Overexpression of PKCα is required to impart estradiol inhibition and tamoxifen-resistance in a T47D human breast cancer tumor model

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We previously reported that stable overexpression of PKCα in hormone responsive T47D:A18 breast cancer cells produces a hormone-independent/tamoxifen (TAM)-resistant and 17β-estradiol (E2)-inhibitory phenotype in vivo. Furthermore, overexpression of PKCα in T47D:A18 cells also results in cross-upregulation of PKCs β and δ. In this study, we further characterized the contribution of PKC isozymes α, β and δ to this complex phenotype. To determine whether down-regulation of PKCα is sufficient to restore the hormone-dependent phenotype in T47D:A18/PKCα cells, PKCα was selectively knocked down using short hairpin RNA (shRNA). To determine the contribution of PKCβ or δ to the hormone-resistant/TAM-resistant and E2-inhibitory phenotype, stable T47D:A18/PKCβ and T47D:A18/PKCδ clones were established. Downregulation of PKCα by shRNA in T47D:A18/PKCα20 cells also resulted in reduced PKCβ protein expression in vitro. Tumors established from a T47D:A18/PKCα/shRNA stable clone exhibit 50% reduction of PKCα protein without concomitant reduction in PKCβ, and exhibit partial reversal of the TAM-resistant and E2-inhibitory phenotype in vivo. Furthermore, stable overexpression of neither PKCβ nor PKCδ in T47D:A18 cells are sufficient to produce hormone-independent growth in vitro or in vivo, nor TAM-resistant and E2-inhibited growth in vivo. Taken together, these results suggest that PKCα is required to impart the TAM-resistant and E2-inhibitory phenotype in vivo.

Introduction

Tamoxifen (TAM) is an effective endocrine therapy in ~60% of all estrogen receptor alpha (ERα) positive/progesterone receptor positive breast cancers. The inhibitory effect of TAM is observed almost exclusively in breast tumors that are ERα positive because estrogen is the major growth stimulator for these types of tumors. However after prolonged antiestrogen hormonal therapy, breast cancer often progresses from an estrogen-sensitive state to an estrogen-insensitive state (1). Although the development of TAM resistance may be a consequence of ERα loss, TAM resistance most often occurs despite expression of ERα (2–4). In these cases, constitutive overexpression of autocrine growth factor or growth factor receptor by tumor cells has been proposed as one possible mechanism of TAM resistance (5–14). Increased autocrine or paracrine growth factor signaling networks bypass the classical ER-mediated signaling pathway in human breast cancer cells and result in failure of antiestrogen therapy. For example, preclinical and clinical studies have reported a decreased efficacy of TAM in tumors overexpressing c-ErbB2 (6,14–16) and PC cell-derived growth factor (PCDGF/GP88) (17). Activation of the ras/mitogen-activated protein kinase (MAPK/ERK1/2) pathway known to lie downstream of growth factor receptor signaling can cause ligand-independent activation of ERα (18–21). Protein kinase C (PKC) isozymes are known to modulate MAPK/ERK1/2 signaling pathways (22,23); PKC-mediated activation of MAPK/ERK1/2 can directly phosphorylate ER Ser-118 (18,21), a site normally phosphorylated in response to estrogen itself by a MAPK-independent mechanism (24). These studies provide additional mechanistic explanations for how signaling by growth factor receptors can result in hormone independence/TAM resistance.

PKC is a family of serine/threonine protein kinases, which mediate a multitude of effects regulating cellular proliferation and differentiation (25). The family consists of 12 isozymes, α, β, βII, γ, δ, ε, ξ, θ, η, μ and ν, which can be classified into three subgroups—conventional, novel and atypical (26). Conventional isozymes are activated by diacylglycerol (DAG), phorbol esters and calcium, and include isozymes α, β, βIII and γ, novel isozymes δ, ε, η, and θ are activated by DAG/phorbol esters but are calcium-independent and atypical isozymes including ξ and η, which can be unresponsive to DAG/phorbol esters and calcium. In addition to differences in their cofactor requirements, these isozymes also differ in tissue expression, sub-cellular localization, and substrate specificity. It is well documented that ER and PKC activity are inversely related in breast cancer cell lines (27) and PKC is elevated in malignant versus normal breast tissue (27–29).

We have shown previously that the ERα-negative/hormone-independent human breast cancer cell line T47D:C42 has elevated PKCα protein expression compared with the ERα-positive/hormone-dependent T47D:A18 clone (30). Stable overexpression of PKCα in T47D:A18 cells produces clones that are able to grow in the absence of hormonal stimulation and, thus, exhibit autonomous growth in vitro (30). Tumors derived from T47D:A18/PKCα clones also display autonomous growth, are TAM-resistant and growth inhibited by 17β-estradiol (E2) in vivo (31). Interestingly, stable overexpression of PKCα also results in increased endogenous expression of PKC isozymes β and δ (30). It was

Abbreviations: ERα, estrogen receptor alpha; NS-shRNA, non-silencing shRNA; PKC, protein kinase C; TAM, tamoxifen; shRNA, short hairpin RNA.
reported previously that stable transfection of PKCα results in upregulation of PKCβ in MCF-7 cells (32,33) and PKCδ in murine lymphoma and promyelocytic cells (34). To study the contribution of the PKCα, β and δ to the growth phenotype of T47D:A18/PKCα cells and tumors, two approaches were employed: (i) PKCα expression in the T47D:A18/PKCα20 clone was selectively knocked down by using short hairpin RNA (shRNA) technology and (ii) Stable PKCs β and δ clones were established in T47D:A18 and the hormone-responsive phenotypes of these clones were examined.

Materials and methods

Cell lines and culture conditions

T47D:A18 is a hormone-dependent human breast cancer cell clone that was described previously (35). T47D:A18/neo and T47D:A18/PKCα20 clones have been described previously (30). T47D:A18, T47D:A18/neo and T47D:A18/PKCα20 clones were maintained in RPMI-1640 phenol-red medium supplemented with 10% fetal bovine serum (FBS), T47D:A18/neo and T47D:A18/PKCα20 clones were also supplemented with G418 (500 \( \mu \)g/ml). Prior to cell proliferation assays, all cell lines were maintained in phenol-red-free RPMI-1640 supplemented with 10% dextran-coated charcoal (DCC)-treated FBS (estradiol-depleted media) for 3 days. Prior to transient transfection experiments for northern blots, all cell lines were grown in complete medium containing 10% FBS.

RNA isolation and northern blot

PKCs α, β and δ cDNAs were cloned in order to synthesize the antisense riboprobes. Total RNA was isolated from the T47D:A18 clones using the Triazol reagent (Invitrogen, Carlsbad, CA). PKCs α, β and δ cDNA fragments were obtained from total RNA by RT-PCR by using the following primers sets: PKCβ-forward (5'-CAGAACGTGAGTTGCAGCTGGG-3') and PKCβ-reverse (5'-GAGTTGGATCCATTGAGGCCTGGC-3') and PKCδ-forward (5'-GGCTCAATGTGATGGTGCTGGC-3') and PKCδ-reverse (5'-GATTTGCGACGATACTACGA-3'). The 1.3 kb EcoRI fragment of the PKCα gene was obtained by digestion of the PKCα clone (obtained from ATCC, Rockville, MD) with EcoRI. The resulting DNA fragments were cloned into the pGEM-T vector (Promega, Madison, WI) to obtain the PKC α, β and δ recombinant plasmids. After sequencing and determination of the insert orientation, the recombinant plasmids were used to synthesize digoxigenin-labeled antisense riboprobes by the RNA labeling kit according the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). For gene expression studies, total RNA (10–20 \( \mu \)g) was isolated from T47D cell lines and electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, transblotted onto nylon membranes using the TransBlot system (BioRad Laboratories, Hercules, CA) and fixed to the membrane by UV cross-linking. Hybridization was carried out at 66 °C for 16 h in 50% formamide hybridization solution containing 20 ng/ml RNA probe. Following hybridization, the membrane was washed twice at room temperature with 2x SSC buffer (30 mM sodium citrate (pH 7.0) and 300 mM NaCl) containing 0.1% SDS and twice at 68 °C with 0.5x SSC buffer containing 0.1% SDS. For detection, anti-DIG-AP antibody was used in conjunction with CDP-star chemiluminescent reagent. Bands were quantified by Scion image software (Scioncorp.com).

Construction of shRNA plasmids

The pSUPER vector, which directs the synthesis of shRNAs, was a gift from Dr Yin Mo (Southern Illinois University) (36). The shRNA sequences of human PKCα corresponded to the coding regions 492–510 relative to the first nucleotide of the start codon (37). The sense oligonucleotide sequence containing the human PKCα shRNA pair (underlined sequence) is as follows: 5'-GATTCCTCCAGGAAATACTGAACAGA-3' and the antisense oligonucleotide sequence containing the human PKCs shRNA sequences is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3', and the antisense oligonucleotide sequences containing the human PKCs shRNA sequence is AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3'. The non-silencing shRNA control sequences were designed according to the non-silencing shRNA (NS-shRNA) sequence available at www.qiagen.com. The sense oligonucleotide sequence containing NS-shRNA pair (underlined sequence) is GATTCCTCCAGGAAATACTGAACAGA-3' and the antisense oligonucleotide containing NS-shRNA pair (underlined sequence) is 5'-AGCTTTTCCAAAA(AAGGCTTGACGGTCTTGTACTTCCAGGAGAGCCAC-TCACGCTTGTTT)TTTGGAAAA-3'.

Overexpression of PKCα in breast cancer

To generate shRNA duplexes, 0.2 μM sense and antisense oligonucleotides were annealed by incubating the mixed oligonucleotides in the PCR thermocycler using the following profile: 95°C for 30 min, 80°C for 30 min and room temperature for another 30 min. Double stranded oligonucleotides were subsequently cloned into the pSUPER plasmid in the frame of the BglII and HindIII sites. The inserts were screened by restriction enzyme digestion with EcoRI and HindIII and confirmed by sequencing with M13 forward and reverse primers (DNA Sequencing Facility, University of Chicago).

Partial PKC purification and western blot

A PKC-enriched fraction was purified from at least 4 × 10⁶ cells by DEAE-cellulose anion exchange column chromatography as previously described (38). Protein concentration was measured using the BCA assay (Pierce, Rockford, IL). An 8% SDS–polyacrylamide gel was loaded with equal amounts of protein (50–100 μg/lane) and biotin-labeled molecular weight standards to approximate protein size. Following electrophoresis, protein was transferred to Hybond-ECL nitrocellulose membranes (Amersham Life Sciences, Buckinghamshire, England) by semi-dry electroblotter transfer. PKC isozyme polyclonal antibodies α, β and δ (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted 1:500 in TBS-T (20 mM Tris (pH 7.6), 137 mM NaCl and 0.1% Tween-20) containing 5% dry milk. The Supersignal western blotting detection system (Pierce, Rockford, IL) was used to visualize immunoreactive bands. Equal loading of total protein was assessed by stripping and reprobing the membrane with a β-actin monoclonal antibody (Sigma Chemical. St. Louis, MO).

Establishment of T47D:A18/PKCβ, PKCS, T47D:A18/PKCa/shRNA#19 and/or shRNA#20 tumors in athymic mice

T47D:A18/neo, T47D:A18/PKCβ13, T47D:A18/PKCG14, T47D:A18/PKC20 and T47D:A18/PKC20/PKCs antibodies#19 or shRNA#20 cells were seeded at 3 × 10⁵ cells/ml estrogen-depleted media into T25 culture tissue flasks. The following day (day 1) medium containing either ethanol (control), 10⁻⁸ M or 4-hydroxytamoxifen (4-OHT) (10⁻⁷ M) was transferred into T47D:A18 cells by cell aspiration. Cells designated T47D:A18/neo, T47D:A18/PKCβ13, T47D:A18/4-OHT, T47D:A18/PKC20/shRNA#19 and/or shRNA#20 were chosen for further characterization.

Proliferation assay

The cell clones T47D:A18/neo, T47D:A18/PKC20, T47D:A18/PKCβ13 or 16, T47D:A18/4-OHT and T47D:A18/PKC20/shRNA#19 or shRNA#20 were seeded at 3 × 10⁵ cells/ml estrogen-depleted media into T25 culture tissue flasks. The following day (day 1) medium containing either ethanol (control), E2 (10⁻⁸ M) or 4-hydroxytamoxifen (4-OHT) (10⁻⁷ M) was added. All compounds were dissolved in 100% ethanol and added to the medium at a 1:100 dilution. Cells were counted on Day 2–10.

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Statistical analysis
When comparing with one group, data were analyzed using unpaired t-test. When comparing groups, data were analyzed using One-way Analysis of Variance (ANOVA) followed by the Dunnett multiple comparison test. All statistics were performed using GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego California USA). Significant differences were indicated when \( P < 0.05 \).

Results

shRNA-mediated degradation of the PKC\(\alpha\) mRNA in T47D:A18/PKC\(\alpha\)20 cells
To determine whether the pSUPER-PKC\(\alpha\)-shRNA construct could produce shRNA specific to PKC\(\alpha\) mRNA degradation, T47D:A18/PKC\(\alpha\)20 cells were transiently transfected with pSUPER, pSUPER-NS-shRNA and pSUPER-PKC\(\alpha\)-shRNA constructs. The cells were harvested 48 or 60 h post-transfection, and PKCs \(\alpha\), \(\beta\) and \(\delta\) mRNA levels were measured by northern blot analysis. More than 80% of the PKC\(\alpha\) mRNA was degraded when cells were transfected with the pSUPER-PKC\(\alpha\)-shRNA construct (10 \(\mu\)g) for 48 h (Figure 1A). PKC\(\delta\) mRNA was not affected by either pSUPER vector or non-silencing RNA construct (pSUPER-NS-shRNA) at the same dose for the same period of time. Interestingly, PKC\(\beta\) mRNA expression was reduced to 61% when the cells were transfected with 10 \(\mu\)g of the pSUPER-PKC\(\alpha\)-shRNA construct compared with the pSUPER-NS-shRNA control vector at 48 h post-transfection and this reduction was statistically significant (Figure 1C). However, no effect on PKC\(\beta\) mRNA expression was observed at 60 h post-transfection suggesting the knockdown was modest and transient. Expression of PKC\(\delta\) mRNA was not affected. (Figure 1A). These results suggest that the PKC\(\alpha\)-shRNA also targeted PKC\(\beta\) mRNA destruction, even though the target sequence was in a region not homologous to PKC\(\beta\). Alternatively, PKC\(\beta\) mRNA may be transcriptionally downregulated secondary to the decrease in PKC\(\alpha\) expression. There is evidence in the literature for cross-regulation between PKC isozymes (32–34).

Generation T47D:A18/PKC\(\alpha\)20/shRNA stable transfectants
To verify that reduction of the PKC\(\alpha\) mRNA corresponded to a decrease in PKC\(\alpha\) protein, pSUPER-PKC\(\alpha\)-shRNA and pcDNA3.1/LacZ were stably co-transfected into T47D:A18/PKC\(\alpha\)20 cells, and individual clones were selected in the medium containing G418 (500 \(\mu\)g/ml) and hygromycin (100 \(\mu\)g/ml) for 2 months. Several stable clones were screened by western blot analysis for PKC isozyme expression. T47D:A18/PKC\(\alpha\)20/shRNA stable clone nos 19 and 20 exhibited a 50% reduction of PKC\(\alpha\) protein expression compared with parental T47D:A18/PKC\(\alpha\)20 cells (Figure 2). Consistent with the observed effect of PKC\(\alpha\)-shRNA on PKC\(\beta\) mRNA expression, endogenous PKC\(\beta\) protein was also decreased in these stable clones when using a PKC\(\beta\)1 antibody from Santa Cruz Biotechnology (Figure 2). However, a PKC\(\beta\) monoclonal antibody from BD Biosciences produced variable expression of PKC\(\beta\) protein in the stable cell lines and in some instances there was no difference in PKC\(\beta\) expression (results not shown). Therefore, in light of the northern blot result that shows PKC\(\alpha\)-shRNA downregulates PKC\(\beta\) mRNA expression, and consistent protein PKC\(\beta\) downregulation with at least one PKC\(\beta\)-specific antibody, we feel investigation of the role of PKC\(\beta\) is important.

T47D:A18/PKC\(\alpha\)20/shRNA stable clones do not reverse autonomous growth in vitro
We have reported that overexpression of PKC\(\alpha\) in T47D:A18 produces hormone-independent growth in cell culture or autonomous growth (30). PKC\(\alpha\)-shRNA clones exhibited down-regulation of both PKCs \(\alpha\) and \(\beta\), therefore reversal of the
autonomous growth phenotype of the T47D:A18/PKCα20/shRNA stable clones were assessed by proliferation assays. As expected, all clones grew in the presence of E2 (Figure 3A). However, in the absence of E2, although both shRNA clones grow at a slower rate, neither of the clones exhibited reversal of autonomous growth (Figure 3B). These results suggest that 50% reduction of PKCs α and β protein expression is not sufficient to reverse the autonomous growth of T47D:A18/PKCα cells in vitro.

T47D:A18/PKCα20/shRNA stable clones exhibit partial reversal of the TAM-resistant and E2 inhibitory phenotypes in vivo

We have reported that tumors derived from the T47D:A18/PKCα20 clone grow in the absence of E2 (autonomous growth), in the presence of TAM, and these tumors are growth inhibited by E2 (31). To determine whether downregulation of PKCs α and β can reverse this phenotype in vivo, we injected T47D:A18/PKCα20/shRNA stable clones into the mammary fat pads of ovariectomized athymic mice. The mice were divided into three treatment groups; no treatment (controls), E2 (1.0-cm capsule) or TAM (1.5 mg/day, p.o. five times per week). The T47D:A18/PKCα20/shRNA#19 clone exhibited partial reversal of the TAM-resistant and E2 inhibitory phenotype in vivo (Figure 4A). However, the T47D:A18/PKCα20/shRNA#20 clone remained TAM-resistant, hormone-independent and growth inhibited by E2 (Figure 4B). The partial phenotypic change exhibited by the T47D:A18/PKCα20/shRNA#19 tumor correlated with 50% reduction of PKCα expression, whereas no reduction of PKCα expression is evident in the T47D:A18/PKCα20/shRNA#20 tumors (Figure 4C and D). Therefore, reduction in PKCα protein correlates with the partial reversal of phenotype observed with the T47D:A18/PKCα20/shRNA#19 tumor. Interestingly, downregulation of PKCβ was not observed in either of the PKCα-shRNA tumors compared with parental T47D:A18/PKCα20 tumors (Figure 4C and D). These results suggest that 50% reduction of PKCα alone is sufficient to partially reverse the TAM-resistant and E2-inhibitory phenotype of T47D:A18/PKCα tumors. However, the partial knockdown

Fig. 2. Western blot analysis of PKC isozyme expression in T47D:A18/PKCα20/shRNA stable cell clones. (A), PKC protein was partially purified from T47D:A18 (lane 1), T47D:A18/neo (lane 2), T47D:A18/PKCα20 (lane 3), T47D:A18/PKCα20/shRNA#19 (lane 4) and T47D:A18/PKCα20/shRNA#20 (lane 5) stable clones by DEAE-cellulose chromatography as described in Materials and methods. Western blot analysis was performed using specific antibodies to PKCs α, β1 and δ isozymes. Equal protein loading was assessed by re-probing with a β-actin monoclonal antibody. (B), Quantitation of the bands in the panel A by Scion image software. The results are expressed as mean ± SE. Asterisk indicates statistical difference compared with T47D:A18/PKCα20 cells.

Fig. 3. Proliferation rate of T47D:A18/PKCα/shRNA clones grown in (A) E2-containing medium and (B) E2-depleted medium. Proliferation rate was assessed by cell counting as described in Materials and methods. The results are expressed as total cell number ± SE for each time point. These results are representative of three independent experiments.
achieved in the T47D:A18/PKCα/shRNA#19 clone may not be sufficient to reverse the autonomous growth phenotype either in vivo (Figure 4A) or in vitro (Figure 3B).

Establishment of PKCs β and δ stable transfectants in T47D:A18

We have reported previously that stable transfection of PKCα in T47D:A18 cells results in concomitant upregulation of PKCs β and δ (30). Other laboratories have also documented cross-regulation of other PKC isozymes by the introduction of PKCα (32–34). To ascertain the contribution of PKCs β and δ in the manifestation of the autonomous, TAM-resistant and E2-inhibitory phenotype, we have established T47D:A18/PKCβ and T47D:A18/PKCδ stable clones and examined PKC isoform expression by western blot analysis. T47D:A18/PKCβ clones were unable to grow in estrogen-depleted medium or in the presence of 4-hydroxytamoxifen (4-OHT) (Figure 6A, C and D). However, these clones grow in response to E2 treatment, thereby exhibiting a hormone-dependent growth phenotype. Only T47D:A18/PKCα20 clones were able to grow in estrogen-depleted medium and in medium containing 4-OHT and E2 (Figure 6B), exhibiting autonomous growth and partial TAM-resistance.

PKCs β or δ stable clones exhibit a hormone-dependent phenotype in vivo

We have shown that overexpression of PKCβ and/or δ in T47D:A18 results in a hormone-dependent phenotype in cell culture. To determine whether this phenotype is maintained in vivo, we injected T47D:A18/PKCβ13 or T47D:A18/PKCδ14 cells into the mammary fat pads of ovariectomized athymic mice. Seven weeks after the injection of T47D:A18/neo cells, E2 significantly stimulated tumor growth compared with the control and TAM groups (Figure 7A), which is consistent with previous findings (31). Eight weeks post-injection of T47D:A18/PKCβ or PKCδ cells, tumor growth was observed only in the E2 group (Figure 7B, C). No tumor was detected in any of the mice in the Control or TAM groups, exhibiting similar growth characteristics as the parental T47D:A18.

Fig. 4. Initiation and growth of tumors in athymic mice. (A), T47D:A18/PKCα20/shRNA#19, or (B), T47D:A18/PKCα20/shRNA#20. A total of 30 mice received injections of each cell line and were treated (10 mice/group) for 7 weeks with a 1 cm E2 capsule (E2 group; open circles), 1.5 mg p.o. TAM (TAM group; closed triangles), or left untreated (control group; closed circles). (C), western blot analysis of PKC isoforms partially purified from tumors derived from T47D:A18/PKCα20 (lane 1); T47D:A18/PKCα20/shRNA#19 (lane 2); T47D:A18/PKCα20/shRNA#20 (lane 3). (D), Bands were quantified using Scion image software. Results represent three independent experiments, and are expressed as mean ± SE. Asterisk indicates statistical difference compared with T47D:A18/PKCα20 cells.
cells. These results indicate that overexpression of PKCβ and/or δ is not sufficient to impart the phenotype observed in the PKCa overexpressing tumors in vivo.

**Discussion**

TAM resistance is a considerable obstacle in the management of breast cancer. Therefore, identification of key molecules involved in TAM-resistance will be the critical first step in the development of a logical targeted therapy (31). We recently reported that elevated PKCa expression may be predictive of TAM treatment failure (40). Our in vivo T47D:A18/PKCα20 tumor model exhibits autonomous, TAM-resistant and E2-inhibitory growth characteristics (31). However, in addition to PKCa, the T47D:A18/PKCα20 clone also exhibits increased endogenous expression of PKCs β and δ (30). To explore the contribution of PKCs α, β and δ to the autonomous, TAM-resistant and E2-inhibitory growth phenotype, two approaches were taken: (i) PKCa was specifically downregulated in T47D:A18/PKCα20 cells by shRNA and (ii) T47D:A18 cells were stably transfected with either PKCβ or δ.

RNA silencing or interference (RNAi) is a process of post-transcriptional gene silencing initiated by double-stranded (dsRNA) molecules (41). To determine whether downregulation of PKCa alone is sufficient to reverse the growth characteristics of T47D:A18/PKCα20 cells, shRNA technology was utilized. The pSUPER-PKCα-shRNA construct produced shRNA that knocked down PKCa mRNA and protein by 80 and 50%, respectively. In cell culture, we found that PKCα-shRNA also resulted in downregulation of PKCβ mRNA and protein, whereas PKCδ expression was unaffected. One possible explanation for this observation is the cross-regulation of PKC isozymes known to exist in cell systems as reported previously (34). Reduction in PKCs α and β in cell culture is not sufficient to reverse autonomous growth in vitro (Figure 3). Interestingly, although tumors were derived from both T47D:A18/PKCα/shRNA constructs, only the T47D:A18/PKCα/shRNA#19 clone produced tumors exhibiting 50% knockdown of PKCa protein. Furthermore, these tumors do not display concomitant PKCβ downregulation (Figure 4C). It is only these T47D:A18/PKCα/shRNA#19 tumors that also exhibit reversal of TAM-resistance and E2-inhibition (Figure 4A). The growth characteristics of

![Fig. 5. Western blot analysis of PKC isozymes expression in T47D:A18/PKCβ and /PKCδ stable cell clones. PKC protein was partially purified from T47D:A18/neo, T47D:A18/PKCα, T47D:A18/PKCβ and T47D:A18/PKCδ stable clones by DEAE-cellulose chromatography as described in Materials and methods. Western blot analysis was performed using specific antibodies to PKCs α, β and δ isozymes proteins. Equivalent protein loading was assessed by re-probing with β-actin antibody.](https://academic.oup.com/carcin/article-abstract/27/8/1538/2476289)

![Fig. 6. Proliferation rate of stable clones. (A), T47D:A18/neo; (B), T47D:A18/PKCα20; (C), T47D:A18/PKCβ13 and (D), T47D:A18/PKCδ14 stable clones grown in E2-depleted medium with or without treatment with E2 or 4-OHT. The results are expressed as total cell number ± SE for each time point. These results are representative of three independent experiments. Asterisk indicates statistical difference among groups at P < 0.05.](https://academic.oup.com/carcin/article-abstract/27/8/1538/2476289)
it is not surprising that a 50% reduction of PKCα would not restore the parental phenotype entirely. The apparent lack of PKCα knockdown in the T47D:A18/PKCα/shRNA#20 tumors may be due to loss of the PKCα-shRNA construct during development of the tumors. These results suggest that PKCα is required to mediate the TAM-resistant and E2-inhibitory phenotype in T47D:A18 cells. The partial reversal of the TAM-resistant and E2-inhibitory phenotype may be due to the incomplete knockdown of PKCα and/or potential synergistic effects with PKCB and/or δ. This possibility is supported by studies showing that both PKCs α and δ are highly expressed in three out of four antiestrogen resistant MCF-7 cell lines (42). However, in our cell and tumor models, PKCα appears to play a dominant role in the acquisition of TAM-resistance, autonomous growth and E2 inhibitory growth characteristics. Interestingly, the PKCα gene, PRKCA, maps to chromosome 17q22–q23.2 according to the genome-draft assignment, and was fine-mapped by fluorescence in situ hybridization (FISH) analysis to be located at 17q24 (43). Several genes involved in the pathogenesis of malignant breast cancer are found on chromosome 17 including BRCA1 and nm23 (44). It is interesting to speculate whether upregulation of PKCα occurs along with alterations of these well-known breast-cancer-associated genes in this highly mutation-susceptible locus in breast cancer. Using dominant negative mutants of PKCα in MCF-7 cells, PKCα-specific downstream signaling targets were identified (45). In these experiments, the dominant negative PKCα construct downregulated the anti-apoptotic bcl-2 protein and sensitized cells to apoptosis in response to TAM. In addition, the cyclin-dependent kinase inhibitor, p21<sup>cip1</sup>, was also downregulated. The authors conclude that PKCα overexpression leads to upregulation of both bcl-2 and p21<sup>cip1</sup>, thus, protecting cells from apoptosis. These effects were not observed using dominant negative PKCε or ζ constructs. Other PKCα downstream signaling molecules implicated in TAM-resistance include AP-1 (46–48), P13K/Akt (49,50), MAPK (51,52) and NFκB (53–55). We demonstrated that the T47D:A18/PKCα<sub>20</sub> cells exhibit elevated basal AP-1 activity compared with the T47D:18/neo vector control cells (30). In addition, elevated phospho-Akt protein levels were observed in TAM-treated T47D:A18/PKCα<sub>20</sub> tumors compared with tumors treated with E2 (manuscript submitted). However no difference in expression of total and phosphorylated ERK1/2 (phospho-p44/42) nor NFκB signaling components (phospho-NFκBp65, Ser32phospho-IκBα) was observed in T47D:A18/PKCα<sub>20</sub> and T47D:A18/neo cells (unpublished observations).

We continue to explore and attempt to connect the molecular signaling pathways initiated by PKCα leading to TAM-resistant, E2-inhibitory, autonomous growth.

In summary, we find that PKCβ and/or δ overexpression is not sufficient to impart autonomous, TAM-resistant and E2-inhibited growth to T47D:A18 cells and tumors. However, partial suppression of PKCα results in partial reversal of TAM-resistance and E2-growth inhibition. This finding may have important therapeutic implications. TAM has been used to treat all stages of ERα-positive breast cancer (56); however, high-dose estrogen also causes tumor regression in post-menopausal women with ERα-positive breast cancer (57,58). We reported previously that overexpression of PKCα may predict TAM treatment failure (40) and, therefore, may be a prognostic indicator for TAM-resistance. Furthermore, the E2-induced tumor regression observed in our pre-clinical xenograft
model suggests that perhaps selection of patients with PKCα-overexpressing tumors may greatly improve the efficacy of high-dose estrogen treatment, a therapeutic approach likely to be superior to TAM in this situation. Perhaps treatment of breast cancer with the PKCα antisense oligonucleotide Affinitak (LY900003/ISIS 3521) (59) can be improved by identification of patients who exhibit PKCα overexpression. Finally, the recent favor of aromatase inhibitor therapy over TAM (60–62) is another potential concern for patients harboring tumors that overexpress PKCα. Eliminating all estrogen may in fact cause tumor growth. We are currently in the process of developing a pre-clinical model to address the utility of aromatase inhibitors in this setting and determining the signaling pathway mediated by PKCα leading to E2-induced tumor regression. Elucidation of the mechanism is likely to lead to additional therapeutic targets for the treatment of breast cancer.

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References


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