Mechanisms that mediate apoptosis resistance are attractive therapeutic targets for cancer. Protein kinase Cδ (PKCδ) is considered a pro-apoptotic factor in many cell types. In breast cancer, however, it has shown both pro-survival and pro-apoptotic effects. Here, we report for the first time that down-regulation of PKCδ per se leads to apoptosis of MDA-MB-231 cells. Inhibition of MEK1/2 by either PD98059 or U0126 suppressed the induction of apoptosis of PKCδ-depleted MDA-MB-231 cells but did not support survival of MCF-7 or MDA-MB-468 cells. Basal ERK1/2 phosphorylation was substantially higher in MDA-MB-231 cells than in the other cell lines. PKCδ depletion led to even higher ERK1/2 phosphorylation levels and also to lower expression levels of the ERK1/2 phosphatase MKP3. Depletion of MKP3 led to apoptosis and higher levels of ERK1/2 phosphorylation, suggesting that this may be a mechanism mediating the effect of PKCδ down-regulation. However, PKCδ silencing also induced increased MEK1/2 phosphorylation, indicating that PKCδ regulates ERK1/2 phosphorylation both upstream and downstream. Moreover, PKCδ silencing led to increased levels of the E3 ubiquitin ligase Nedd4, which is a potential regulator of MKP3, because down-regulation led to increased MKP3 levels. Our results highlight PKCδ as a potential target for therapy of breast cancers with high activity of the ERK1/2 pathway.

A crucial step in cancer progression is the ability of the cells to escape apoptosis. There are numerous mechanisms that can mediate apoptosis resistance, and they vary between cancers and cancer subtypes. The identification of such mechanisms would be highly valuable in the search for novel therapeutic targets. For breast cancer, there are several potential signaling pathways that can be targeted to remove the survival support. One of these is the protein kinase C (PKC) family of serine/threonine kinases.

The PKC family is divided into three subgroups depending on the structure of the regulatory domain: classical (PKCα, PKCβI, PKCβII, and PKCγ), novel (PKCδ, PKCe, and PKCd), and atypical (PKCζ and PKCμ/λ) isoforms. Classical and novel PKCs contain diacylglycerol-binding C1 domains and are therefore regulated by activation of pathways that lead to diacylglycerol generation. Classical PKCs also possess a Ca2+-sensitive C2 domain. The novel C2-like domain is Ca2+-insensitive. Atypical PKCs are diacylglycerol- and Ca2+-insensitive and regulated in a different manner (1). The functions of PKC isoforms are cancer and cell type-specific, but several studies have implicated PKCδ and PKCe in the regulation of cancer cell survival.

PKCδ is generally considered to have anti-tumorigenic roles because it has pro-apoptotic effects in many cell types (2, 3). Proteolytic activation of PKCδ by caspases is a common event during apoptosis, and expression of its isolated catalytic domain induces apoptosis (4, 5). This cofactor-independent activation of PKCδ further activates caspase-3 and function as a positive feedback loop (6, 7). However, in breast cancer, the role of PKCδ is more ambiguous. It has been suggested to have less favorable effects by conferring resistance to tamoxifen and irradiation in breast cancer cells (8, 9). PKCδ has also been shown to promote both metastasis (10–12) and proliferation (13) of murine mammary cancer and epithelial cells. On the other hand, PKCδ has also been shown to be crucial for induction of apoptosis in breast cancer cells (14, 15).

Contrary to PKCδ, PKCe has generally been assigned pro-survival and anti-tumorigenic effects (16) for most cell types, including breast cancer cells. In MDA-MB-231 cells, down-regulation of PKCe reduces the tumor growth and metastatic capacity in mice (17). Moreover, inhibition or silencing of PKCe in breast cancer cell lines makes them more susceptible to apoptotic insults (18–20), and overexpression or activation of PKCe protects against apoptosis (18, 20, 21).

Another class of proteins that regulate survival and death induction is the MAPK family. The ERK1/2 pathway has been assigned oncogenic effects because it is associated with the ability of cancer cells to grow independently of normal proliferation signals and is deregulated in many human cancers. On the other hand, the p38 and JNK pathways have been associated with tumor suppressor effects because of their roles in stress-induced apoptosis (22). MAPKs are activated by phosphorylation of essential threonine and tyrosine residues in their activation loop by the dual-speci
ficity MAPK kinases and are inactivated by dephosphorylation of these residues by the dual specificity MKPs (23). In this study, we show that PKCδ silencing per se induces apoptosis of MDA-MB-231 breast cancer cells. Apoptosis is induced via activation of the ERK1/2 pathway through increased phosphorylation of MEK1/2 and degradation of MKP3.

MATERIALS AND METHODS

Cell Culture—All cell lines were obtained from American Type Culture Collection. MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells were maintained in RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum (Invitrogen), 1 mM sodium pyruvate (PAA Laboratories GmbH), 100 IU/ml penicillin, 100 μg/ml streptomycin (Invitrogen). T47D cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 mM HEPES (PAA laboratories GmbH), 100 IU/ml penicillin, and 100 μg/ml streptomycin. The media for MCF-7 and T47D cells were additionally supplemented with 0.01 mg/ml insulin (Nово Nordisk A/S).

Transfections—For small interfering RNA (siRNA) transfections, cells were seeded at 35–50% confluency and grown in complete medium without antibiotics for 24 h. Cells were thereafter transfected for 48 h using 4 μl/ml Lipofectamine 2000 (Invitrogen) and 40 nm siRNA (Invitrogen) (Table 1) in Opti-MEM I (Invitrogen) according to the supplier’s protocol. Where indicated, 17β-estradiol (10 nM), 4-ОH-tamoxifen (1 μM), ICI 182,780 (50 nM), benzoyloxyxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethyl ketone (Z-VA DNAase; 20 μM), U0126 (20 μM), PD98059 (50 μM), SB203580 (10 μM), SP600125 (20 μM), (all from Sigma), Sorafenib (0.3 or 1 μM; LC Laboratories), MG132 (10 μM; Calbiochem), or an equal volume of dimethyl sulfoxide was present from the initiation of transfection or for 6 h. For 17β-estradiol treatment, cells were transfected in RPMI 1640 medium without phenol red supplemented with 10% charcoal-stripped fetal bovine serum (both from Invitrogen).

For plasmid transfections, cells were seeded at approx. 80% confluency, cultured for 24 h, and thereafter transfected for 5 h by replacing normal medium with Opti-MEM I containing 2 μl/ml Lipofectamine 2000 and 2 μg/ml DNA. Cells were transfected with plasmids encoding full-length PKCδ and PKCe or the isolated catalytic domains of the isoforms fused to enhanced green fluorescent protein (EGFP), which have been described previously (24, 25). At the end of transfection, the Lipofectamine 2000-containing Opti-MEM I was removed and replaced with regular growth medium.

Sample Preparation and Western Blotting—Cells were lysed using radioimmune precipitation assay buffer (10 mM Tris-HCl (pH 7.2), 160 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, and 1 mM EGTA) containing 40 μl/ml Complete protease inhibitor (Roche Applied Science) and incubated on ice for 30 min. Lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C, diluted in sample buffer containing β-mercaptoethanol, and boiled for 5 min. Proteins were electrophoretically separated on 10% NuPAGE Novex BisTris gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked with phosphate-buffered saline containing 5% nonfat milk and probed with antibodies to PKCδ (1:500), PKCe (1:500), and PKCα (1:3000) (Santa Cruz Biotechnology); caspase-3, phospho-ERK1/2, ERK1/2, phospho-MEK1/2, MEK1/2, phospho-Akt, and Akt (all at 1:500; Cell Signaling); Nedd4 (1:500; Millipore); SMURF2 (1:1000; Upstate); MKP3 (2 μg/ml) (26); and actin (1:2000; MP Biomedicals). Proteins were visualized with horseradish peroxidase-labeled secondary antibody (Amersham Biosciences) using the SuperSignal system (Pierce) as substrate. Chemiluminescence was detected with a CCD camera (Fujifilm).

Analysis of Viable Cell Number—Cells were seeded at a density of 2000 cells/well in 96-well culture plates, transfected with siRNA, and thereafter incubated for 24 h. The amount of viable cells was assessed by a WST-1 cell viability assay (Roche Applied Science). Absorbance was measured using a Tecan Infinite 200 microplate reader (Tecan). Cells were transfected with siRNA, and thereafter incubated for 24 h. Floating cells, pooled with trypsinized adherent cells, were stained with annexin V-ALP (APC; Pharmingen) according to the supplier’s protocol, and the amount of bound annexin V-ALP was quantified with a FACSCalibur cytometer (BD Biosciences). 10,000 events were acquired on the FL-4 channel for the annexin V-ALP signal. Cells stained with an APC-conjugated goat anti-mouse antibody (Pharmingen) were used as a negative control.

Nuclear Morphology Analysis—For siRNA transfections, MDA-MB-231 cells were seeded on glass coverslips at a density of 100,000 cells/35-mm cell culture dish and transfected with siRNA. For plasmid transfections, 250,000 cells were seeded on glass coverslips.

Cells were grown for 24 h after transfections, fixed in 4% paraformaldehyde; washed with phosphate-buffered saline; and incubated with a solution containing with 3.5 μM Tris-HCl (pH 7.6), 10 mM NaCl, 50 μg/ml propidium iodide, 20 μg/ml RNase, and 0.1% IGEPAL CA-630 for 20 min in the dark. Cells were washed with phosphate-buffered saline and mounted as described (25). 200 transfected cells were scored for apoptotic nuclei using fluorescence microscopy.

Statistics—The significance of differences between multiple variables was assessed by analysis of variance followed by Duncan’s multiple range test. The significance of difference between two

| siRNA    | Oligonucleotides       |
|----------|------------------------|
| Control  | GACAGUUGAACGUCGAUUUGCAUUG |
| PKCa    | CCAGAUGCACCCUGACACUCAUU |
| PKCδ#1  | CCAGAUGCCGCCCCGACACCUCAU |
| PKCδ#2  | CCAGAUGCACCCUGACACUCAUU |
| MKP3#1  | GCCUCUCACUACGACACACAUU |
| MKP3#2  | UCUUGGUACAUUGCUUGGCAUCC |
| Nedd4#1 | UAUUGGUGACAACUACUUGCAUCC |
| Nedd4#2 | UUAUUGGUGACAACUACUUGCAUCC |

PKCδ Suppresses ERK1/2 and Apoptosis
variables was assessed by Student’s t test. The difference was considered significant if the p value was <0.05. All statistical calculations were performed using SPSS Version 11.0.

RESULTS

Down-regulation of PKCα or PKCe Suppresses Growth of MDA-MB-231 Cells—To examine whether individual PKC isoforms are important for growth of breast cancer cells, the effects of PKC down-regulation were studied (Fig. 1). PKCα, PKCδ, and PKCe levels were specifically down-regulated with cognate siRNA (Fig. 1, A and C). Down-regulation of PKCα did not influence the growth of MDA-MB-231 cells, whereas depletion of PKCδ and PKCe reduced cell growth, as indicated by the amount of viable cells, to 45 and 58%, respectively, of control cells (Fig. 1B). For MCF-7 cells, down-regulation of PKCα or PKCδ did not influence cell growth, and down-regulation of PKCe caused only a slight growth reduction of MCF-7 cells (Fig. 1D).

PKCδ Suppresses ERK1/2 and Apoptosis

Because a reduction in cell growth can result from either reduced proliferation or increased cell death and because cell cycle analyses indicated that PKCδ and PKCe do not promote proliferation (data not shown), we analyzed cell death following PKC down-regulation. Depletion of PKCδ in MDA-MB-231 cells resulted in 52% cell death as indicated by annexin V positivity, compared with 13% under control conditions. PKCε depletion also led to increased cell death, whereas knockdown of PKCα did not alter cell survival (Fig. 1E). Knockdown of PKCδ in MCF-7 cells did not influence their survival, whereas PKCe down-regulation induced cell death (Fig. 1F).

To establish that the MDA-MB-231 cell death induced by PKC silencing is not due to off-target effects of the siRNA, cells were transfected with additional siRNAs targeting PKCδ and PKCe before annexin V analysis (Fig. 1, G and H). For PKCδ, both siRNAs led to increased annexin V positivity. For the
additional PKCe siRNA (e#2), there was a tendency for increased cell death, but it did not reach statistical significance. However, PKCe was not as efficiently down-regulated with this siRNA.

Because an increase in annexin V positivity does not distinguish between apoptotic and necrotic cells, we investigated whether the cell death induced by PKCδ and PKCe in MDA-MB-231 cells is apoptotic. For this purpose, the nuclear morphology of the cells was analyzed following propidium iodide staining. In addition, we analyzed the effect of caspase inhibition by applying the pan-caspase inhibitor Z-VAD-fmk (Fig. 1, I and J). Only 1% of the control cells had apoptotic morphology, i.e. condensed or fragmented nuclei. For MDA-MB-231 cells with down-regulated PKCδ and PKCe, the corresponding amounts were 12 and 6%, respectively. These effects were abolished by Z-VAD-fmk treatment, indicating that down-regulation of PKCδ or PKCe induces apoptosis of MDA-MB-231 cells. The percentage of apoptotic cells is lower than the number of dead cells obtained in the annexin V analyses. This is conceivably largely due to the fact that the nuclear morphology analyses will capture only those cells that are apoptotic but have not yet detached from the coverslip. In the annexin V assay, both floating and attached cells are analyzed. In addition, down-regulation of PKCδ and PKCe induced cleavage of caspase-3 (Fig. 1K), further establishing that the cell death is apoptotic. The caspase-3 cleavage was abolished if cells were treated with Z-VAD-fmk.

**Free Catalytic Domains of PKCδ and PKCe Induce Apoptosis of Breast Cancer Cells**—Our results implicate PKCδ and PKCe as survival factors in MDA-MB-231 breast cancer cells. However, in many cell types, PKCδ (and in particular, its free catalytic domain, which is released following caspase activation) is pro-apoptotic (2, 3). To evaluate whether this is the case in breast cancer cells as well, MDA-MB-231 and MCF-7 cells were transfected with vectors encoding full-length (FL) PKCδ and PKCe and their free catalytic domains and analyzed for apoptotic morphology. Expression of the isolated catalytic domain of PKCδ or PKCe resulted in a 3.5–4-fold increase in apoptosis compared with MDA-MB-231 cells expressing EGFP alone (Fig. 2A). The free catalytic domains of PKCδ and PKCe also significantly increased apoptosis in MCF-7 cells compared with EGFP-expressing cells (Fig. 2B). Thus, as for most other cell types, the free catalytic domain of PKCδ induces apoptosis in breast cancer cells. However, overexpression of full-length PKCδ did not induce apoptosis in either cell line.

**Cell Death Induced by PKCδ Down-regulation Is Mediated by the ERK1/2 Pathway**—The magnitude of cell death induction by depletion of PKC isoforms indicates PKCδ as the most robust suppressor of apoptosis in MDA-MB-231 cells. We fur-
ther investigated potential mechanisms for the apoptotic effect of PKCδ silencing in MDA-MB-231 cells by inhibiting MAPK signaling. Inhibition of MEK by PD98059 or U0126 rescued cells from apoptosis induced by PKCδ down-regulation (Fig. 3, A and B). Furthermore, treatment with Sorafenib, an inhibitor of Raf, which is a kinase upstream of ERK1/2, at concentrations that diminish ERK1/2 phosphorylation in these cells (27) also reduced the cell death induced by PKCδ silencing (Fig. 3D). The effect of Sorafenib was smaller than that obtained with the MEK inhibitors. However, Sorafenib will influence a number of other pathways because Raf is upstream of MEK and can phosphorylate several other substrates and because Sorafenib also inhibits other kinases than Raf. It is therefore not unreasonable that the magnitude of the effects of Sorafenib and MEK inhibitors differs. On the other hand, inhibition of p38 (SB203580) or JNK (SP600125) did not influence the apoptotic effect of PKCδ down-regulation (Fig. 3D). Moreover, PKCδ depletion led to a >3-fold increase in ERK1/2 phosphorylation (Fig. 3, E and F), indicating that PKCδ exerts its survival support by suppressing the ERK1/2 pathway.

A comparison between different breast cancer cell lines demonstrated that MDA-MB-231 cells have highly phosphorylated...
ERK1/2 under normal growth conditions compared with the other breast cancer cell lines investigated (Fig. 3G). Thus, PKCδ down-regulation enhances ERK1/2 phosphorylation in MDA-MB-231 cells from an already high level.

The PI3K pathway has recently been shown to be negatively regulated by the ERK1/2 pathway in a number of breast cancer cell lines, including MDA-MB-231 cells (28). Furthermore, PKCδ has been shown to support survival by inducing activation of Akt in cells with a constitutively activated Ras pathway (29). These data together indicate that Akt may be a mediator of the PKCδ effects. However, we could not observe a decrease in Akt phosphorylation after down-regulation of PKCδ (Fig. 3H). Thus, reduced Akt signaling cannot explain the apoptosis induced by PKCδ silencing in MDA-MB-231 cells.

**PKCδ Suppresses ERK1/2 Phosphorylation by Counteracting MKP3 Degradation and Suppressing MEK1/2 Phosphorylation**—The relatively high level of ERK1/2 phosphorylation in MDA-MB-231 cells, which is further elevated by PKCδ depletion, indicates that PKCδ influences the levels or activity of ERK1/2 regulators. Increased ERK1/2 phosphorylation could be due to either higher activity of kinases or lower activity of phosphatases that target the phosphorylation sites. When activated, ERK1/2 can be dephosphorylated by MKP3, a dual specificity phosphatase that inactivates ERK1/2 by dephosphorylation of the tyrosine and threonine in the activation loop (23). To investigate whether PKCδ modulates MKP3 levels and thereby ERK1/2 phosphorylation, we analyzed the expression of MKP3 after down-regulation of PKCδ. Depletion of PKCδ, but not of PKCa or PKCe, led to decreased protein levels of MKP3 (Fig. 4, A and B). Of the breast cancer cell lines investigated, MKP3 was detected only in MDA-MB-231 cells (Fig. 4C), which makes this cell line unique among these cell lines and can explain the cell-specific effects of PKCδ depletion in MDA-MB-231 cells.

Our data indicate that down-regulation of MKP3 may be the mechanism through which PKCδ depletion leads to increased ERK1/2 phosphorylation and cell death. We therefore investigated if down-regulation of MKP3 alone (Fig. 4D) could induce cell death as seen after down-regulation of PKCδ. MKP3 depletion significantly increased cell death compared with control cells (Fig. 4E). However, the effect of MKP3 down-regulation did not have the same magnitude as PKCδ depletion. Furthermore, although MKP3 depletion increased ERK1/2 phosphorylation, it did not reach the levels of phosphorylation in PKCδ-depleted cells (Fig. 4F). Thus, down-regulation of MKP3 by PKCδ depletion does not seem to fully explain the effect of PKCδ depletion on ERK1/2 phosphorylation and cell survival.

For this reason, we also investigated MEK1/2 phosphorylation after down-regulation of PKCδ. PKCδ depletion led to a 2.5-fold increase in MEK1/2 phosphorylation (Fig. 4, G and H). These results suggest that PKCδ induces apoptosis by regulating ERK1/2 phosphorylation upstream (MEK1/2) and downstream (MKP3) of ERK1/2 activation.

Because MKP3 levels are largely regulated by ubiquitinylia- tion and subsequent proteasome-mediated degradation (30), we treated PKCδ-depleted cells with the proteasome inhibitor MG132 during the last 6 h of incubation (Fig. 4I). This led to a marked increase in MKP3 levels and also a reversal of the up-regulated ERK1/2 phosphorylation in PKCδ-depleted cells. This suggests that PKCδ depletion potentiates the proteasomal degradation of MKP3. The remaining difference in MKP3 levels between control and PKCδ-depleted cells after MG132 treatment is conceivably due at least in part to the fact that the cells were exposed to MG132 only during the last 6 h of incubation. Any difference in MKP3 levels that may have arisen during the time period before the last 6 h would therefore not be influenced by MG132.

Proteasome-mediated MKP3 degradation can be regulated by ERK1/2 phosphorylation itself, where ERK1/2 can phosphorylate MKP3 at Ser199 and Ser207, which targets it for proteasomal degradation (31). However, we did not observe increased MKP3 levels when cells were simultaneously treated with the MEK inhibitor U0126 (Fig. 4J), suggesting that the increased phosphorylation of ERK1/2 caused by down-regulation of PKCδ is not the reason for MKP3 degradation.

**Nedd4 Is a Potential Mediator of MKP3 Degradation**—Because MKP3 is degraded by the proteasome, our aim was to find potential E3 ubiquitin ligases that could mediate the degradation of MKP3 induced by PKCδ depletion. By analyzing data from a microarray analysis of mRNA from PKCδ-down-regulated and control MDA-MB-231 cells,3 we found indications that the mRNAs for E3 ubiquitin lages SMURF2 (SMAD ubiquitination regulatory factor 2) and Nedd4 (neural precursor cell expressed developmentally down-regulated 4) were up-regulated after down-regulation of PKCδ. We therefore investigated the protein levels of these proteins. The Nedd4 protein levels were up-regulated by >3-fold compared with control cells (Fig. 5, A and B). This was confirmed with an additional PKCδ siRNA oligonucleotide (Fig. 5C). However, the protein level of SMURF2 was not up-regulated after PKCδ depletion. As for MKP3, Nedd4 was detected only in MDA-MB-231 cells of the four breast cancer cell lines investigated (Fig. 5D).

We further investigated whether Nedd4 regulates MKP3 stability. Down-regulation of Nedd4 in MDA-MB-231 cells led to increased levels of MKP3 (Fig. 5E), suggesting that Nedd4 is involved in destabilization of MKP3. Nedd4 has several substrates (32), which can explain why we observed slightly increased MDA-MB-231 cell death following Nedd4 down-regulation (data not shown). This prevented us from investigating whether depletion of Nedd4 was sufficient to rescue cells from apoptosis induced by down-regulation of PKCδ or MKP3.

**Inhibition of the ERK1/2 Pathway Does Not Support Survival of MCF-7 or MDA-MB-468 Cells**—The basal ERK1/2 phosphorylation is substantially lower in the other cell lines investigated compared with MDA-MB-231 cells. To examine whether the ERK1/2 pathway may promote apoptosis also under these conditions, we investigated the effect of PD98059 on the survival of MDA-MB-468 and MCF-7 cells (Fig. 6). In MCF-7 cells, which do not die in response to PKCδ down-regulation (Fig. 1F), PD98059 did not lead to reduced cell death. Instead, it had a death-promoting effect (Fig. 6A). In MDA-MB-468 cells, PKCδ depletion led to increased cell death (Fig. 6C), albeit not with the same magnitude as in MDA-MB-231 cells. In contrast to

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3 G. K. Lenne and C. Larsson, unpublished data.
the effect in MDA-MB-231 cells, PD98059 did not suppress cell death in PKCδ-depleted MDA-MB-468 cells (Fig. 6D).

**DISCUSSION**

Many studies have implicated PKCδ as a pro-apoptotic protein in a wide range of cell types (2, 3). However, for breast cancer cells, there are indications of PKCδ having both pro-apoptotic and pro-survival effects. PKCδ has been shown to promote resistance to tamoxifen, γ-irradiation, retinoic acid, and TRAIL (8, 9, 33, 34). On the other hand, apoptosis induced by UV light is mediated via PKCδ (14, 15). Here, we report that down-regulation of PKCδ per se leads to apoptosis in the MDA-MB-231 cell line. This has not previously been reported, and one reason for this may be that a rather long period with lowered levels is necessary for death induction to be detectable.

As mentioned previously, PKCδ is generally considered to be pro-apoptotic because it is cleaved by caspases during apoptosis and because expression of the isolated PKCδ catalytic domain induces apoptosis (4, 5). Our data demonstrate that although PKCδ has a survival role in breast cancer cells, as opposed to most other cell types, the free catalytic domain induces apoptosis. This latter effect therefore seems to be universal, whereas upstream PKCδ effects on cell survival are more cell-specific.

We also observed increased apoptosis of MDA-MB-231 and MCF-7 cells following expression of the free catalytic domain of PKCε. Although PKCε is considered to support survival by protecting cells against apoptotic insults (18, 20), cleavage at Asp383 by caspases creates an active catalytic fragment, and depending on the intracellular context and cell type, the released catalytic domain can promote or protect against apoptosis (21, 35–37). In addition, Lee et al. (38) showed that overexpression of the catalytic domain of PKCε in mouse fibroblasts enhances irradiation-induced cell death. Several of
PKCδ Suppresses ERK1/2 and Apoptosis

these observations are in line with our finding that transfection with the isolated catalytic domain of PKCe induces apoptosis of breast cancer cells. However, the siRNA experiments support a pro-survival role for PKCe in both MDA-MB-231 and MCF-7 cells. The finding that the free catalytic domains of PKCδ and PKCe induce apoptosis whereas at the same time down-regulation of the endogenous proteins has the same effect can potentially explain the sometimes opposing effects on cell survival of these isoforms.

Our results further suggest that PKCδ supports survival via inhibition of the ERK1/2 pathway in MDA-MB-231 cells. This is also the cell line with the highest basal ERK1/2 phosphorylation compared with the other breast cancer cell lines investigated, which conceivably can be explained by the fact that it harbors an activated K-Ras because of a mutation in codon 13 of the KRAS gene (39). PKCδ silencing led to increased ERK1/2 phosphorylation in MDA-MB-231 cells, which is in line with a previous report showing that treatment with GF109203X, an inhibitor of classical and novel PKC isoforms, induces ERK1/2 phosphorylation in this cell line (33). In addition, the ERK1/2 pathway has been connected to apoptosis induction in other cell types as well (40, 41).

Oncogene-induced accelerated cell cycle progression can be halted by checkpoints that activate failsafe mechanisms or tumor suppressor networks, i.e. mechanisms like senescence and apoptosis that are activated to prevent further progression toward malignancy. However, cells can evade this by enhancing prosurvival signaling that increases the threshold for apoptosis induction (42, 43). It has been reported, for example, that NFκB suppresses apoptosis induced by oncogenic Ras in mouse fibroblasts (44). Because PKCδ silencing apparently induced apoptosis by higher activity in the ERK1/2 pathway, PKCδ might be crucial to prevent the activation of failsafe mechanisms induced by Ras-Raf-MEK-ERK signaling in MDA-MB-231 cells.

One signaling pathway that is often involved in escaping oncogene-induced failsafe mechanisms is the PI3K/Akt pathway (43). It has been reported that PKCδ is required for survival of mouse fibroblasts with mutated constitutively activated Ras by mediating Akt activation (29). Furthermore, ERK1/2 has been shown to suppress Akt phosphorylation in many breast cancer cell lines (28). It might therefore be expected that decreased Akt phosphorylation, as a consequence of either lower PKCδ levels or increased ERK activity, mediates induction of apoptosis. However, we did not observe a decrease in Akt phosphorylation after PKCδ silencing. Thus, PI3K/Akt signaling is likely not involved in the PKCδ-regulated survival of MDA-MB-231 cells.

PKCδ conceivably regulates ERK phosphorylation both upstream and downstream of ERK activation because PKCδ silencing resulted in increased MEK1/2 phosphorylation and decreased expression levels of the ERK1/2 phosphatase MKP3. Reduction of MKP3 alone by siRNA did not induce ERK1/2 phosphorylation and cell death to the same extent as PKCδ silencing. Thus, both regulation points of ERK1/2 activation are most probably crucial for the pro-survival effect of PKCδ in MDA-MB-231 cells.

We also observed that MKP3 is expressed only in MDA-MB-231 cells and not in any of the other cell lines investigated. MKP3 is encoded at a chromosomal region that exhibits loss of heterozygosity and has reduced expression in some cancers. This and functional studies support a role for MKP3 as a tumor suppressor. However, MKP3 can be up-regulated in cancers with deviant receptor tyrosine kinase and Ras/Raf signaling (30). In the human breast epithelial cell line MCF-10A, which does not express MKP3,

FIGURE 4. PKCδ depletion leads to MKP3 down-regulation. MDA-MB-231 cells were mock-treated or transfected with siRNA targeting PKCδ (#1), PKCδ (#2), PKCε (#3) or a control oligonucleotide (c) or with two different siRNAs targeting MKP3 ( #1 and #2) and incubated for 24 h in complete medium before being harvested for Western blotting. Data in B (mean ± S.E., n = 3) are quantifications of control and PKCδ siRNAs in A. Data in E (mean ± S.E., n = 3) show the percentage of annexin V-positive cells. Data in H (mean ± S.E., n = 3) are quantifications of G. Western blots are representative of three independent experiments. Asterisks indicate statistically significant differences (*, p < 0.05; **, p < 0.01) compared with control-transfected cells. p-ERK, phoshpo-ERK.

FIGURE 5. PKCδ depletion leads to up-regulation of Nedd4, which suppresses MKP3 levels. MDA-MB-231 cells were transfected with siRNAs targeting PKCδ (#1) or #2), Nedd4 (#1 or #2), or a control oligonucleotide (c) and incubated for 24 h in complete medium before being harvested for Western blotting. In D, whole cell lysates of the indicated breast cancer cell lines grown in complete medium were subjected to Western blotting. Data in B (mean ± S.E., n = 3) are quantifications of A. Western blots (A and C–E) are representative of three independent experiments. Asterisks indicate statistically significant differences (**, p < 0.01) compared with control-transfected cells.
PKCδ Suppresses ERK1/2 and Apoptosis

FIGURE 6. Inhibition of the ERK1/2 pathway does not increase the survival of MCF-7 or MDA-MB-468 breast cancer cells. A, MCF-7 cells were mock-treated or transfected with siRNA targeting PKCα (α), PKCβ (δ), or PKCε (ε). Where indicated, 50 nM PD98059 was present from the initiation of transfection. B, MDA-MB-468 cells were transfected with control (α) or PKCδ siRNA, and where indicated, 50 nM PD98059 was present from the initiation of transfection. The cells were then analyzed by Western blotting for cell death by annexin V staining. Data are means ± S.E. (n = 2 [A and D] or n = 3 [C]) and are expressed as percent annexin V-positive cells. Asterisks indicate statistically significant differences (**, p < 0.01) compared with control-transfected cells (C). DMSO, dimethyl sulfoxide.

stable overexpression of H-Ras results in MKP3 expression (45). Our finding that MDA-MB-231 is the only cell line expressing MKP3 is therefore in line with these observations.

MKP3 levels are largely regulated by ubiquitinylination and subsequent proteasome-mediated degradation (30). We observed that inhibition of the proteasome led to increased MKP3 levels in MDA-MB-231 cells. For this reason, we searched for potential E3 ubiquitin ligases involved in the MKP3 degradation induced by PKCδ silencing and found Nedd4 to be up-regulated in cells with down-regulated PKCδ. Furthermore, Nedd4 silencing led to reduced levels of MKP3, suggesting that Nedd4 mediates MKP3 degradation and might be an E3 ubiquitin ligase for MKP3. Nedd4 has several known substrates that are targeted for proteasomal degradation, including PTEN (32, 46). This unspecific substrate selectivity of Nedd4 may be the reason that we observed slightly increased cell death after Nedd4 silencing, which prevented us from studying the effect of simultaneous down-regulation of Nedd4 and PKCδ.

In conclusion, PKCδ is a pro-survival factor in MDA-MB-231 breast cancer cells. PKCδ conceivably prevents activation of fail-safe mechanisms leading to apoptosis by suppressing the Ras oncogene-induced constitutively activated MEK/ERK pathway. This is regulated both upstream and downstream of ERK1/2 activation by inhibiting MEK1/2 activity and stabilizing MKP3. Finally, Nedd4 is a potential E3 ubiquitin ligase for MKP3. Our results highlight PKCδ and other suppressors of the ERK1/2 pathway as potential targets for therapy of breast cancers with high activity of the Ras pathway.

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