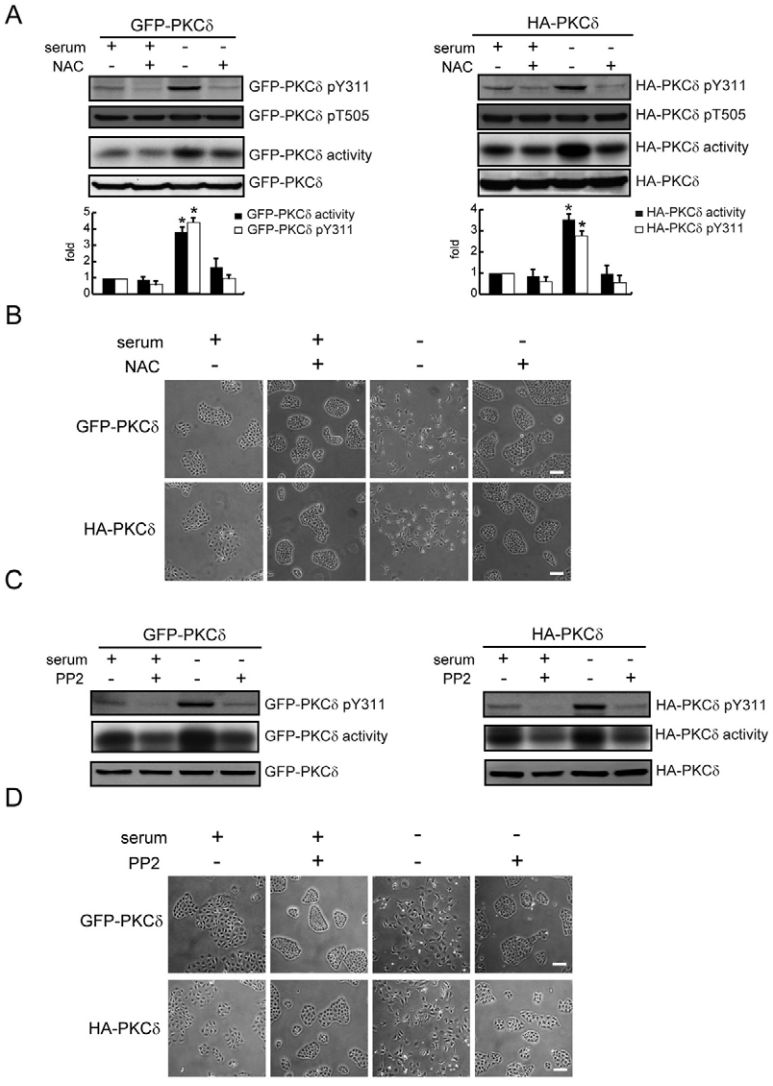

Fig. 3. PKCδ activation and cell scattering triggered by serum deprivation is dependent on ROS and Src. (**A**) MDCK cells stably expressing GFP-PKCδ (left panels) or HA-PKCδ (right panels) were maintained in medium supplemented with (+) or without (–) 10% serum and 25 mM NAC for 24 hours. The catalytic activity of ectopically expressed PKCδ proteins and their phosphorylation on Y311 and T505 were analyzed as described in Fig. 2. The activity and Y311 phosphorylation of PKCδ were quantified and expressed as fold relative to the control cells in the presence of serum. Values (means ± s.d.) are from three independent experiments. **P*<0.05. (**B**) MDCK cells stably expressing GFP-PKCδ (top row) or HA-PKCδ (bottom row) were allowed to grow as colonies and then maintained in medium supplemented with (+) or without (–) 10% serum and 25 mM NAC. After 24 hours, photographs were taken under a phase-contrast microscope. Scale bars: 200 μm. (**C**) MDCK cells stably expressing GFP-PKCδ (left panels) or HA-PKCδ (right panels) were maintained in medium with (+) or without (–) 10% serum and 10 μM PP2 for 24 hours. The activity and Y311 phosphorylation of PKCδ proteins were analyzed as described in Fig. 2. (**D**) MDCK cells stably expressing GFP-PKCδ (top row) or HA-PKCδ (bottom row) were allowed to grow as colonies and then maintained in medium with (+) or without (–) 10% serum and 10 μM PP2. After 24 hours, photographs were taken under a phase-contrast microscope. Scale bars: 200 μm.

for its catalytic activity under certain circumstances (Le Good et al., 1998; Stempka et al., 1999). However, PKCδ was constitutively phosphorylated on T505 in MDCK cells, regardless of serum deprivation (Fig. 2C,D).

ROS has been shown to induce Y311 phosphorylation and activation of PKCδ (Konishi et al., 2001; Rybin et al., 2004). To examine the role of ROS in serum-deprivation-induced activation of PKCδ, the cells were serum-starved in the presence or absence of *N*-acetyl-L-cysteine (NAC), a potent ROS scavenger. Our results clearly showed that NAC inhibited Y311 phosphorylation and activation of PKCδ (Fig. 3A) as well as inhibiting cell scattering in response to serum deprivation (Fig. 3B). Src has been reported to act upstream of PKCδ in response to oxidative stress (Konishi et al., 2001; Rybin et al., 2004). To examine whether Src is involved in PKCδ activation and cell scattering upon serum deprivation, the Src-specific inhibitor PP2 was employed. Like NAC, PP2 was able to inhibit the Y311 phosphorylation and activation of PKCδ (Fig. 3C) as well as cell scattering upon serum deprivation (Fig. 3D). These results not only suggest that the activation of PKCδ upon serum deprivation is ROS- and Src-dependent, but also underscore the significance of the ROS-Src-PKCδ axis in cell scattering in response to serum deprivation.

Phosphorylation of PKCδ at Y311 and Y332 by Src is essential for its activation and ability to promote cell scattering upon serum deprivation

In addition to Y311, the Y332 of PKCδ has been identified as being phosphorylated by Src (Lu et al., 2007). To examine the significance of the phosphorylation of Y311 and Y332 in PKCδ activation and its ability to promote cell scattering upon serum deprivation, PKCδ mutants including Y311F, Y332F, and Y311F/Y332F were stably expressed in MDCK cells (Fig. 4A). Mutation at Y311 and Y332 did not affect the PKCδ subcellular localization of PKCδ (Fig. 4B). Mutation at Y311 or Y332 partially impaired PKCδ activation upon serum deprivation. Importantly, the Y311F/Y332F mutant with substitution at both sites was refractory to activation (Fig. 4A) and failed to promote cell scattering upon serum deprivation (Fig. 4C). These results further support a role for Src in the phosphorylation and activation of PKCδ as well as in cell scattering upon serum deprivation.

PKCδ promotes ROS production, at least partially, through NADPH oxidases in response to serum deprivation

As ROS play an essential role in PKCδ activation and cell scattering upon serum deprivation (Fig. 3), the level of intracellular ROS was measured by flow cytometry. Serum deprivation increased intracellular ROS to some extent in the control cells, which was significantly (~60%) enhanced by overexpression of HA-PKCδ (Fig. 5A). In addition, the serum-deprivation-induced production of ROS was suppressed by rottlerin in both control cells and the cells overexpressing HA-PKCδ (Fig. 5B). These results together suggest a role for PKCδ in promoting ROS production in response to serum deprivation and raise an intriguing possibility for a positive feedback loop between ROS and PKCδ.

To identify oxidase(s) that mediate the effect of PKCδ on promoting cell scattering, selective inhibitors for various oxidases were used. Among the inhibitors examined, the flavonoid inhibitor diphenylene iodinium (DPI), a non-scavenging inhibitor of NADPH oxidase, was found to significantly inhibit the scatter response of the PKCδ-overexpressed MDCK cells to serum deprivation (Fig.

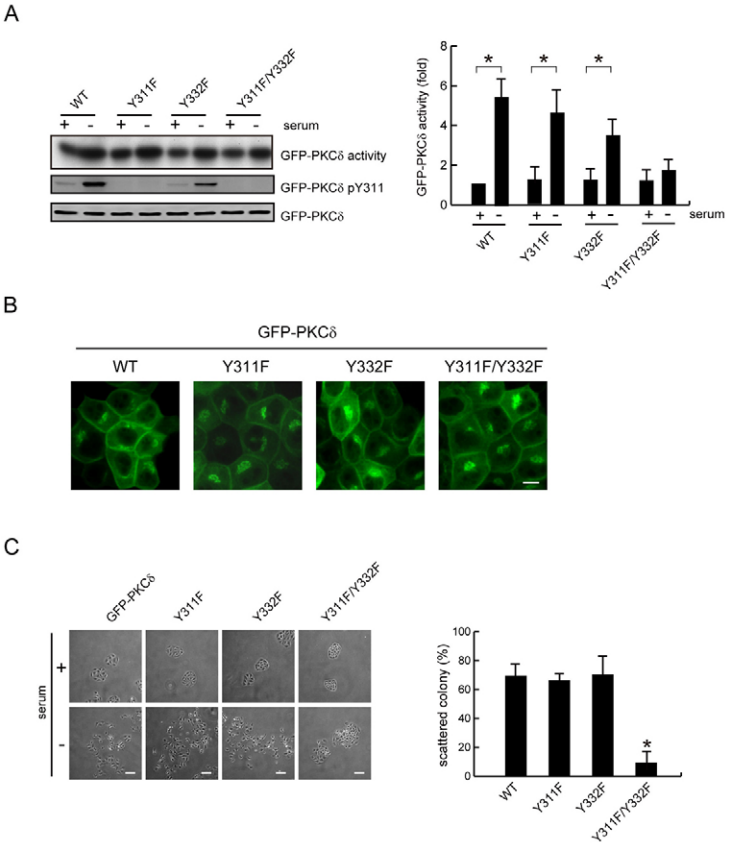

Fig. 4. Phosphorylation of PKCδ at Y311 and Y332 is essential for its activation and ability to promote cell scattering upon serum deprivation. (**A**) GFP-PKCδ or its mutants (Y311F, Y332F, and Y311F/Y332F) were stably expressed in MDCK cells. The cells were maintained in medium with (+) or without (–) 10% serum for 30 hours. The catalytic activity and Y311 phosphorylation of GFP-PKCδ were analyzed. The catalytic activity of GFP-PKCδ and its mutants was quantified and expressed as fold relative to the level of the wild type (WT) in the presence of serum. Values (means ± s.d.) are from three independent experiments. **P*<0.05. (**B**) The MDCK cells were allowed to grow to confluence and the localization of GFP-PKCδ and its mutants were visualized under a confocal microscope. Scale bar: 20 µm. (**C**) MDCK cells stably expressing GFP-PKCδ were allowed to grow as colonies and then maintained in medium with (+) or without (–) serum. After 30 hours, photographs were taken under a phase-contrast microscope and the percentage of scattered colonies in total counted colonies (*n*>200) was measured. Values (means ± s.d.) are from three independent experiments. **P*<0.05 (compared to the wild type). Scale bars: 150 µm.

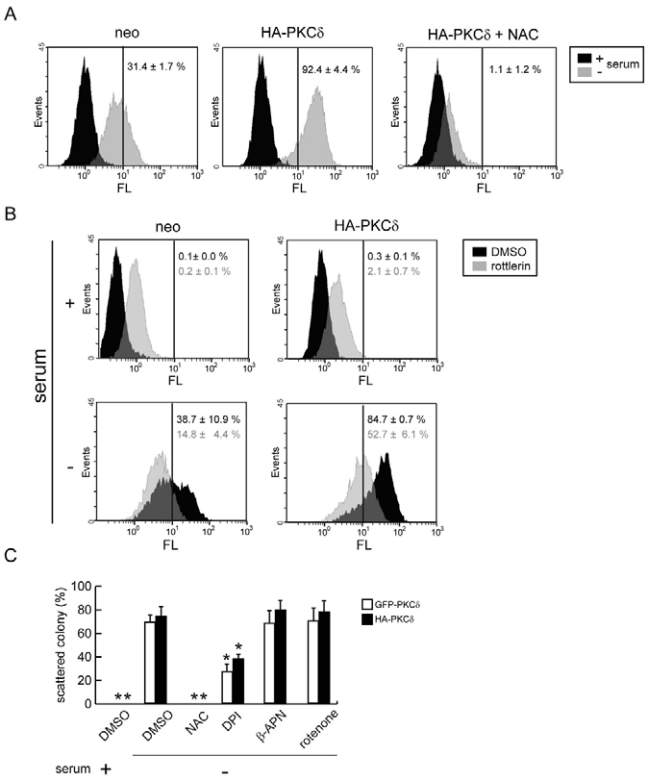

Fig. 5. PKCδ promotes ROS production upon serum deprivation. (**A**) Control MDCK cells (neo) or those stably expressing HA-PKCδ were maintained in medium with (+) or without (–) 10% serum and 25 mM NAC for 30 hours, labeled with 5 μM CM-H_2DCFDA, and subjected to flow cytometry. The percentage of cells with fluorescence intensity above the level of 10 FL (an arbitrary unit) was measured. Values (means ± s.d.) are from three independent experiments. (**B**) Control MDCK cells (neo) or those stably expressing HA-PKCδ were maintained in medium with (+) or without (□) 10% serum and 0.2 μM rottlerin for 30 hours, labeled with CM-H_2DCFDA, and subjected to flow cytometry. The solvent dimethyl sulfoxide (DMSO) was used as a control. The percentage of cells with fluorescence intensity above the level 10 FL (an arbitrary unit) was measured. Values (means ± s.d.) are from three independent experiments. (**C**) MDCK cells stably expressing GFP-PKCδ or HA-PKCδ were allowed to grow as colonies and then maintained in medium with (+) or without (–) 10% serum and various inhibitors (25 mM NAC, 1 μM DPI, 500 μM β-APN, or 5 μM rotenone) for 30 hours. The percentage of scattered colonies in total counted colonies (n>200) was measured. Values (means ± s.d.) are from three independent experiments. *P<0.05.

5C). By contrast, the inhibitors for lysyl oxidase (□-amino-propionitrile) and mitochondrial oxidases (rotenone) had no such effects. These results suggest that NADPH oxidase(s) might, at least in part, mediate the effect of PKCδ on ROS production and cell scattering upon serum deprivation.

JNK acts downstream of ROS and PKCδ to induce cell scattering upon serum deprivation

The mitogen-activated protein kinase (MAPK) family members c-Jun N-terminal kinase (JNK) and p38 have been shown to be activated in response to oxidative stress (Choi et al., 2004; Luo et al., 1998). We found that the JNK inhibitor SP600125, but not the p38 inhibitor SB200350, completely inhibited cell scattering upon serum deprivation (Fig. 6A), which thus suggests a potential role of JNK in this event. Serum deprivation led to activation of both PKCδ and JNK in an ROS-dependent manner (Fig. 6B). In our effort to clarify the relationship between JNK and PKCδ, we found that the PKCδ inhibitor rottlerin inhibited serum-deprivation-induced activation of JNK (Fig. 6C), but that the JNK inhibitor SP600125 did not affect the activation of PKCδ by serum deprivation (Fig. 6D). Furthermore, increased expression of PKCδ potentiated the activation of JNK upon serum deprivation but, by contrast, the kinase-deficient mutant of PKCδ suppressed it (Fig. 6E). It is worth noting that serum-deprivation-induced activation of p38 was independent of PKCδ (Fig. 6E). In contrast to JNK and p38, extracellular signal-regulated kinase (ERK) was apparently suppressed by serum deprivation (Fig. 6E). These data together indicate that JNK acts downstream of ROS and PKCδ to induce cell scattering in response to serum deprivation.

The localization of PKCδ to the Golgi complex is required for it to trigger cell scattering in response to serum deprivation

To identify the domain(s) of PKCδ required for its ability to induce cell scattering upon serum deprivation, PKCδ mutants with a deletion of the C2 domain (designated as ΔC2 with deletion of amino acids 1–123), the C1A domain (designated as ΔC1A with deletion of amino acids 159–208), or the C1B domain (designated as ΔC1B with deletion of amino acids 232–280) were stably expressed in MDCK cells (Fig. 7A). Like the wild-type PKCδ, the ΔC2 and ΔC1A mutants were capable of inducing cell scattering upon serum deprivation. By contrast, the ΔC1B mutant had no such effect (Fig. 7B). Furthermore, the ΔC1B mutant was refractory to activation and phosphorylation of Y311 upon serum deprivation (Fig. 7C,D). The C1B domain of PKCδ has been shown to be crucial for its localization at the Golgi complex (Kajimoto et al., 2004; Schultz et al., 2004). As expected, the ΔC1B mutant did not localize at the Golgi (supplementary material Fig. S3), raising a possibility that the localization of PKCδ to the Golgi complex might be important for its activation by Src upon serum deprivation. Indeed, we found that the Golgi complex became more condensed at the perinuclear region in the cells overexpressing GFP-PKCδ upon serum deprivation (Fig. 8A,B) and, more importantly, that a significant fraction of GFP-PKCδ, but not the ΔC1B mutant, was colocalized with Src at the Golgi complex (Fig. 8C). Hepatocyte growth factor, a potent growth factor known to induce cell scattering, also induces condensation of the Golgi complex in MDCK cells (supplementary material Fig. S4). Although the biological significance of the Golgi condensation is currently not clear, it might be associated with cell scattering.

Depletion of PKCδ restores cell–cell contacts in bladder carcinoma T24 cells

Our experiments to this point suggest that increased expression of PKCδ might render epithelial cells susceptible to scatter upon growth-factor deprivation. As cell scattering resembles epithelial-to-mesenchymal transition (EMT), characterized by loss of cell–cell adhesions and a scattered growth pattern, it raises a possibility that PKCδ might play a role in EMT. To examine this possibility, PKCδ was depleted in human bladder carcinoma T24 cells, which displayed a scattered growth pattern and expressed a high level of endogenous PKCδ (Fig. 9). Short-hairpin RNA (shRNA)-mediated knockdown of PKCδ in T24 cells restored cell–cell contacts and allowed them to grow as discrete colonies (Fig. 9B). The cell colonies formed by PKCδ-depleted T24 cells did not disperse upon serum deprivation (Fig. 9B). Consistent with our notion that PKCδ

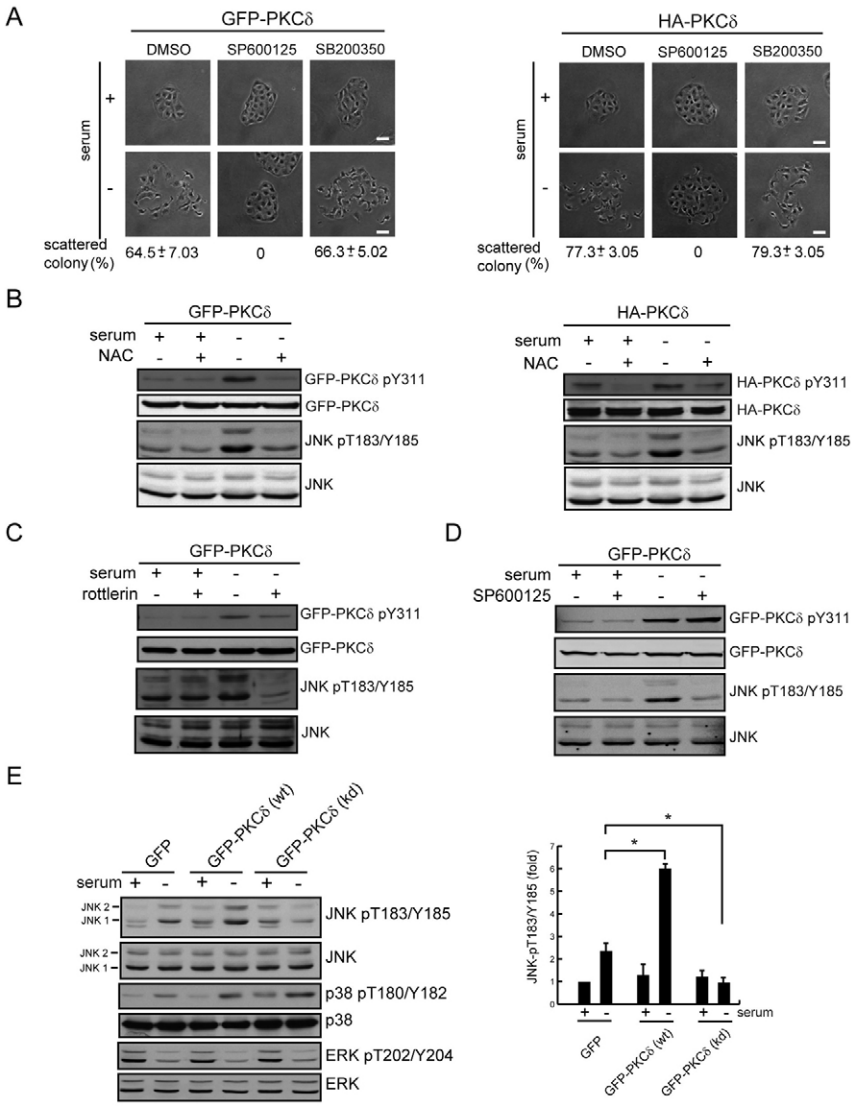

Fig. 6. JNK acts downstream of ROS and PKCδ to induce cell scattering upon serum deprivation. (**A**) Cell colonies formed by MDCK cells stably expressing GFP-PKCδ or HA-PKCδ were maintained in medium with (+) or without (–) 10% serum and the inhibitors. SP600125 (a specific inhibitor for JNKs, 10 μM); SB200350 (a specific inhibitor for p38, 10 μM). The solvent dimethyl sulfoxide (DMSO) was used as a control. After 24 hours, the percentage of scattered colonies in total counted colonies (n>200) was measured. Values (means ± s.d.) are from three independent experiments. Micrographs shown are representative of three independent experiments. Scale bars: 50 μm. (**B**) MDCK cells stably expressing GFP-PKCδ or HA-PKCδ were maintained in the medium with (+) or without (–) 10% serum and 25 mM NAC for 24 hours. The Y311 phosphorylation of ectopically expressed PKCδ and the T183/Y185 phosphorylation of JNK were analyzed by immunoblotting with phospho-specific antibodies. (**C**) MDCK cells stably expressing GFP-PKCδ were maintained in medium with (+) or without (–) 10% serum and 1 μM rottlerin for 24 hours. The Y311 phosphorylation of GFP-PKCδ and the T183/Y185 phosphorylation of JNK were analyzed. (**D**) MDCK cells stably expressing GFP-PKCδ were maintained in the medium with (+) or without (–) 10% serum and 10 μM SP600125 for 24 h. The Y311 phosphorylation of GFP-PKCδ and the T183/Y185 phosphorylation of JNK were analyzed. (**E**) MDCK cells stably expressing GFP, GFP-PKCδ, or GFP-PKCδ kinase-deficient (kd) mutant were maintained in medium with (+) or without (–) 10% serum for 24 hours. The expression and phosphorylation of JNK, p38, and ERK were analyzed by immunoblotting with antibodies as indicated. The T183/Y185 phosphorylation of JNK was quantified and expressed as fold relative to the level of the GFP control in the presence of serum. Values (means ± s.d.) are from three independent experiments. *P<0.05.

is important for ROS production upon serum deprivation, PKCδ depletion in T24 cells suppressed ROS production upon serum deprivation (Fig. 9C).

Discussion

In this study, we demonstrate that elevated expression of PKCδ in MDCK epithelial cells allows them to scatter in response to serum deprivation. Conversely, those scattered cells can restore cell–cell contacts upon replenishment of serum to the medium (supplementary material Fig. S1). This serum-deprivation-induced cell scattering relies on the activity of PKCδ, because overexpression of the PKCδ kinase-deficient mutant or of other kinases such as PKCα and FAK has no such effect (Fig. 1). Intriguingly, the effect of PKCδ on promoting cell scattering appears to be triggered only by serum deprivation, because other stresses including hypoxia, deprivation of nutrients (glucose and

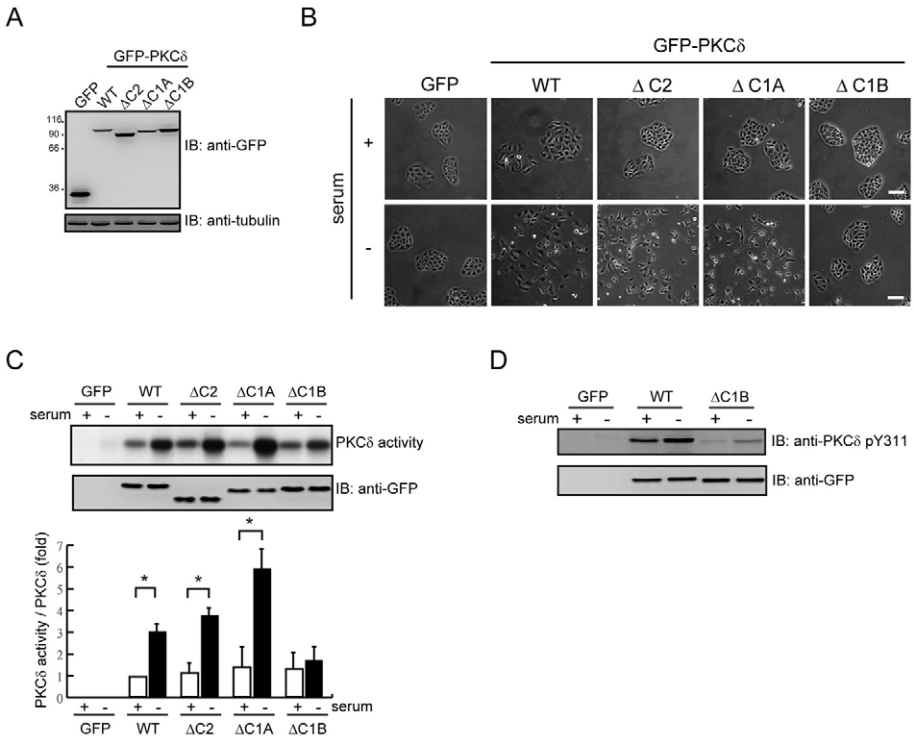

Fig. 7. The C1B domain of PKCδ is required for its activation and ability to promote cell scattering upon serum deprivation. (**A**) An equal amount of whole cell lysate from MDCK cells stably expressing GFP, GFP-PKCδ, or its mutants with a deletion of the C2 domain (ΔC2), the C1A domain (ΔC1A), or the C1B domain (ΔC1B) were analyzed by immunoblotting with anti-GFP or anti-β-tubulin. (**B**) MDCK cells (as described for A) were allowed to grow as colonies and maintained in medium with (+) or without (–) 10% serum for 36 # hours. Micrographs were taken under a phase-contrast microscope. Results shown are representative of three independent experiments. Scale bars: 200 µm. (**C**) MDCK cells (as described for A) were maintained in medium with (+) or without (–) 10% serum for 30 hours. GFP-PKCδ and its mutants were immunoprecipitated by anti-GFP. The washed immunocomplexes were subjected to an in vitro kinase assay using MBP as a substrate, or to immunoblotting with anti-GFP. The kinase activity of GFP-PKCδ and its mutants was quantified and expressed as fold relative to the level of the wild type (WT) in the presence of serum. Values (means ± s.d.) are from three independent experiments. *P<0.05. (**D**) MDCK cells stably expressing GFP, GFP-PKCδ, or its ΔC1B mutant were maintained in medium with (+) or without (–) 10% serum for 30 hours. An equal amount of whole cell lysate was analyzed by immunoblotting with anti-phospho-PKCδ (Y311-*P*) or anti-GFP.

amino acids), osmotic stress, heat shock, and ultraviolet (UV) irradiation do not induce such a scatter response in MDCK cells, regardless of whether PKCδ is overexpressed or not (data not shown). Therefore, the scatter response observed in this study might be a unique phenomenon involving both PKCδ overexpression and serum deprivation. Hypoxia is well known to stimulate invasion and metastasis of tumor cells through hypoxia-inducible factors (HIFs) (Bertout et al., 2008; Semenza, 2003). PKCδ has been shown to modulate the stability of HIFs and to have enhanced transcriptional activity under hypoxic conditions in HeLa cells (Lee et al., 2007). We found that serum deprivation slightly induces expression of HIF-1α in a PKCδ-independent manner (supplementary material Fig. S5). However, the role of HIFs in serum-deprivation-induced cell scattering remains to be examined.

Increased expression of PKCδ has been shown to be associated with malignant tumor progression (Alonso-Escolano et al., 2006; Kharait et al., 2006; Kiley et al., 1999; Kruger and Reddy, 2003; Villar et al., 2007). EMT is now recognized as a significant step in the acquisition of an aggressive, invasive phenotype in tumor cells. Our laboratory previously demonstrated that overexpression of PKCδ suppresses E-cadherin-mediated adherens junctions, but not tight junctions, in MDCK cells (Chen and Chen, 2009). This suppression relies on the catalytic activity of PKCδ and its localization to cell–cell junctions (Chen and Chen, 2009). It is apparent that although an elevated expression of PKCδ by itself is able to suppress adherens junctions, it is not sufficient to cause cell scattering. In this study, we further demonstrate that catalytic activation of PKCδ upon serum deprivation facilitates disruption of cell–cell adhesions, an essential step for cell scattering. Moreover, given its positive role in cell motility (Chen et al., 2007; Gliki et al., 2002; Iwabu et al., 2004; Li et al., 2003), PKCδ might also contribute to the scattered phenotype by promoting cell motility. Thus, our data support a role for PKCδ in promoting EMT. Consistent with this notion, we show in this study that depletion of PKCδ in bladder carcinoma T24 cells restores their cell–cell contacts, which thereby reverses a scattered mesenchymal-like growth pattern to an epithelial-like growth pattern (Fig. 9).

Previous studies have identified PKCδ as an oxidative stress-sensitive kinase that acts as a critical downstream mediator of ROS signaling (Frank et al., 2003; Gopalakrishna and Jaken, 2000; Konishi et al., 2001; Hu et al., 2007). In this study, we show that PKCδ is activated upon serum deprivation in an ROS-dependent manner (Figs 2 and 3). In addition, we demonstrate that both ROS production and PKCδ activation are required for cell scattering upon serum deprivation (Figs 2 and 3). Because other stresses such

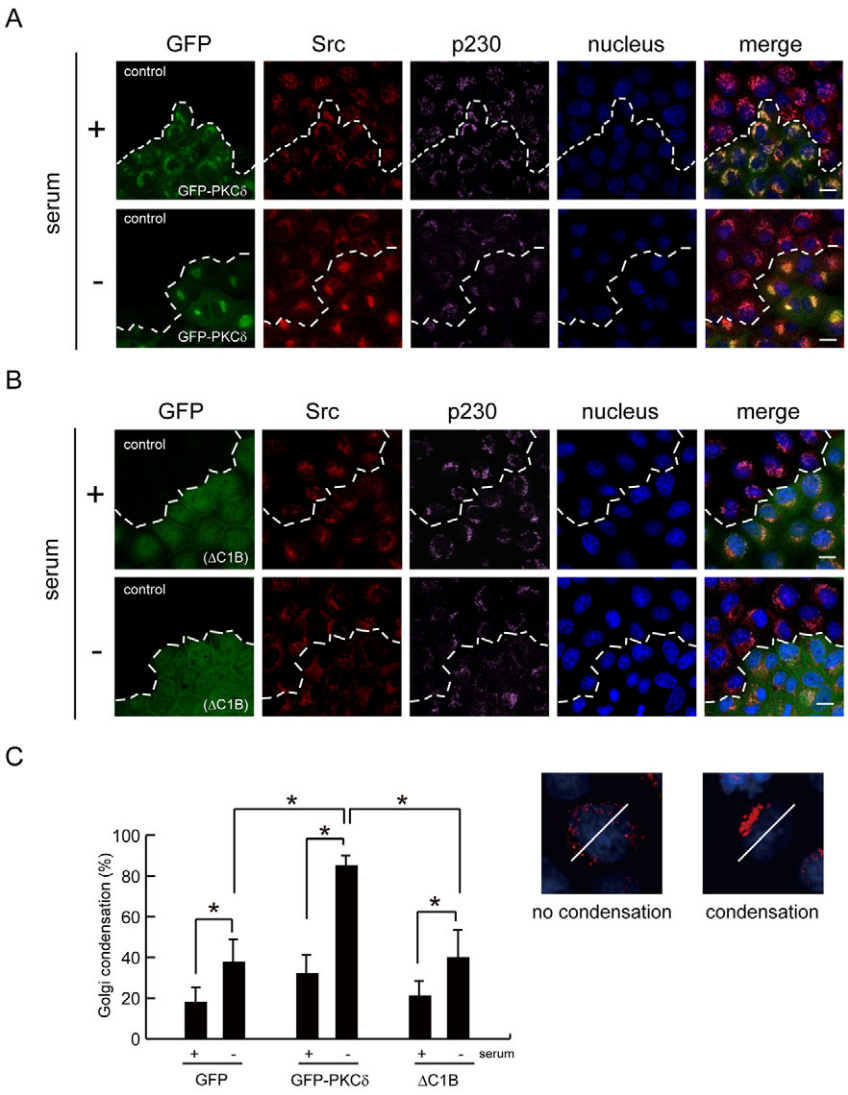

Fig. 8. Colocalization of PKCδ and Src at the Golgi apparatus. (**A**) MDCK cells stably expressing GFP-PKCδ were cocultured with parental MDCK cells (control) and then grown to confluence. The cells were maintained in medium with (+) or without (–) 10% serum for 30 hours and then stained for Src, the trans-Golgi marker p230, and the nucleus. Dashed lines indicate the boundary between control cells and the cells expressing GFP-PKCδ. Scale bars: 20 μm. (**B**) MDCK cells stably expressing GFP-PKCδ ΔC1B mutant were cocultured with parental MDCK cells (control) and then grown to confluence. The cells were maintained in medium with (+) or without (–) 10% serum for 30 hours and then stained as described for A. Dashed lines indicate the boundary between control cells and the cells expressing GFP-PKCδ ΔC1B mutant. Scale bars: 20 μm. (**C**) The percentage of the cells with condensed Golgi complex in total counted cells (n>200) was measured. The Golgi complex was judged as being condensed when its distribution at the perinuclear region was limited within a half of the nuclear periphery. Values (means ± s.d.) are from three independent experiments.*P<0.05.

as UV irradiation also increase intracellular ROS, why they fail to trigger scatter of PKCδ-overexpressed MDCK cells is an intriguing issue. The answer to this question is not clear, but it might be related to the magnitude and duration of oxidative stress and to the corresponding mechanism for PKCδ activation. For example, UV irradiation induces acute oxidative stress, which leads to PKCδ activation through a caspase-mediated cleavage (Koriyama et al., 1999; Denning et al., 2002). The cleaved, constitutively active catalytic fragment of PKCδ exerts a pro-apoptotic function, leading to cell death (Ghayur et al., 1996; Reyland et al., 1999; Kaul et al., 2005). By contrast, serum deprivation might induce chronic oxidative stress, which activates PKCδ through a mechanism other than proteolytic activation. In this study, we show that PKCδ mainly remains intact and is slowly activated in response to serum deprivation (Fig. 2). However, prolonged (>48 hours) serum deprivation leads to cleavage of PKCδ and cell death (data not shown).

How is PKCδ activated by ROS in response to serum deprivation? Our results support a role for the Src family kinases (SFKs) in this process (Fig. 3). SFKs have been shown to phosphorylate and thereby activate PKCδ in cells treated with H_2O_2 (Konishi et al., 1997; Konishi et al., 1997, 2001). In particular, the phosphorylation of PKCδ at Y311 in the hinge region by one or more SFKs correlates with increased catalytic activity of PKCδ (Konishi et al., 2001; Rybin et al., 2004). In addition, the phosphorylation of PKCδ at Y332 by Src is essential for the association of PKCδ and Src (Lu et al., 2007). Importantly, the Src-phosphorylated form of PKCδ is constitutively active, and displays a lipid-independent kinase activity with altered substrate specificity (Rybin et al., 2004). In this study, we demonstrate that the catalytic activation of PKCδ upon serum deprivation is accompanied by increased phosphorylation of Y311 in a Src-dependent manner (Fig. 3). Moreover, mutation at both Y311 and Y322 of PKCδ abolishes its activation and ability to promote cell scattering upon serum deprivation (Fig. 4). Thus, our results support a crucial role of the ROS-Src-PKCδ axis in cell scattering upon serum deprivation.

Another interesting finding in this study is that, once activated by ROS, PKCδ itself activates production of ROS at least in part through NADPH oxidase (Fig. 5), thereby establishing a positive feedback loop to sustain ROS-PKCδ signaling. In fact, PKCδ has an established role in activating NADPH oxidase through phosphorylating $p47^{phox}$, an essential component of NADPH oxidase (Bey et al., 2004; Talior et al., 2005). Accordingly, smooth

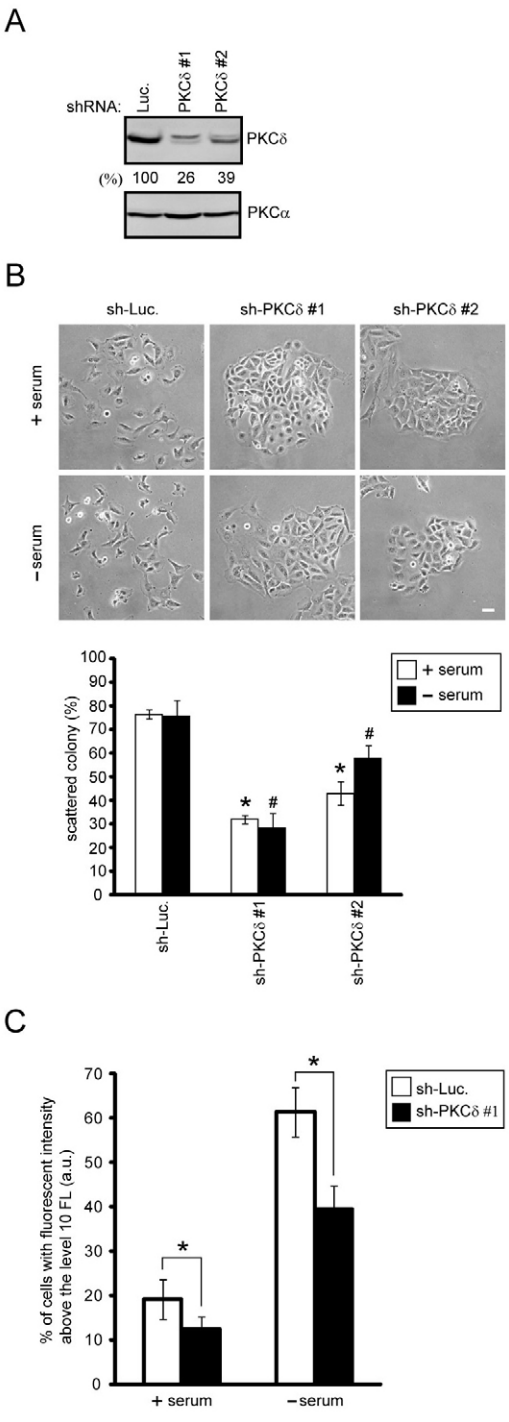

Fig. 9. Depletion of PKCδ in human bladder carcinoma T24 cells restores their cell–cell contacts. (**A**) T24 cells were infected by recombinant lentiviruses expressing shRNAs specific to PKCδ (sh-PKCδ) or luciferase (sh-Luc) as a control. Two distinct PKCδ-specific shRNAs (designated as sh-PKCδ #1 and sh-PKCδ #2) were expressed. An equal amount of whole cell lysate was analyzed by immunoblotting with anti-PKCδ or anti-PKCα. (**B**) The cells (8×10^3) were grown in 6-cm dishes in medium with 10% serum. Three days later, the medium was replaced by serum-free medium for another 30 hours. Note that control T24 cells exhibit a scattered growth pattern, whereas most of PKCδ-depleted T24 cells grow as discrete colonies in the medium with (+) or without (–) serum. The percentage of scattered colonies in total counted colonies (n>200) was measured. Values (means ± s.d.) are from three independent experiments.*P<0.05 (compared to the control in the presence of serum); #P<0.05 (compared to the control in the absence of serum). Scale bar: 50 μm. (**C**) The cells were maintained in medium with (+) or without (–) 10% serum for 30 hours and the intracellular level of ROS was measured by flow cytometry. The percentage of cells with fluorescence intensity above the level of 10 FL (an arbitrary unit) was measured. Values (means ± s.d.) are from three independent experiments.*P<0.05.

muscle cells derived from PKCδ$^{-/-}$ mice produce significantly less ROS in response to UV irradiation and are markedly resistant to H_2O_2-induced cell death (Leitges et al., 2001). Recently, PKCδ was found to promote ROS production through the adapter protein p66Shc in cardiomyocytes (Guo et al., 2009). The p66Shc has been implicated as a major regulator of ROS production (Nemoto and Finkel, 2002). All these studies support a pivotal role of PKCδ in ROS production.

The MAPK family kinases p38 and JNK have been shown to be activated in response to oxidative stress (Choi et al., 2004; Luo et al., 1998). In this study, we demonstrate that serum deprivation induces activation of both p38 and JNK via ROS, but only the activation of JNK is related to PKCδ overexpression and involved in cell scattering upon serum deprivation (Fig. 6). Overexpression of PKCδ enhanced the effect of serum deprivation on JNK activation, but the PKCδ kinase-deficient mutant suppressed this event (Fig. 6E). Moreover, inhibition of PKCδ by rottlerin suppressed the JNK activation by serum deprivation (Fig. 6C), but inhibition of JNK by SP600125 did not affect the activation of PKCδ upon serum deprivation (Fig. 6D). These results together indicate that JNK acts downstream of PKCδ to promote cell scattering in response to serum deprivation. It has been shown that PKCδ is essential for JNK activation in response to DNA damage (Yoshida et al., 2002). In addition to its established nuclear functions, JNK is found to localize in cytoplasmic compartments and play a role in some cytoplasmic processes such as cell migration. Several cytoskeleton-associated proteins have been identified as JNK substrates, including paxillin, the actin-binding protein Spir, the microtubule-associated proteins MAP1B and MAP2, etc. (Huang et al., 2004). Of these, the phosphorylation of paxillin by JNK promotes cell migration (Huang et al., 2003). It is not known whether JNK phosphorylates any of these proteins upon serum deprivation, which might thereby facilitate cell scattering.

It is well known that the GTPase Rho family proteins play a crucial role in regulation of cell–cell junctions (reviewed by Iden and Collard, 2008) and cell motility (reviewed by Ridley, 2001). In this study, we found that overexpression of PKCδ induces an increase in Rac activity and sustains this activation upon serum deprivation (supplementary material Fig. S6). By contrast, the activity of Rho is suppressed by serum deprivation, regardless of PKCδ overexpression (supplementary material Fig. S6). Thus, it is likely that increased Rac activity and decreased Rho activity contribute to PKCδ-promoted cell scattering upon serum deprivation. Moreover, because the activation of Rac has been shown to lead to activation of NADPH oxidases (Sarfstein et al., 2004) and JNK (Coso et al., 1995; Minden et al., 1995), it is possible that Rac plays an important role in mediating the effects of PKCδ on activation of NADPH oxidases and JNK.

In this study, we demonstrate that the C1B domain of PKCδ and its localization to the Golgi complex are essential for PKCδ to be

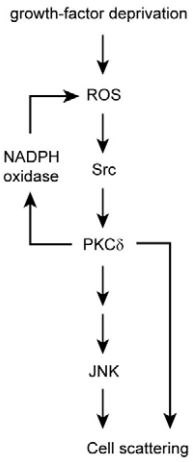

Fig. 10. Model illustrating that growth-factor deprivation could cause a scatter response of epithelial cells through the ROS-Src-PKCδ-JNK signaling pathway. Growth-factor deprivation evokes production of ROS, which leads to Src activation. Activated Src phosphorylates and activates PKCδ at the Golgi complex. Once activated, PKCδ promotes production of ROS at least in part through NADPH oxidase, thereby establishing a positive feedback loop to sustain ROS-PKCδ signaling. Activated PKCδ subsequently contributes to activation of JNK through an unknown mechanism. The activation of both PKCδ and JNK causes disruption of cell–cell adhesions and subsequently promotes cell motility, finally leading to cell scattering.

able to promote cell scattering upon serum deprivation (Fig. 7). The C1B domain of PKCδ has been shown to be required for PKCδ to localize at the Golgi complex (Chen and Chen, 2009; Kajimoto et al., 2004; Schultz et al., 2004). Why is the localization of PKCδ at the Golgi complex essential for its ability to promote cell scattering upon serum deprivation? Our results show that the PKCδ ΔC1B mutant is refractory to activation and phosphorylation of Y311 upon serum deprivation (Fig. 7C,D). Thus, the failure of the ΔC1B mutant to promote cell scattering can be interpreted by its deficiency in catalytic activation upon serum deprivation. Because of the role of SFKs in PKCδ activation, it is possible that activation of PKCδ by SFKs upon serum deprivation might occur at the Golgi complex. In accordance with this notion, we demonstrate that Src is mainly present at the Golgi complex, where it is colocalized with PKCδ upon serum deprivation (Fig. 8). Thus, our study highlights the Golgi complex as the subcellular compartment in which Src activates PKCδ upon serum deprivation. In conclusion, our study provides a model (Fig. 10) to illustrate that growth factor deprivation could cause a scatter response of epithelial cells through the ROS-Src-PKCδ-JNK signaling pathway.

Materials and Methods

Materials

Polyclonal anti-phospho-JNK (T183-*P*/Y185-*P*), anti-phospho-p38 (T180-*P*/Y182-*P*), anti-phospho-ERK (T202-*P*/Y204-*P*), and anti-phospho-PKCδ (T505-*P*) and (Y311-*P*) were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-Src, anti-JNK, anti-p38, and anti-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-p230 trans-Golgi marker was purchased from BD Transduction Laboratories (Lexington, KY). Monoclonal anti-GFP was purchased from Roche. Mouse ascites containing monoclonal anti-hemagglutinin (anti-HA) produced by hybridoma (12CA5) were prepared in our laboratory. Monoclonal anti-tubulin, myelin basic protein (MBP), NAC (a potent ROS scavenger), diphenylene iodinium (DPI; an inhibitor of NADPH oxidase), β-aminopropionitrile (β-APN; an inhibitor of lysyl oxidase), rotenone (an inhibitor of oxidases involved in the electron transport chain in mitochondria), and protein-A-Sepharose were purchased from Sigma-Aldrich. Fetal bovine serum and Lipofectamine were purchased from Invitrogen Life Technologies. SP600125 (a specific inhibitor for JNKs), SB200350 (a specific inhibitor for p38), PP2 (a specific inhibitor for Src), and G418 sulfate were purchased from Calbiochem (San Diego, CA). The ROS detection reagent CM-H_2DCFDA (chloromethyl-2□,7□-dichlorodihydrofluorescein diacetate) was purchased from Molecular Probes.

Plasmids and mutagenesis

Plasmid pHACE-PKCδδwas kindly provided by Jae-Won Soh (Inha University, Korea) and described previously (Soh and Weinstein, 2003). Plasmids pEGFP-PKCα, pEGFP-PKCδ, and the PKCδ mutants (kinase-deficient, ΔC2, ΔC1A, and ΔC1B) were described previously (Chen and Chen, 2009). Plasmid pcDNA3.1-HA-FAK was described previously (Chen and Chen, 2006). All mutagenesis was carried out using QuikChange site-direct mutagenesis kit (Stratagene). The mutagenic primers used were: PKCδ Y311F: 5′-CACAACAGAGTCTGTCGGAATATTCCAGGGATTTGA-GAAGAAGCC-3′; PKCδ Y332F: 5′-CATCCTAGACAACAACGGGACCTTTG-GCAAGATCTGGGAGGG-3′. The positions of substituted codons are underlined. The desired mutations were confirmed by dideoxy DNA sequencing.

Cell culture and transfections

MDCK cells and human bladder carcinoma T24 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and cultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air atmosphere. To generate MDCK cells stably expressing GFP-fusion proteins or HA-tagged proteins, MDCK cells were transfected with plasmids encoding the desired proteins using Lipofectamine. Two days after transfection, the cells were selected in the medium containing 0.5 mg/ml G418 for one week. Those neomycin-resistant cells were collected and analyzed by immunoblotting with the monoclonal anti-GFP or anti-HA.

shRNA-mediated knockdown

The lentiviral expression system was provided by the National RNAi Core Facility (Academia Sinica, Taiwan). Target sequences of siRNAs for human PKCδ are 5′-GGCCGCTTTGAACTCTACCGT-3′ (designated as #1) and 5′-GCAGGGATTAAAGTGTGAAGA-3′ (designated as #2). To produce recombinant lentiviruses, HEK293T cells were co-transfected with pLKO-AS2.puro-shRNA (2.5 μg), pCMV-ρR8.91 (2.25 μg), and pMD.G (0.25 μg) by Lipofectamine. After 72 hours, the medium was harvested, aliquoted, and stored at □80°C. Human bladder carcinoma T24 cells were infected with recombinant lentiviruses encoding shRNAs specific to PKCδ or luciferase (as a control) in the presence of 8 μg/ml polybrene (Sigma) for 24 hours. The cells were rinsed with DMEM and allowed to grow in the growth medium for another 48 hours. Subsequently, the cells were selected in the growth medium containing 2 μg/ml puromycin (Calbiochem) for one week. The puromycin-resistant T24 cells were collected, and the expression of PKCδ was determined by immunoblotting with anti-PKCδ antibody.

Cell scatter assay

Cells were allowed to grow as colonies on 60-mm dishes. When a colony contained 20–30 cells, the growth medium was replaced by serum-free medium. After various intervals, the percentage of scattered colonies in all counted colonies was determined. To quantify cell scattering, a cell colony was judged as a 'scattered' one when one third of the cells in it had lost contact with their neighbors and exhibited a fibroblast-like phenotype.

Immunoprecipitation and immunoblotting

Immunoprecipitation and immunoblotting were carried out as described previously (Chen and Chen, 2006). Chemiluminescent signals were detected and quantified using the Fuji LAS-3000 luminescence image system.

In vitro protein kinase assay

To perform in vitro kinase assays, GFP-PKCδ or HA-PKCδ were immobilized on protein A beads with anti-GFP or anti-HA. The kinase reactions were carried out in 40 μl of kinase buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1.25 μg phosphatidylserine) containing 10 μCi of [γ-^{32}P] ATP (3000 Ci/mmol; PerkinElmer Life Sciences) and 5 μg MBP at 25°C for 10 minutes. Reactions were terminated by addition of sodium dodecyl sulfate (SDS) sample buffer, and the ^{32}P-incorporated proteins were fractionated by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. The radioisotope activity was quantified by a phosphoimager system (Pharmacia).

Detection of intracellular ROS

To detect intracellular ROS, cells were incubated in DMEM containing 5 μM CM-H_2DCFDA for 30 minutes, collected by trypsinization, washed three times with cold phosphate-buffered saline (PBS), and suspended in 750 μl cold PBS. The fluorescence intensity (Ex/Em: 488/525 nm) from 2×10^4 cells was analyzed using a Beckman Coulter FC500 flow cytometer.

Laser-scanning confocal fluorescent microscopy

Cells were allowed to grow as colonies on 12-mm glass coverslips. If necessary, the cells were serum-starved for 24 hours before fixation. Cells were fixed for 15

minutes in PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.2% Triton X-100 for 15 minutes, stained with primary antibodies for 2 hours, and followed by rhodamine-conjugated or Cy5-conjugated secondary antibodies (Jackson ImmunoReseach Laboratories) at 4 μg/ml for 2 hours. The primary antibodies used in immunofluorescence staining were diluted before use: monoclonal anti-p230 (1:100) and polyclonal anti-Src (1:100). Coverslips were mounted in anti-fading solution with DAPI and viewed using a Zeiss LSM510 laser-scanning confocal microscope image system with a Zeiss 63× Plan-Apochromat objective. Wavelengths of 488 nm, 543 nm, and 633 nm were used to excite GFP, rhodamine, and Cy5 respectively. Beam path filters (BP 505–530 nm) and (BP 560–615 nm), and a long path filter (LP 560 nm) were used to acquire images for the emission from GFP, rhodamine, and Cy5, respectively, in a multi-track channel mode.

To measure the condensation of the Golgi complex, MDCK cells were maintained in medium with or without serum for 30 hours and stained for the Golgi complex with anti-p230. The Golgi complex was judged as being condensed when its distribution at the perinuclear region was limited within a half of the nuclear periphery.

Statistics

Student's *t*-tests were used to determine whether there was a significant difference between two means ($P<0.05$); statistical differences are indicated with an asterisk.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/??/????/DC1

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