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Expression of the Antisense cDNA for Protein Kinase Cα Attenuates Resistance in Doxorubicin-Resistant MCF-7 Breast Carcinoma Cells

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SUMMARY

Multidrug resistance is functionally associated with the expression of a plasma membrane energy-dependent drug efflux pump termed P-glycoprotein, the product of the *mdr1* gene. Transfection of P-glycoprotein-expressing doxorubicin-resistant MCF-7 cells with an expression vector containing the cDNA for protein kinase Cα in the antisense orientation reduces protein kinase Cα levels and decreases total protein kinase C activity by 75%. This is accompanied by reduced phosphorylation of P-glycoprotein, a 2-fold increase in drug retention, and a 3-fold increase in doxorubicin cytotoxicity. These results provide further evidence that protein kinase Cα can positively regulate multidrug resistance in MCF-7 cells through posttranslational phosphorylation of P-glycoprotein.

MDR is associated with the elevated expression of the plasma membrane protein drug efflux pump PGP, the product of the *mdr1* gene (1–4). A wide variety of MDR cells exhibit amplification and overexpression of the *mdr1* gene (5–9), and transfection studies with both genomic and cDNA copies of the *mdr1* gene have confirmed that it confers the MDR phenotype to recipient cells (10–15). Although there is overwhelming evidence that PGP is a major factor in MDR, the role of its posttranslational modification in the expression of resistance is less certain. One of the first noted metabolic changes of PGP was its phosphorylation in colchicine- or doxorubicin-resistant cells but not in the parental or revertant cell lines (16–18), and this characteristic has been shown subsequently in a variety of MDR cell lines (19–22). Although the protein kinase responsible for the phosphorylation of PGP has not been unequivocally established, there is considerable evidence that PKC may be involved in this process. The PKC activator phorbol dibutyrate stimulates the phosphorylation of PGP in intact cells (19), and most cell lines selected for MDR contain increased levels of PKC (23–30). Transfection of MCF-7 breast carcinoma cells expressing *mdr1* with the cDNA encoding PKCα confers increased resistance to doxorubicin and vinblastine, which is associated with decreased drug retention and increased phosphorylation of PGP (31). In contrast, transfection with the atypical γ isoform of PKC, which is not normally expressed in MCF-7/ADR cells, does not modify resistance (32).

In an effort to see whether down-regulation of a single isoform of PKC could affect drug resistance, doxorubicin-resistant MCF-7 cells were transfected with an expression vector containing the cDNA for PKCα in the antisense orientation, and their doxorubicin sensitivity, drug retention, and expression of PKC were determined.

Experimental Procedures

Cells. The doxorubicin-resistant cell line MCF-7/ADR was selected by growth in increasing concentrations of doxorubicin (33) and was obtained from Dr. Kenneth H. Cowan, National Cancer Institute. These cells were further subcloned and a high *mdr1*-expressing isolate exhibiting a uniform content of PGP was used throughout the present investigation. The doubling time for the clonal line or various transfectants was approximately 24 hr, similar to that for the original MCF-7/ADR cell line.

Cytotoxicity assays. Cytotoxicity by continuous exposure to doxorubicin was determined by clonogenic assay (31). After removal of the medium, the cells were re-fed with fresh Iscove's medium containing varying concentrations of doxorubicin. After 2 weeks, colonies were fixed in methanol, stained with Giemsa stain, and counted.

Transfection of MCF-7/ADR cells with antisense PKCα. The expression vectors pRSVneo and pRSV-PKCα were generously provided by Dr. Shigeo Ohno, Yokohama University School of Medicine (34). The antisense expression vector pRSV-αPKCα was prepared by

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ABBREVIATIONS: MDR, multidrug resistant (or resistance); PGP, P-glycoprotein; PKC, protein kinase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

digestion of pRSV-PKCα with *Hind*III, followed by religation and selection of transformants with the PKCα cDNA in the antisense orientation. Lipofectin (Life Technologies, Inc.) was used for transfection according to the instructions of the manufacturer, as modified previously (32). Stable transfectants were initially selected in the presence of 1 mg/ml G418 (Life Technologies, Inc.), which was subsequently increased to 1.5 mg/ml in the second through fourth weeks of selection. Cells surviving selection were expanded in the absence of G418 for the present studies.

PKC assay. Logarithmic phase cells ($1–2 \times 10^7$) were harvested 3 days after seeding and cell extracts were assayed for PKC activity as described previously (31), except that PKC was assayed with the PKC-specific peptide [Ser^{25}]$PKC_{19–31}$ (Life Technologies, Inc.).

Immunoblotting for PKC. DEAE-Sepharose-purified protein (50 μg) was separated by SDS-PAGE in 10% polyacrylamide gels (Novex Inc.) and transferred to nitrocellulose by electroblotting, as described previously (31). Blots were incubated with affinity-purified rabbit polyclonal antibodies against the α, β, γ, δ, ε, and ζ isoforms of PKC (Life Technologies, Inc.). Immunoreactive protein was visualized with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad), with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma Chemical Co.) as the substrates.

PGP detection. PGP was quantitated by flow cytometry using monoclonal antibody MRK16, which was generously provided by Dr. Takashi Tsuruo, Japanese Foundation for Cancer Research (Tokyo, Japan), as described previously (31, 35). Negative controls were performed with each cell line, using fluorescein-conjugated goat anti-mouse IgG without MRK16.

***In vivo* phosphorylation of PGP.** Cells were incubated for 30 min in 175-cm^2 plastic flasks in 10 ml of phosphate-free Iscove's modified Dulbecco's minimal essential medium containing 40 mM HEPES, pH 7.4, and 50 μg/ml gentamicin, before labeling for 4 hr with 0.25 mCi/ml $H_3{}^{32}PO_4$. Cells were harvested and immunoprecipitated with monoclonal antibody C219 (36). Immunoprecipitates were quantitated using an AMBIS radiodensitometer.

Drug retention. Cells were incubated overnight in a 24-well plate at a density of 10^4 cells/well, and each cell line was plated into eight wells. The medium was replaced with serum-free medium, and 1 μM [^{3}H]vinblastine (0.9 Ci/mmol; Moravek Biochemicals) was added to four wells and incubated for 2 hr at 37°. Cells were then rinsed three times with ice-cold Dulbecco's phosphate-buffered saline, and the cells were lysed with 200 μl of 1 N NaOH. The NaOH lysate was removed, neutralized with acetic acid, and counted in a scintillation counter. The other four wells of each cell line were used to determine the cell number in a Coulter counter.

Results

MCF-7/ADR cells were subcloned from the original isolate to obtain a population of cells that expressed PGP uniformly. Transfection was carried out with either pRSVneo or the same plasmid containing the cDNA for PKCα in the antisense orientation. Transfected cells were selected for their resistance to G418 and were analyzed for PGP levels by flow cytometry (Fig. 1). The median fluorescence values were equivalent in both *neo* and antisense PKCα populations of transfected cells.

MCF-7/ADR cells transfected with pRSV-αPKCα exhibited a 4-fold reduction in total PKC activity, in comparison with *neo*-expressing cells, using the peptide substrate [Ser^{25}]$PKC_{19–31}$ (Fig. 2A). PKC activity was also assessed by autophosphorylation and SDS-PAGE under varying assay conditions (Fig. 2B). In the absence of cofactors, there was no autophosphorylation of PKC in extracts of cells transfected with antisense PKCα (Fig. 2B, *lane 1*), whereas autophosphorylation was readily apparent in *neo*-expressing cells (Fig. 2B, *lane 4*). Antisense PKCα-expressing cells exhibited 25% of the Ca^{2+}- and

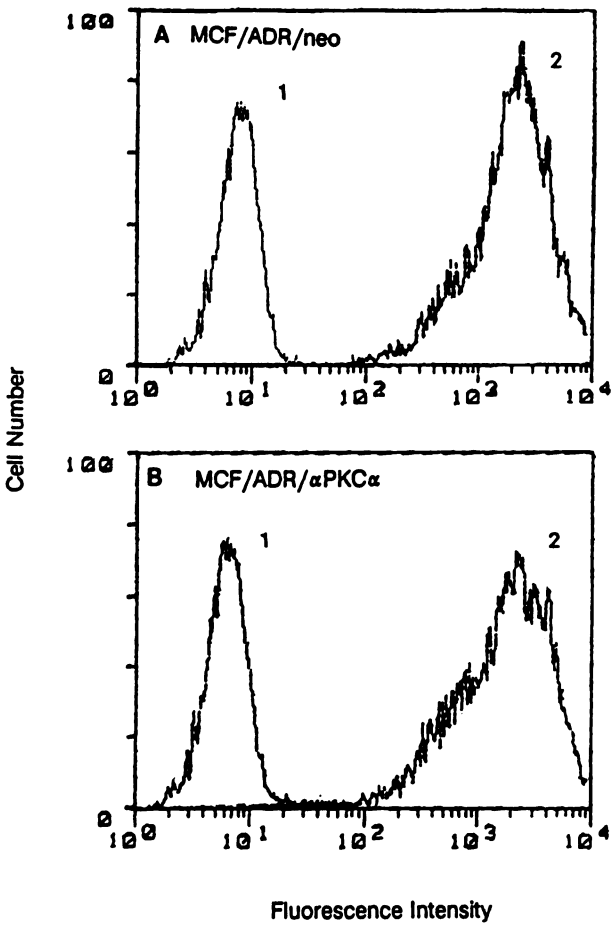


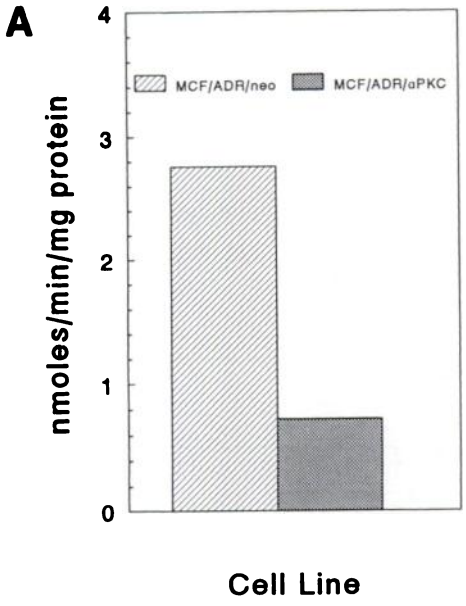
Fig. 1. Flow cytometry analysis of PGP in transfected MCF-7/ADR cells. Flow cytometric analysis was carried out using logarithmic phase cells and monoclonal antibody MRK16. *1*, Cells incubated with fluorescein-conjugated goat anti-mouse IgG alone; *2*, cells incubated with MRK16 and fluorescein-conjugated goat anti-mouse IgG.

phosphatidylserine-dependent autophosphorylation (Fig. 2B, *lane 2*) of *neo*-transfected cells (Fig. 2B, *lane 5*). In the presence of tetradecanoyl phorbol acetate and phosphatidylserine, autophosphorylation of PKC was slightly greater in MCF-7/ADR/αPKCα cells (Fig. 2B, *lane 3*), in comparison with MCF-7/ADR/neo cells (Fig. 2B, *lane 6*), although the degree of autophosphorylation was far less than that observed in the presence of Ca^{2+}.

MCF-7/ADR/αPKCα cells also contained decreased levels of PKCα, as determined by immunoblotting with an isoform-specific polyclonal antibody (Fig. 3). Immunoblot analysis with other isoform-specific antibodies against PKC indicated that neither cell line contained significant levels of the β, γ, δ, or ζ isozymes of PKC (data not shown), whereas the amount of PKCε was greater in antisense PKCα-expressing cells, in comparison with control cells.

To establish whether reduction in PKCα activity resulted in a change in PGP phosphorylation, cells were metabolically labeled with $H_3{}^{32}PO_4$ and PGP was immunoprecipitated from equal amounts of membrane protein with monoclonal antibody C219 (Fig. 4). Despite the presence of equal levels of PGP in both transfected cell lines (Fig. 1), the phosphorylation of PGP was reduced by 60% in cells expressing antisense PKCα.

Drug retention in MCF-7 cells and both MCF-7/ADR isolates was measured by incubation for 2 hr with 1 μM [^{3}H]



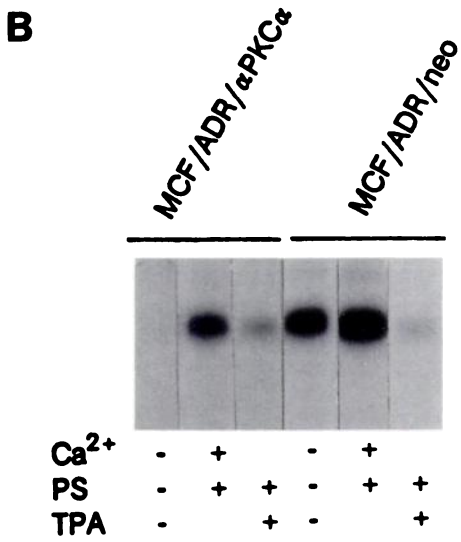


Fig. 2. Activity and levels of PKC. A, PKC activity was measured in total cellular extracts after partial purification with DEAE-Sepharose. Assays utilized 2–3 μg of protein and the PKC-specific peptide substrate [Ser^{25}] PKC_{19-31}. Each value is the mean of three separate experiments. B, Autophosphorylation of PKC *in vitro* was measured using the preparations in A. Assays were carried out for 10 min at 30° in the presence (+) and absence (−) of cofactors in the absence of peptide substrate. Each *lane* contains 6 μg of protein. *Lane* designations in text are from *left* to *right*. The autoradiograph of the dried gel is shown. *PS*, phosphatidylserine; *TPA*, tetradecanoyl phorbol acetate.

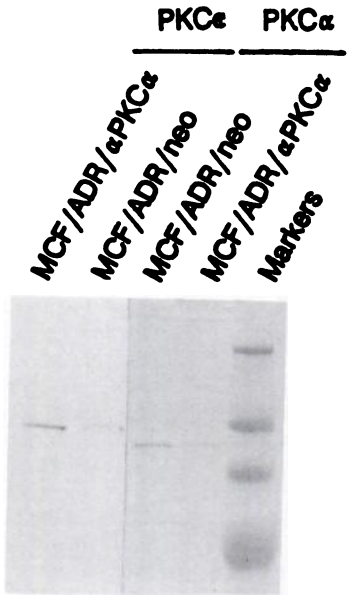


Fig. 3. Immunoblot of PKCα and -ε. Cell extracts were partially purified with DEAE-Sepharose, separated by SDS-PAGE in 10% polyacrylamide gels, and transferred to nitrocellulose. Each *lane* contains 50 μg of protein. PKCα and -ε were identified using isoform-specific affinity-purified rabbit polyclonal antibodies, as described in Experimental Procedures.

Fig. 4. Phosphorylation of PGP in transfected MCF-7/ADR cells. Cells were metabolically labeled for 4 hr with $H_3{}^{32}PO_4$ in phosphate-free medium, and solubilized membrane preparations were immunoprecipitated (100 μg of protein) with monoclonal antibody C219. Samples were separated by SDS-PAGE in an 8% polyacrylamide gel and were identified by autoradiography.

vinblastine (Fig. 5). MCF-7/ADR/neo cells accumulated 20-fold less drug than did wild-type cells, whereas MCF-7/ADR/αPKCα cells showed 2-fold more drug retention than did MCF-7/ADR/neo cells.

Clonogenic assays were performed to determine the sensitivity of the *neo*- and antisense PKCα-expressing cells after continuous exposure to doxorubicin (Fig. 6). The IC_{50} and IC_{90} values in cells expressing antisense PKCα were 3- and 2-fold lower, respectively, than those in *neo*-expressing cells. The respective IC_{50} values for antisense PKCα- and *neo*-expressing cells were 1.6 and 4.8 μM, and the respective IC_{90} values were 6.9 and 15.1 μM.

Discussion

The involvement of PKC in the MDR phenotype has been implied by previous studies that demonstrated that protein kinase inhibitors could partially reverse MDR (21, 29, 36). However, the interpretation of these results is complicated by

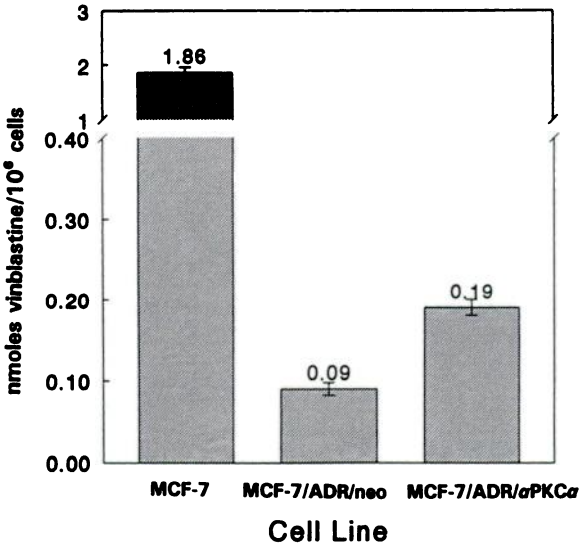


Fig. 5. Retention of [^{3}H]vinblastine in MCF-7, MCF-7/ADR/neo, and MCF-7/ADR/αPKCα cells. Cells were grown in 24-well plates and drug retention was measured after incubation for 2 hr with 1 μM [^{3}H]vinblastine (0.9 Ci/mmol). Each value is the mean ± standard error of four determinations.

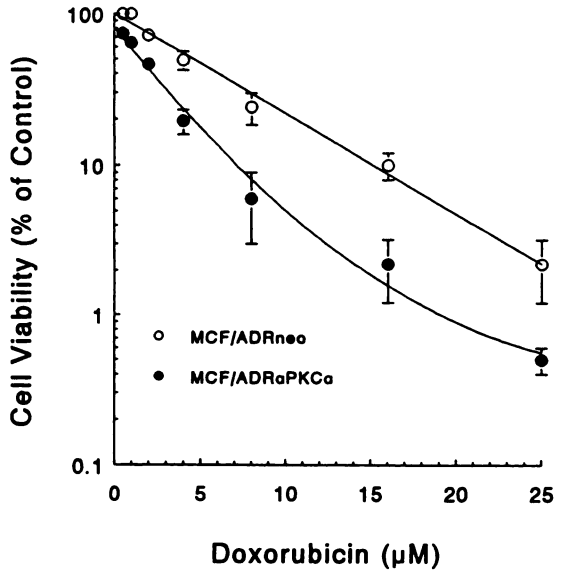


Fig. 6. Cytotoxicity of doxorubicin in transfected MCF-7/ADR cells. MCF-7/ADR/neo or MCF-7/ADR/αPKCα cells were exposed continuously for 10 days to different concentrations of doxorubicin, and colonies were determined. Each value is the mean ± standard error of four determinations.

the fact that several of these inhibitors, such as staurosporine and H-7, can also compete for drug binding to PGP (37, 38). Thus, the effects of these inhibitors on drug accumulation (36) and cytotoxicity (29) may be attributable to interference with drug efflux rather than inhibition of PKC. On the other hand, increased phosphorylation of PGP after treatment of doxorubicin- (19, 31) or vincristine-resistant cells (39, 40) with phorbol ester activators of PKC or the protein phosphatase inhibitor okadaic acid (36), as well as its direct phosphorylation *in vitro* by PKC (39), strongly suggests that PKC plays a role in the phosphorylation of PGP and in the maintenance of MDR.

The present study demonstrates that down-regulation of the predominant PKCα isoform expressed in MCF-7/ADR cells, by the expression of antisense PKCα, leads not only to reduced PKC activity and phosphorylation of PGP but also to greater drug retention and increased sensitivity to doxorubicin. These data support the thesis that MDR in this cell line is modulated, in part, by PKCα and extend our previous results demonstrating that overexpression of PKCα in wild-type MCF-7 cells transfected with the *mdr1* gene confers increased MDR (31). The 4-fold lower activity of PKC was reflected in a 2.5-fold lower level of PGP phosphorylation, a 2-fold greater retention of vinblastine, and a 2–3-fold greater sensitivity to doxorubicin. Although the reduction in PKC activity produced only a moderate effect on drug accumulation and drug sensitivity, there was an almost stoichiometric relationship between these three parameters. Therefore, these results suggest that a more effective means of reducing PKCα activity, possibly with antisense oligodeoxynucleotides or another construct of antisense PKCα cDNA, might be a more efficient way of increasing drug sensitivity in this MDR cell line.

An additional consideration is the contribution of other isoforms of PKC to the observed drug resistance. There was a moderate up-regulation of the level of PKCε that corresponded to increased Ca^{2+}-independent autophosphorylation of PKC in cells expressing antisense PKCα. Therefore, PKCε may have compensated to some degree for the reduction in PKCα and may have compromised drug accumulation and doxorubicin cytotoxicity in the antisense-expressing cells. Interestingly, overexpression of the atypical PKCγ in MCF-7 cells transfected with *mdr1* (BC-19 cells) led to decreased levels of PKCε (32), and Blobe *et al.* (43) have also noted that in MCF/ADR cells the higher level of PKCα is associated with a lower level of PKCε, compared with wild-type cells. Therefore, there is compelling evidence that there is a reciprocal modulation of PKCε and the more typical Ca^{2+}- and phospholipid-dependent α and γ PKC isoforms. Because PKCε belongs to the recently described family of PKC genes that lack a Ca^{2+}-binding domain and that probably function in signal transduction in a very tissue-specific manner (41, 42), it will be interesting to determine whether this PKC isoform can directly phosphorylate and modulate the activity of PGP or affect the transcription of the *mdr1* gene itself.

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