Transcriptional Regulation of Oncogenic Protein Kinase Cε (PKCε) by STAT1 and Sp1 Proteins*

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Overexpression of PKCε, a kinase associated with tumor aggressiveness and widely implicated in malignant transformation and metastasis, is a hallmark of multiple cancers, including mammary, prostate, and lung cancer. To characterize the mechanisms that control PKCε expression and its up-regulation in cancer, we cloned an approximately 1.6-kb promoter segment of the human PKCε gene (PRKCE) that displays elevated transcriptional activity in cancer cells. A comprehensive deletional analysis established two regions rich in Sp1 and STAT1 sites located between −777 and −105 bp (region A) and −921 and −796 bp (region B), respectively, as responsible for the high transcriptional activity observed in cancer cells. A more detailed mutagenesis analysis followed by EMSA and ChIP identified Sp1 sites in positions −668/−659 and −269/−247 as well as STAT1 sites in positions −880/−869 and −793/−782 as the elements responsible for elevated promoter activity in breast cancer cells relative to normal mammary epithelial cells. RNAi silencing of Sp1 and STAT1 in breast cancer cells reduced PKCε mRNA and protein expression, as well as PKCε promoter activity. Moreover, a strong correlation was found between PKCε and phosho-Ser-772 (active) STAT1 in levels in breast cancer cells. Our results may have significant implications for the development of approaches to target PKCε and its effectors in cancer therapeutics.

Background: PKCε, a kinase widely implicated in tumorigenesis and metastasis, is overexpressed in many cancers.

Results: Transcription factors Sp1 and STAT1 control the expression of PKCε in cancer cells.

Conclusion: Up-regulation of PKCε is mediated by dysregulated transcriptional mechanisms.

Significance: Our results may have significant implications for the development of approaches to target PKCε and its effectors in cancer therapeutics.

The serine-threonine kinase protein kinase Cε (PKCε), a phorbol ester receptor, has been widely implicated in numerous cellular functions, including cell cycle progression, cytokinesis, cytoskeletal reorganization, ion channel control, and transcription factor activity regulation (1–6). This ubiquitously expressed kinase has been associated with multiple disease conditions, including obesity, diabetes, heart failure, neurological diseases, and cancer (7–10). PKCε is primarily activated by the lipid second messenger diacylglycerol (11), a product of phosphatidylinositol 4,5-bisphosphate hydrolysis by phospholipase C, which, like phorbol esters, binds to the C1 domain located in the N-terminal regulatory region. Receptors coupled to diacylglycerol generation, including tyrosine kinase and G-protein-coupled receptors, cause the intracellular mobilization of PKCε to the plasma membrane and other intracellular compartments, where it associates with interacting partners and phosphorylates specific substrates (12).

It is widely recognized that distinct members of the diacylglycerol/phorbol ester-regulated PKCs act either as promoters or suppressors of growth and tumorigenesis (13, 14). In that regard, work from several laboratories identified PKCε as an oncogenic kinase and established important roles for this kinase in the development and progression of cancer. Early studies revealed that ectopic overexpression of PKCε leads to malignant transformation in some cell types (11, 15, 16). PKCε confers growth advantage and survival through the activation of Ras/Raf/ERK, PI3K/Akt, STAT3, and NF-κB pathways (17, 18). PKCε also mediates resistance to chemotherapeutic agents and ionizing radiation, and inhibition of its activity or expression sensitizes cancer cells to cell death-inducing agents (19–21). Most remarkably, PKCε emerged as a cancer biomarker, as it is markedly up-regulated in most epithelial cancers (22, 23). For example, the vast majority of prostate tumors, in particular those from advanced and recurrent patients, display elevated PKCε levels (24). Prostate-specific PKCε transgenic mice develop prostatic neoplastic lesions with elevated Akt, STAT3, and NF-κB activity (17). Another remarkable example of PKCε up-regulation is in lung cancer; the vast majority (>90%) of primary human non-small cell lung cancers show significant PKCε overexpression compared with normal lung epithelium, and knockdown of PKCε from non-small cell lung cancer cells impairs their ability to form tumors and metastasize in nude mice (25). Likewise, depletion of PKCε from breast cancer cells impairs growth, tumorigenicity, and invasiveness. Accordingly, PKCε up-regulation has been associated with poor disease-free and overall survival of breast cancer patients (22). More
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recently, a PKCe ATP mimic inhibitor was found to impair the growth of breast cancer cells in vitro and in vivo, highlighting the potential of PKCe as a breast cancer therapeutic target (26). Regardless of the well accepted fact that disregulation in PKCe expression plays a causative role in cancer progression, little is known regarding the mechanisms that control the expression of this pro-oncogenic and metastatic kinase. To our knowledge, the transcriptional mechanisms controlling the expression of the PRKCe promoter in humans or other species have not yet been studied. To characterize the regulation of PKCe expression, we cloned a fragment of the promoter region of the human PRKCe gene and investigated the critical determinants controlling transcriptional activation of this gene. Our analysis revealed key cis-acting elements in the PRKCe promoter and candidate transcription factors, particularly Sp1 and STAT1, that contribute to PKCe overexpression in breast cancer. Furthermore, we identified a self-controlled mechanism that significantly contributes to the up-regulation of PKCe in breast cancer cells.

EXPERIMENTAL PROCEDURES

Cell Culture—Mammary (MCF-10A, MCF-7, T-47D, BT-474, HCC-1419, MDA-MB-231, MDA-MB-453, and MDA-MB-468), prostate (RWPE-1, LNCaP, C2, C2-4, DU145, and PC3), and lung (HBE, H358, H1975, H1650, HCC827, PHC9, H4006, H460, and A549) cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). PC3-ML cells were a kind gift of Dr. Alessandro Fatatis (Drexel University). Breast cancer cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 medium supplemented with 10% FBS, 1-glutamine (500 μM), and penicillin/streptomycin (100 units/100 μg/ml). Normal immortalized MCF-10A, HBE, and RWPE-1 cell lines were cultured as described previously (18, 27). All cells were grown at 37 °C in a humidified 5% CO₂ incubator.

Reagents—The PKC inhibitor GF 109203X was purchased from Biomol (Plymouth Meeting, PA). Actinomycin D, mithramycin A, 5-aza-2’-deoxycytidine, and trichostatin A were obtained from Sigma.

Cloning of the Human PRKCe Promoter and Generation of Luciferase Reporter Constructs—All primers used for PCR were purchased from Integrated DNA Technologies (IDT, Coralville, IA). PRKCe promoter truncated fragments (−1933/−219, −1416/+219, −808/+219, −531/+219, −401/+219, −320/+219, and −105/+219) were amplified by PCR from human genomic DNA prepared from T-47D cells using BglII and NheI-flanked following primers and subcloned into the pGL3 vector. The pGL3−1416/+219 vector was used as a template to generate a series of pPRKCe promoter truncated luciferase reporter vectors (−1319/+219, −1224/+219, −1121/+219, −1032/+219, −1028/+219, −921/+219, −877/+219, −873/+219, −619/+219, −796/+219, and −777/+219) with the Erase-a-Base kit (Promega, Madison, WI). pGL3−644/+219 was generated by digestion of pGL3−808/+219 vector with PfIMI and NheI and subsequent religation. All constructs were verified by DNA sequencing.

Site-directed mutagenesis—For PCR-based mutagenesis, we used the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). pGL3−921/+219 was used as a template to generate deletional mutations of STAT1 sites using the following primers: 1) CTATCGATCTCATTTCATGTTGCTCCCC (forward) and GGGAGCAATAACGAAAGTGTGAGATCGATA (reverse); 2) GGCAAAACCTTCTATCCCAACACATCGCC (forward) and CCAGTGTTTGGGATGAGAACTTTTGGC (reverse); 3) GACGTCTTCCTGCGACCTGCTTGGAGGAG (forward) and CTCCTCTTAATCGAGATGGAAGCG (reverse) and CTCCCTCTACATGGGAACTCCGAG (reverse); and 5) CTCGCAGAGATCGATGCTCGGAATACCGGGAGTTATGTTGCC (forward) and GGAACATATAACCCCAGAGTGATCGCTCAGG (reverse). All mutant constructs were confirmed by DNA sequencing.

Transient Transfection and Luciferase Assays—Cells in 12-well plates (~2 × 10⁵ cells/well) were co-transfected with 450 ng of a PRKCe promoter Firefly luciferase reporter vector and 50 ng of the Renilla luciferase expression vector (pRL-TK) using Lipofectamine 2000 (Invitrogen) or X-tremeGENEHP DNA transfection reagent (Roche Applied Science). After 48 h, cells were lysed with passive lysis buffer (Promega, Madison, WI). Luciferase activity was determined in cell extracts using the Dual-Luciferase™ reporter assay kit (Promega). Data were expressed as the ratio between Firefly and Renilla luciferase activities. In each experiment, the pGL3−positive control vector (Promega) was used as a control. Promoter activity of each PRKCe promoter luciferase reporter construct was expressed as follows: (Firefly (sample)/Renilla (sample))/([Firefly (positive)/Renilla (positive)]) × 100%.

Western Blot—Western blot analysis was carried out essentially as described previously (28). Bands were visualized by the ECL Western blotting detection system. Images were captured using a FujiFilm LAS-3000 system. The following antibodies were used: anti-PKCε and anti-Sp1 (1:1000, Santa Cruz Bio-technology Inc., Santa Cruz, CA); anti-STAT1 and anti-phospho-STAT1 (Ser-727) (1:1000, Cell Signaling Technology Inc., Danvers, MA); and anti-vinculin and anti-β-actin (1:50,000,
were expressed as (2) for the analysis of presence of CpG islands in the human PRKCE genes. PKC
TaqMan reverse transcription reagent kit (Applied Biosystems). cDNA was synthesized using the TRIzol (Invitrogen). cDNA was synthesized using the sequence detection system software version 1.7 for the analysis of mRNA expression after demethylation, MCF-10A cells were treated with different concentrations (1–100 μM) of 5-aza-2’-deoxycytidine (96 h or 7 days) and/or trichostatin A (100 ng/ml, 24 h). Total mRNA was extracted, and PKCe mRNA levels were determined by qPCR as described above.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as described elsewhere (18). Briefly, nuclear and cytosolic fractions were obtained after cell lysis using the NE-PER nuclear protein extraction kit (Pierce). The following probes were used: STAT1-2 oligonucleotide probes (sense 5’-AGCTTTTCTATTTCCAAAAACCTGCGG-3’ and antisense, 5’-AATCTCGGAGATGTTCGGGAAATAGAAA-3’); Sp1-2 oligonucleotide probe (sense 5’-AGCTTACGCCGGGAGGGGGCGGC-3’ and antisense, 5’-AATTCCGCGGCGGACC-3’); STAT1 consensus probe (sense, 5’-AGCTTTCATGTATGCATATTCTGTAGAAGGTTTGGGGAAATAGAAA-3’); Sp1 consensus probe (sense, 5’-AGCTTACGCCGGGAGGGGGCGGC-3’ and antisense, 5’-AATTCCGCGGCGGACC-3’). Probes were labeled with [α-32P]deoxyadenosine triphosphate using Klenow enzyme and purified on a Sephadex G-25 column. The binding reaction was carried out at 25 °C for 10 min with or without nuclear proteins (5 μg), poly(dI-dC) (1 μg), and labeled probe (106 cpm) in 20 μl of binding buffer (10X buffer: 100 mM Tris-HCl, pH 7.5, 500 mM NaCl, 50 mM MgCl₂, 100 mM EDTA, 10 mM DTT, 1% Triton X-100, and 50% glycerol). Binding specificity was confirmed by cold competition with 50-fold molar excess of cold STAT1 or Sp1 oligonucleotides. Cold AP-1 oligonucleotides (AP-1 sense 5’-AGCTTCTGGCTGTACTCAGCCGAA-3’ and antisense 5’-AATCTCGGCGGACC-3’) were used as negative controls. DNA–protein complexes were separated on a 6% nondenaturing polyacrylamide gel at 200 V. The gel was fixed and dried, and DNA–protein complexes were visualized by autoradiography.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed essentially as described previously (30). Briefly, 2 × 10⁶ cells were fixed in 1% formaldehyde for 15 min to cross-link DNA with associated proteins. The cross-linking reaction was termination by the addition of 125 mM glycine, and cells were then washed and harvested in PBS containing protease/phosphatase inhibitors. The pelleted cells were lysed on ice in a buffer containing 50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, and protease/phosphatase inhibitors. Cells were sonicated for 10 s (six times). DNA was fragmented in a range of 200–1000 bp. Equal amounts of chromatin were diluted in ChIP buffer (16.7 mM Tris-HCl, pH 8.1, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, and 167 mM NaCl) and incubated overnight at 4 °C with anti-STAT1 or anti-Sp1 antibodies (Abcam, Cambridge, MA) or control rabbit IgG (Cell Signaling Inc.), followed by 1 h of incubation with salmon sperm DNA/protein A-agarose beads. 10% of the sample was kept as input. Protein A-agarose beads were washed sequentially with a low salt buffer (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 150 mM NaCl), a high salt buffer (20 mM Tris-HCl, pH 8.1, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, and 500 mM NaCl), LiCl wash buffer (10 mM Tris-HCl, pH 8.1, 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA), and TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). Protein-DNA complexes were eluted in a buffer containing 1% SDS and 0.1 mM NaHCO₃. Cross-linking was reversed with 0.1 M Tris HCl, pH 8.0, and 1 M NaCl. The eluted DNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and was used as a template for PCR amplification using the TaqMan reverse transcription reagent kit (Applied Biosystems).
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mm NaCl overnight at 65 °C, followed by incubation in a buffer containing 40 mM Tris-HCl, pH 6.5, 10 mM EDTA, and 20 μg of proteinase K for 2 h at 55 °C. DNA was then extracted with QiAquick PCR purification kit (Qiagen) and analyzed by PCR. For STAT1-2/3 sites −880/−869 and −793/−782 bp, we used primers 5’-GGAGACCTGCTGGAGGTCTTC (forward) and 5’-GTCCGTGTTGCAGGCTCCTGC (reverse). For Sp1-5 site −347/−338 bp, we used primers 5’-GCGAGAAGAGGATCC (forward) and 5’-GGTGTGCGAAGAGGATCC (reverse). For Sp1-2 site −269/−260 bp and −256/−247 bp, we used primers 5’-GCCAGCTCTCCACCGTTGTC (forward) and 5’-GGTGTGCGCAGGATCC (reverse).

Cell Migration Assay—Cell migration was determined with a Boyden chamber, as described previously (31). Briefly, MCF-7 cells (3 × 10⁴ cells/well) were seeded in the upper compartment of a Boyden chamber (Neuro Probe). A 12-μm pore polycarbonate filter (Neuro Probe) coated overnight with type IV collagen in cold PBS was used to separate the upper and lower compartments. In the lower chamber, 0.1% BSA/DMEM with or without FBS (5%) was used. After 24 h of incubation at 37 °C, nonmigrating cells on the upper side of the membrane were wiped off the surface, and migrating cells on the lower side of the membrane were fixed, stained with Diff Quik Stain Set (Dade Behring), and counted by contrast microscopy in five independent fields.

Statistical Analysis—Results are the means ± S.E. of at least three individual experiments. Student's t test was used for statistical comparison. A p value < 0.05 was considered statistically significant.

RESULTS

Overexpression of PKCe in Breast Cancer Cells and Initial Characterization of the PRKCE Promoter—PKCe, a kinase broadly implicated in tumorigenesis and metastasis, is overexpressed in multiple cancers. Elevated PKCe levels have been associated with poor outcome in prostate, breast, lung, and head and neck cancer (22, 24, 32, 33); however, the mechanisms behind the control of PKCe expression remain to be determined. A comparative analysis of PKCe protein levels by Western blot shows that this kinase is overexpressed in multiple breast cancer cell lines (MCF-7, T-47D, BT-474, HCC-1419, MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells) relative to MCF-10A cells, an immortalized nontumorigenic mammary cell line (Fig. 1A). qPCR assays also revealed significantly higher PKCe mRNA levels in breast cancer cells compared with MCF-10A cells (Fig. 1B). To determine whether overexpression of PKCe is associated with altered mRNA stability, we assessed mRNA levels at different times after treatment with the transcriptional inhibitor actinomycin D. As shown in Fig. 1C, the decay in mRNA levels is essentially the same in breast cancer cell lines (MCF-7, T-47D, and MDA-MB-453) and MCF-10A cells. Thus, the differential expression of PKCe may involve a dysregulation of transcriptional mechanisms. Likewise, and in agreement with previous studies (18, 27), PKCe is overexpressed in lung and prostate cancer cell lines relative to corresponding normal “nontumored” cell lines (Fig. 1A).

To investigate the transcriptional mechanisms involved in PKCe expression, we cloned a 2.1-kb fragment of the human PKCe gene from genomic DNA using PCR. This fragment includes 1933 bp of the putative PRKCE promoter as well as 219 bp after the putative transcription start site. We also cloned four fragments encompassing shorter regions of the putative PRKCE promoter (1416/+ 219 bp, 808/+ 219 bp, 320/+ 219, and 105/+ 219 bp, respectively). The different DNA fragments were subcloned into the pGL3-enhancer luciferase reporter vector to generate the plasmids pGL3−1933/+219, pGL3−1416/+219, pGL3−808/+219, pGL3−320/+219, and pGL3−105/+219. Plasmids were transiently transfected into MCF-7 breast cancer cells along with pRL-TK (Renilla luciferase) vector for normalization of transfection efficiency. The pGL3−1416/+219 reporter construct exhibited the highest luciferase activity, which was ~40 times higher than pGL3-enhancer empty vector, therefore confirming that it possesses functional PRKCE promoter activity. A progressive loss in luciferase activity was observed upon deletions of fragments −1416/−809, −1416/−321, and −1416/−106. A significant loss of promoter activity was also observed with pGL3−1933/+219, suggesting repressive transcriptional elements within the −1933/−1417 bp region (Fig. 1D). A comparison of PRKCE promoter activity in different cell lines using pGL3−1416/+219 revealed a manifest elevation in luciferase activity in breast cancer cells relative to normal immortalized MCF-10A cells. Similarly, lung and prostate cancer cell lines exhibited higher promoter activity than the corresponding nontumorigenic counterparts (Fig. 1E).

A comparative analysis of PRKCE gene expression in 48 breast cancer cell lines (24 luminal-like and 24 basal-like) obtained from three independent studies (GSE10843, GSE12777, and GSE41445) was performed using inSilicoDb and inSilicoMerging R/Bioconductor packages (29). This analysis showed no statistically significant differences between luminal and basal-like breast cancer cell lines (p = 0.673) (Fig. 1F).

Differential Expression of PKCe Is Not Related to Promoter Methylation—It is well established that epigenetic mechanisms control the expression of key oncogenic and tumor-suppressing proteins. To determine whether methylation of the PRKCE promoter could be implicated in the differential expression between normal mammary and breast cancer cells, we first examined if the promoter was rich in CpG islands using the Methyl Primer Express software (Applied Biosystems). This analysis revealed two regions in the PRKCE promoter that were very rich in CpG islands, a proximal region between −2.6 and +0.9 kb and a distal region between −8.9 and −7.7 kb (Fig. 2A). To determine whether the reduced PKCe expression in MCF-10A cells could be due to promoter methylation, we used the demethylating agent 5-aza-2‘-deoxycytidine (AZA). qPCR analysis revealed that PKCe mRNA levels remain essentially unchanged in MCF-10A cells treated with different concentrations of AZA, either in the presence or absence of the HDAC inhibitor trichostatin A (Fig. 2B). A similar treatment in MCF-10A cells caused a significant rescue in the expression of the oncogenic protein P-Rex1, a gene that is regulated by methyla-
Therefore, overexpression of PKCe in breast cancer cells does not seem to be related to demethylation of the PRKCE gene promoter.

**Identification of Key Transcriptional Regions in the Human PKCe Promoter**—To characterize the human PRKCE promoter in more detail and to identify positive regulatory elements responsible for transcriptional activation, a series of 5'-uni-directional deletions was generated from the pGL3-1416/+219 luciferase reporter vector using the Erase-a-Base system. The resulting constructs were transfected into MCF-7 cells, and luciferase activity was determined. Fig. 3 shows that promoter activities of pGL3-1319/+219, pGL3-1224/+219, pGL3-1121/+219, pGL3-1032/+219, pGL3-1028/+219, and pGL3-921/+219 constructs were essentially similar to that of pGL3-1416/+219. However, a significant...
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\( \text{bp} \ -9000 \ )

\begin{figure}[h]
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\caption{Deletional analysis of the human PRKCE promoter. MCF-7 cells were co-transfected with pGL3 vectors coding different PKCe promoter fragments generated with the Erase-a-Base kit (Promega) and pRL-TK plasmid. Luciferase activity was measured 48 h later. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave similar results.}
\end{figure}

reduction in transcriptional activity was observed upon serial deletions starting from \( \text{bp} \ -887 \). Indeed, pGL3−887/+219, pGL3−873/+219, and pGL3−819/+219 display 77, 58, and 37% activity, respectively, compared with that of pGL3−1416/+219. No additional changes in reporter activity were observed with pGL3−808/+219. Constructs pGL3−796/+219 and pGL3−777/+219 display slightly lower luciferase activity than pGL3−808/+219. Luciferase activity drops significantly with constructs pGL3−320/+219 (91% reduction) and pGL3−105/+219 (98% reduction). To summarize these initial observations, the deletional analysis delineated two prominent regions in the PRKCE promoter containing positive regulatory elements that we defined as region A (−777 to −105 bp) and region B (−921 to −796 bp). In subsequent sections, a more detailed characterization of the cis-acting elements in these two regions will be shown.

Analysis of Region A Revealed a Crucial Role for Sp1 in PKCe Transcription—To identify putative transcriptional elements in region A of the PRKCE promoter, we initially used the PROMO software. This analysis revealed the presence of seven putative Sp1-responsive elements that we named Sp1-1 (the most distal site, \( \text{bp} \ -716 \) to −707) to Sp1-7 (the most proximal site, \( \text{bp} \ -256 \) to −247) (Fig. 4A, left panel). The putative Sp1-binding sequences are shown in Fig. 4A, right panel. To define the relevance of the different Sp1-binding sites, additional truncated mutants for region A were generated using pGL3−777/+219 as a template (pGL3−644/+219, pGL3−531/+219, and pGL3−401/+219), and we examined for their luciferase activity upon transfection into MCF-7 cells. Fig. 4B shows that deletion of region comprising \( \text{bp} \ -777 \) to −664 (which includes Sp1-1 and Sp1-2 sites) caused a 65% drop in luciferase activity. No additional changes in reporter activity were observed upon deletions of regions comprising \( \text{bp} \ -644/-532, -644/-402, \) and −644/−321, which include sites Sp1-3, Sp1-4, and Sp1-5. However, when fragment −320/−105 (which includes Sp1-6 and Sp1-7) was deleted, an additional reduction in luciferase activity was observed. These results suggest that multiple Sp1 sites in region A contribute to the transcriptional activity of the PRKCE promoter.
To further determine the contribution of the different Sp1 sites in the transcriptional activation of the PRKCE promoter, we performed site-directed mutagenesis of these sites in the context of the pGL3−777/+219 construct. Essential residues GGCG in Sp1 sites were mutated to TTAT, and luciferase activities of the corresponding constructs were determined after transfection into MCF-7 cells. As shown in Fig. 4C, mutation of Sp1-1 in pGL−777/+219 had no effect; however, mutation of Sp1-2 caused a 62% reduction in reporter activity. Sp1-6 and Sp1-7 were only 4 bp apart, and therefore we decided to mutate them together. When we mutated Sp1-6/7 in pGL3−777/+219, a significant reduction (50%) in luciferase activity was observed. We further mutated Sp1-6/7 sites in pGL3−320/+219, and observed a significant reduction in reporter activity.

FIGURE 4. Sp1 elements in region A of the PRKCE promoter control its transcriptional activity. A, schematic representation of putative Sp1 sites (black boxes) in the PRKCE gene promoter. Seven putative Sp1-binding sites (Sp1-1 through Sp1-7) were identified (left panel). The corresponding sequences are shown (right panel). TSS, putative transcription starting site; ATG, start codon. B, deletion analysis of region A. Luciferase (Luc) activity of truncated constructs was determined 48 h after transfection into MCF-7 cells. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave similar results. *, p < 0.05; **, p < 0.01 versus control vector. C, schematic representation of mutated PRKCE promoter reporter constructs. The nonmutated Sp1 sites are indicated with black square boxes, and the mutated sites are marked with X on the black box. Luciferase activity of truncated constructs was determined 48 h after transfection into MCF-7 cells. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave similar results. *, p < 0.05 versus wild-type vector. D, MCF-7 cells were transfected with pGL3−777/+219 or pGL3−320/+219 reporter vectors and 24 h later treated with the Sp1 inhibitormithramycin A (MTM, 100 nM) or vehicle for 16 h. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave similar results. *, p < 0.05, **, p < 0.01 versus control E, ChIP assay. Upper panel, ChIP assay for Sp1-2 sites (fragment comprising bp −668/+659). Middle panel, ChIP assay for Sp1-5 site (fragment comprising bp −347/+338). Lower panel, ChIP assay for Sp1-6/7 sites (fragment comprising bp −269/+260 and bp −256/+247). F, MCF-7, T-47D, MDA-MB-231, and BT-474 cells were transiently transfected with Sp1 or nontarget control (NTC) RNAi duplexes. PKCe expression was determined by Western blot after 72 h. G, PKCe mRNA expression was determined by qPCR 72 h after transfection with either Sp1 or nontarget control RNAi duplexes. Data are expressed as fold-change relative to nontarget control and represent the mean ± S.D. of triplicate samples. *, p < 0.05 versus control. Similar results were observed in two independent experiments.
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compared with the wild-type pGL3–320/+219 construct. However, it did not reach complete inhibition, thus arguing for the presence of other relevant transcriptional element(s) within the −320/−105 region that remain to be identified. The dele- tional and mutational analyses of region A indicate that multi plple Sp1 sites control the transcriptional activation of the PRKCE promoter.

To confirm the relevance of the Sp1-binding sites in tran- scriptional activation of the PRKCE gene, we used a number of additional approaches. First, we examined the effect of mithra mycin A (MTM), an agent that prevents binding of Sp1 to its transcription binding site (34, 35). As shown in Fig. 4D, MTM markedly reduced luciferase activity of reporters pGL3−777/+219 and pGL3−320/+219. As a second approach, and to address whether Sp1 proteins associate with the PRKCE pro mter in vivo, we performed a chromatin immunoprecipitation (ChIP) assay using an anti-Sp1 antibody. As a negative control, we used IgG. Three sets of primers were utilized in these exper iments as follows: one encompassing bp −772 to −615 (for site Sp1-2); a second encompassing bp −320 to −186 (for Sp1-6 and Sp1-7), and a third for bp −443 to −286 (for site Sp1-5). Sp1 immunoprecipitation revealed the expected bands for regions −772/−615 and −320/−186, and no band was observed for region −443/−286 (Fig. 4E). Thus, the Sp1 trans cription factor binds in vivo to the sites identified in our dele tional/mutational analysis. Finally, to confirm the involvement of Sp1, we knocked down this transcription factor using RNAi. Sp1 RNAi depletion from MCF-7, T-47D, MDA-MB-231, and BT-474 breast cancer cell lines significantly reduced the expression of PKCe protein (Fig. 4F) and PKCe mRNA, as determined by qPCR (Fig. 4G). Altogether, these results demon strate the relevance of Sp1 in transcriptional activation of the PRKCE promoter.

STAT1-binding Sites in Region B Control PKCe Transcriptional Activation—As established in the deletional analysis shown in Fig. 3, region B located between bp −921 and −796 plays a positive role in transcriptional activation of the PRKCE promoter. Analysis using the PROMO program revealed two putative STAT1 sites in this region, which we named STAT1-1 (−916 to −905 bp) and STAT1-2 (−880 to −869 bp). There is also a third STAT1 site (STAT1-3) at the edge of region B (−793 to −782 bp) (Fig. 5A). To determine the potential rele vance of these sites, essential residues TTTC in STAT1 sites were mutated to T→C in pGL3−921/+219. The resulting mut ant constructs were transfected into MCF-7 cells and assessed for their luciferase reporter activity. As shown in Fig. 5B, mutation of the most distal STAT1 site (STAT1-1) had no significant effect on luciferase activity. Conversely, mutation of STAT1-2 site caused a 44% reduction in reporter activity. A slight, yet statistically significant reduction in luciferase activity was observed upon mutation of the STAT1-3 site. A double mutant for STAT1-2 and STAT1-3 sites was generated, and its activity was examined in MCF-7 cells, which revealed a 61% reduction in luciferase activity compared with the pGL3−921/+219 construct. Therefore, the STAT1-2 and STAT1-3 sites are involved in the regulation of PKCe promoter activity.

The program PROMO also identified two additional STAT1 sites outside region B, which were named STAT1-4 (−401 to −390 bp) and STAT-5 (−227 to −216 bp). These two sites were actually located within the region A and in close proximity to Sp1 sites (Fig. 5A). We mutated STAT1-4 and STAT1-5 sites and found these mutations do not alter reporter activity (Fig. 5B), suggesting that only STAT1-2 and STAT1-3 sites are involved in transcriptional control of the PRKCE promoter in breast cancer cells.

Next, to confirm the relevance of STAT1 in the control of PKCe transcriptional activity, we used RNAi (Fig. 5C). MCF-7 cells were transfected with a STAT1 SMARTpool®RNAi, which caused >90% depletion in STAT1 levels (Fig. 5C, inset), or a SMARTpool® control RNAi and then transfected with the pGL3−921/+219 luciferase reporter vector. As expected from the deletional and mutational analyses, silencing STAT1 inhib ited transcriptional activity of the PKCe reporter (54% reduc tion, which is in the same range as the reduction in activity observed upon mutation of STAT1-2 and STAT1-3 sites combined, see Fig. 5B). Moreover, when we assessed the activity of the STAT1-2/3-mutated pGL3−921/+219 construct, STAT1 RNAi depletion failed to cause an additional reduction in luciferase activity (Fig. 5C), thus confirming the importance of STAT1-2 and STAT1-3 sites in the control of PRKCE promoter activity. To further confirm the relevance of the STAT1 sites, we used ChIP. For this analysis, we used a set of primers encompassing −949 to −751 bp in the PRKCE promoter, a region that includes both STAT1-2- and STAT1-3-binding sites. Results shown in Fig. 5D revealed a band of the expected size (199 bp) when an anti-STAT1 antibody was used in the immunoprecipitation, whereas no band was observed using control IgG, thus suggesting direct binding of STAT1 to the −949 to −751-bp promoter region. Furthermore, STAT1 RNAi depletion from MCF-7 cells caused a significant reduction in PKCe mRNA (Fig. 5E) and protein levels (Fig. 5F). Altogether, these results indicate that STAT1-2- and STAT1-3-binding sites are involved in the transcriptional control of the PRKCE promoter. An additive effect between STAT1 RNAi depletion and MTM treatment was observed (Fig. 5F).

STAT1 and Sp1 Contribute to the Elevated PKCe Transcriptional Activity in Breast Cancer Cells—Once we identified relevant Sp1 and STAT1 sites in the PRKCE promoter, we asked if these sites mediate PKCe up-regulation in breast cancer cells relative to non-tumorigenic mammary cells. To address this issue, we compared the activities of the different deleted report ers between MCF-7 versus MCF-10A cells. As shown previ ously in Fig. 1E with reporter pGL3−1416/+219, activity of pGL3−921/+219 reporter was also higher in MCF-7 cells relative to MCF-10A cells (Fig. 6A). Deletion of fragment −921 to −777 bp, which includes STAT1-2/3 sites in region B, diminished luciferase activity in MCF-7 cells by 61%, an effect that was not seen in MCF-10A cells (Fig. 6A and B). To verify the relevance of the STAT1 sites in PKCe up-regulation in breast cancer cells, we compared the activity of pGL3−921/+219 (wild type) versus pGL3−921/+219 (STAT1-2/3-mutated) in MCF-7 and MCF-10A cells. Whereas mutation of STAT1-2 and STAT1-3 sites failed to reduce reporter activity in MCF-10A cells, a marked reduction in activity (~70% reduction) was observed in MCF-7 cells (Fig. 6C) as well as in T-47D cells (data not shown). To validate the relevance of the STAT1-2/3 sites in
PKCε up-regulation, we used an EMSA approach. Nuclear extracts from MCF-10A, MCF-7, or T-47D cells were incubated with 25-bp double-stranded radiolabeled probes for either the STAT1-2 site or a standard STAT1 binding consensus. As shown in Fig. 6D, a shift protein-DNA complex band was detected after incubation of nuclear extracts from either probe both in MCF-7 (lanes 3 and 6) and T-47D cells (lanes 4 and 7). However, this effect was not seen in nontumorigenic MCF-10A cells (Fig. 6D, lanes 2 and 5). The shift band was competed by co-incubation with an excess (50-fold molar) of...
unlabeled probes for either STAT1-2 (Fig. 6D, lane 8) or a standard STAT1-binding consensus sequence (lane 9) but not with an excess unlabeled probe for AP-1 (lane 10), thereby confirming the specificity of the interaction. A similar result was observed using a probe for site STAT1-3 (data not shown). Thus, STAT1-2/3 sites contribute to the up-regulation of PKC\(\text{e}\) transcriptional activity in breast cancer cells.

Next, we carried out similar experiments to determine whether the Sp1-2 site was implicated in PKC\(\text{e}\) up-regulation in breast cancer cells relative to nontumorigenic MCF-10A cells. As shown in Fig. 6A, deletion of fragment \(-777\) to \(-531\) bp, which includes relevant Sp1-2 site in region A (position \(-668\) to \(-659\)), reduced luciferase reporter activity in MCF-7 cells but not in MCF-10A cells. No additional changes were found upon deletion of region \(-531\) to \(-320\) bp in either cell line. To verify the relevance of the Sp1-2 site in PKC\(\text{e}\) up-regulation in breast cancer cells, we compared the activity of pGL3\(-777/\text{+219}\) (wild type) versus pGL3\(-777/\text{+219}\) (Sp1-2-mutated) in MCF-7 and MCF-10A cells. Fig. 7A shows that mutation of Sp1-2 significantly reduced luciferase activity in MCF-7 cells, whereas this mutation had no effect in MCF-10A cells. As expected, mutation of the Sp1-1 site, which was dispensable for transcriptional activity (see Fig. 4C), did not alter reporter activity in MCF-7 or MCF-10A cells. To further verify the relevance of the Sp1-2 site in PKC\(\text{e}\) up-regulation in breast cancer, we used an EMSA approach. Nuclear extracts from MCF-10A, MCF-7, or T-47D cells were incubated with radiolabeled probes for either the Sp1-2 site or a standard Sp1 binding consensus. As shown in Fig. 7B, a shift protein-DNA complex band was detected after incubation of nuclear extracts from either probe both in MCF-7 (lanes 3 and 6) and T-47D cells (lanes 4 and 7) but not in nontumorigenic MCF-10A cells (lanes 2 and 5). The specificity of the interaction was confirmed by competition of the shift band with an excess (50-fold molar) of unlabeled probes for either Sp1-2 (Fig. 7B, lane 8) or a standard Sp1 binding consensus (lane 9) but not with an unlabeled probe for AP-1 (lane 10).

We also found that deletion of fragment \(-320\) to \(-105\) bp, which comprises proximal Sp1-binding sites (Sp1-6-7), essentially abolished luciferase activity both in MCF-7 and MCF-10A...
PKCe Controls Its Own Expression in Breast Cancer Cells—

There is evidence that PKCe controls the phosphorylation status and activity of STAT1 in several cellular models (36–38). Ser-727 phosphorylation in STAT1 is required for its maximal transcriptional activity (39). Likewise, we found that PKCe controls the activation status of STAT1 in breast cancer cells, as judged by the reduction in phospho-Ser-727-STAT1 levels upon PKCe depletion in MCF-7, T-47D, MDA-MD-231, MDA-MB-453, and MDA-MB-468 breast cancer cell lines (Fig. 8A). Similar results were observed in prostate and lung cancer models (data not shown). Treatment of MCF-7 cells with the pan-PKC inhibitor GF 109203X or the specific PKCe inhibitor εV1-2 also reduced phospho-Ser727-STAT1 levels (Fig. 8B). Given our finding that STAT1 transcriptionally regulates PKCe expression, we speculated that PKCe controls its own expression via STAT1. Treatment of MCF-7 cells with εV1-2 (Fig. 8C) or GF 109203X (data not shown) significantly reduced pGL3−320/+219 luciferase reporter activity. To examine the potential involvement of PKCe in controlling its own promoter activity, we used PKCe RNAi. PKCe expression was silenced from MCF-7 cells by 90% upon delivery of two different PKCe RNAi duplexes (ε1 and ε2), as we did previously in other models (18, 25). Notably, luciferase activity of the pGL3−320/+219 reporter was significantly decreased in PKCe-depleted MCF-7 cells (Fig. 8D), indicating that the elevated levels of PKCe in breast cancer cells positively control its own expression at a transcriptional level. The results described above argue for a mutual dependence between PKCe expres-

FIGURE 7. Contribution of Sp1-2 site to PKCe overexpression in breast cancer cells. A, mutation of Sp1-2 site decreases PKCe promoter activity in MCF-7 breast cancer cells but not in MCF-10A cells. Luciferase activity of pGL3−777/+219 (wild-type, Sp1-1 site mutant, or Sp1-2 site mutant) was determined 48 h after transfection. Data are expressed as mean ± S.E. of three individual experiments. Luciferase activity of wild-type pGL3−777/+219 construct was set as 1. **, p < 0.01 versus pGL3−777/+219 (WT). B, elevated Sp1-DNA binding activity in MCF-7 and T-47D breast cancer cells, as determined by EMSA. Similar results were observed in two independent experiments. C, mutation of Sp1-6/7 sites reduces PRKCE promoter activity both in MCF-7 and MCF-10A cells. Luciferase activity of pGL3−320/+219 (wild-type or Sp1-6/7 sites mutant) was determined 48 h after transfection. Data are expressed as mean ± S.E. of three individual experiments. Luciferase activity of wild-type pGL3−320/+219 construct was set as 1. **, p < 0.01 versus pGL3−320/+219 (wt).
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FIGURE 8. Correlation between PKCe expression levels and STAT1 activation status. A, PKCe RNAi depletion reduces phospho-Ser-727-STAT1 levels in breast cancer cell lines. MCF-7, T-47D, MDA-MB-231, MDA-MB-453, and MDA-MB-468 cells were transiently transfected with PKCe (1 or 2) or nontarget control (NTC) RNAi duplexes. After 72 h, levels of phospho-Ser-727-STAT1 and total STAT1 were determined by Western blot. A second experiment gave similar results. *, $p < 0.05$; **, $p < 0.01$ versus control. C, inhibition of pGL3–1416/+219 reporter activity in MCF-7 cells by εV1-2 (1 μM, 24 h). Luciferase activity of construct pGL3–1416/+219 was determined 48 h after transfection. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave same results. *, $p < 0.05$ versus control. D, inhibition of pGL3–1416/+219 reporter activity by PKCe RNAi. MCF-7 cells were transiently transfected with PKCe (1 or 2) or nontarget control RNAi duplexes. After 24 h, pGL3–1416/+219 was transiently transfected into MCF-7 cells along with the pRL-TK Renilla luciferase vector. Luciferase activity was determined 48 h later. Data are expressed as mean ± S.D. of triplicate samples. Two additional experiments gave same results. *, $p < 0.05$ versus control. Insert, PKCe expression, as determined by Western blot. E, PKCe and phospho-Ser-727-STAT1 levels in mammary cell lines, as determined by Western blot. Similar results were observed in three independent experiments. F, correlation between expression levels of PKCe and phospho-Ser-727-STAT1 levels in mammary cell lines.

expression and STAT1 activation. We decided to formally test this hypothesis in mammary cellular models (Fig. 8E). We observed that normal immortalized MCF-10A cells, which express low PKCe levels, display low levels of phospho-Ser-727-STAT1. Conversely, breast cancer cell lines with very high PKCe levels (MCF-7, T-47D, MDA-MB-231, MDA-MB-453, and MDA-MB-468) show high levels of phospho-Ser-727-STAT1. Breast cancer cell lines with intermediate PKCe levels (BT-474 and HCC-1419) show intermediate phospho-Ser-727-STAT1 signals by Western blot. Upon densitometric quantification of Western blots, we found a strong correlation between PKCe and phospho-Ser-727-STAT1 levels ($R^2 = 0.90$) (Fig. 8F). Altogether, these results argue for a positive feedback between PKCe expression and STAT1 activation in breast cancer cells.

PKCe Mediates Migration of Breast Cancer Cells—PKCe has been implicated in tumor initiation, progression, and metastasis (22, 25, 27). Fig. 9A shows that PKCe RNAi depletion significantly reduced the motility of cells in response to 5% FBS, as determined with a Boyden chamber. The Sp1 inhibitor MTM, which significantly reduces PKCe expression (Fig. 9B, see also
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PKCe, a member of the novel PKCs, has been extensively characterized as a mitogenic/survival kinase that activates pathways linked to malignant transformation and metastasis, including Ras/Raf/Erk, PI3K/Akt, and NF-kB (17, 18). Pharmacological inhibition or RNAi silencing of PKCe expression impairs the ability of cancer cells to form tumors in nude mice and metastasize to distant sites (22). Overexpression of PKCe in nontransformed cells confers growth/survival advantage or leads to malignant transformation (16). In an in vivo scenario, transgenic overexpression of PKCe in the mouse prostate leads to a neoplastic phenotype, and skin transgenic overexpression of this kinase leads to the development of metastatic squamous carcinoma (40). Therefore, there is significant evidence that overexpression of PKCe is causally associated with the development of a malignant and metastatic phenotype. This is highly relevant in the context of human cancer, as a vast majority of cancers displays PKCe up-regulation, including breast, prostate, and lung cancer (18, 22, 25). Increased PKCe expression in breast cancer correlates with high histological grade, positive ErbB2/Her2 status, and hormone-independent status (22). Despite the wealth of functional information regarding PKC and cancer, both in vitro and in vivo, as well as the established mechanistic links with proliferative pathways, the causes behind the up-regulation of PKCe in human cancer remained elusive.

In this study we report that PKCe up-regulation in breast cancer cells occurs through dysregulation of transcriptional mechanisms. An ~1.6-kb fragment of human genomic DNA encompassing the 5'-flanking region and part of the first exon (~1.4 to +0.2 kb) of the PRKCE gene was isolated and cloned into a luciferase reporter vector. This fragment displayed significantly higher transcriptional activity when expressed in breast cancer cells relative to normal immortalized MCF-10A cells. However, the elevated PKCe mRNA levels in breast cancer cells do not seem to be related to changes in mRNA stability. Our deletional and mutagenesis studies combined with in silico analysis identified key positive regulatory cis-acting Sp1 and STAT1 elements in two regions (regions A and B) that we defined as responsible for the up-regulation of PKCe transcriptional activation in breast cancer cells, and their functional relevance was confirmed by EMSA and ChIP. A region that negatively regulates transcription located upstream from the 1.6-kb fragment, specifically between −1.4 and −1.9 kb, was also identified. Studies to dissect and characterize these negative elements are underway.

From the seven putative Sp1-responsive elements located in region A of the PRKCE gene, only one located between bp −668 and −659 contributes to the differential overexpression of PKCe in MCF-7 cells. The two most proximal Sp1 sites located in positions −269/−260 and −256/−247 contribute to transcriptional activation of the PRKCE gene both in MCF-7 and MCF-10A cells, suggesting that these sites control basal expression both in normal and cancer cells. The Sp1 transcription factor has been widely implicated in cancer and is up-regulated in human tumors. For example, it has been reported that Sp1 protein and binding activity are elevated in human breast carcinoma (41, 42). Sp1 is highly expressed both in estrogen receptor-positive and -negative cell lines (43), and its depletion using RNAi leads to reduced G1/S progression of breast cancer cells (44). Sp1 controls the expression of genes implicated in breast tumorigenesis and metastatic dissemination, including ErbB2 (45), EGF receptor (46), IGF-IR (47, 48), VEGF (49, 50), cyclin D1 (51), and urokinase-type plasminogen activator receptor (42). The transcription factor Sp1 binds to GC-rich motifs in DNA, and DNA methylation of CpG islands can inhibit Sp1 binding to DNA (52–54). Nevertheless, our studies show that the demethylating agent AZA could not up-regulate PKCe mRNA levels in MCF-10A cells. Thus, despite the presence of CpG-rich regions in the PRKCE promoter, repression by methylation does not seem to take place in normal mammary cells. It is interesting that a recent study in ventricular myocytes showed PRKCE gene repression through methylation of Sp1 sites via reactive oxygen species in response to norepinephrine or hypoxia (55, 56), suggesting that epigenetic regulation of the PRKCE gene can take place in some cell types under specific...
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conditions. Notably, functional Sp1-binding sites have been identified in the promoters of PKCβ and PKCδ isoforms, and Sp1 binding to the PKCβ gene is repressed by hypermethylation and re-expressed by AZA treatment (57, 58).

The most notable characteristic of region B in the PKRCE promoter is the presence of three STAT1-binding sites. Two of those sites located in position −880/−869 and −793/−782 are functionally relevant in breast cancer cells. Indeed, a marked reduction (>50%) of promoter activity was observed upon mutation of these sites. Moreover, STAT1 RNAi caused a significant reduction in PKCe mRNA and protein levels. The elevated PKCe levels in breast cancer cell lines strongly correlate with the activation status of STAT1. Activation of STAT tran-
scription factors involves the phosphorylation of tyrosine resi-
dues either by JAK or independently of JAK by tyrosine kinase receptors such as EGF receptor (59). To date, the role of STAT1 in cancer progression remains controversial. Based on its canonical role in IFN-γ signaling and loss of function studies using STAT1 knockout mice, it has been postulated that STAT1 acts as a tumor suppressor (60). However, a large num-
ber of studies link STAT1 with tumor promotion as well as with resistance to chemotherapy and radiotherapy. Moreover, STAT1 is up-regulated and/or hyperactive in many cancers, including breast cancer (61, 62). STAT1 up-regulation in human breast cancer is associated with metastatic dissemination and poor outcome in patients (62–64). In addition, STAT1 overexpression has been linked to aggressive tumor growth and the induction of proinflammatory factors, whereas STAT1 knockdown delays tumor progression (61). Inhibition of STAT1 in breast cancer prevents the homing of suppressive immune cells to the tumor microenvironment and enables immune-mediated tumor rejection (61). ErbB receptor activa-
tion, a common event in human breast cancer, significantly enhances STAT1 expression (65). In other models, such as mel-
anoma, suppression of STAT1 expression reduces cell motility, invasion, and metastatic dissemination (66). STAT1 expression correlates with resistance to chemotherapeutic agents such as doxorubicin, docetaxel, and platinum compounds and is ele-
vated in resistant tumors (67–72). STAT1 also promotes radioresistance of breast cancer stem cells (73). Notably, PKCe has been linked to chemo- and radio-resistance (19, 20); thus, it is conceivable that PKCe up-regulation mediated by STAT1 may play a role in this context. The fact that PKCe controls its own expression in breast cancer cells suggests the possibility of a vicious cycle that contributes to the overexpression of this kinase. It is unclear at this stage what pathways are controlled by PKCe that lead to its own transcriptional activation. One possibility is that PKCe controls the expression of factors that influence STAT1 activation status, such as growth factors or cytokines that signal via this transcription factor.

In summary, this study identified relevant mechanisms that control PKCe expression in breast cancer cells. As PKCe over-
expression has been linked to an aggressive phenotype and metastatic dissemination, our study may have significant therapeutic implications. In this regard, several studies sug-
gested that targeting PKCe could be an effective anticancer strategy. Indeed, the PKCe translocation inhibitor eV1-2 has anti-tumorigenic activity in non-small cell lung cancer and head and neck squamous cell carcinoma models (25, 27). More recently, an ATP mimic inhibitor with selectivity for PKCe was shown to impair the growth of MDA-MB-231 breast cancer xenografts in mice as well as to reverse Ras-driven and epi-
thelial-mesenchymal transition-dependent phenotypes in breast cancer cells (26). Thus, targeting PKCe or the mecha-
nisms responsible for its up-regulation in tumors may provide novel means for the treatment of cancer types driven by PKCe overexpression.

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