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Activation of Phospholipase C Induces the Expression of the Multidrug Resistance (*MDR*1) Gene through the Raf-MAPK Pathway

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ABSTRACT

Resistance to multiple, unrelated cancer chemotherapeutic drugs can be mediated by P-glycoprotein, the *MDR*1 gene product. Numerous substances, including chemotherapeutic drugs, heavy metals, growth factors, activated oncogenes, or changes in temperature increase *MDR*1 gene expression. Because several of these factors regulate cellular function through the activation of phospholipase C (PLC), we postulated that PLC-mediated signaling could be central to regulating the expression of *MDR*1. Transfection of NIH 3T3 cells with a pMJ30-PLC-γ1 expression vector increased the activity of the *MDR*1 promoter by 2- to 10-fold. PLC-mediated activation required a region between −106 and −99 of the *MDR*1 promoter. Treatment of cotransfected cells with platelet-derived growth factor further enhanced the activity of the *MDR*1 promoter. The stimulatory effect of PLC on the *MDR*1 promoter was increased by cotransfection with constitutively active v-raf and was blocked by the dominant-negative mutant, c-Raf-C4. The activity of mitogen-activated protein kinase (MAPK) was also increased in PLC-γ1-transfected cells. Furthermore, PD-98059 and U0126, two MAPK inhibitors, blocked PLC-γ1-induced expression of *MDR*1. The results of Northern blot analysis showed that activation of PLC by heat shock and growth factors increased expression of endogenous MDR1 mRNA in human renal carcinoma cells. These effects were blocked by inhibitors of the PLC-MAPK pathway. In summary, our results indicate for the first time that activation of PLC by a variety of cellular stimuli can regulate the expression of *MDR*1 and that the transcriptional modulation of *MDR*1 expression by PLC is mediated by the Raf-MAPK pathway.

Resistance to multiple chemotherapeutic drugs occurs in cancer and infectious disease. One form of multidrug resistance common to both is characterized by enhanced drug efflux mediated by P-glycoprotein (P-gp), a 150- to 180-kDa plasma membrane phosphoglycoprotein that functions as an energy-dependent drug transporter with broad substrate specificity (Gottesman and Pastan, 1993). Several important characteristics of P-gp include homology with bacterial transport proteins (Chen et al., 1986), ATP binding (Cornwell et al., 1987b), and hydrolysis (Hamada and Tsuruo, 1988), drug binding (Cornwell et al., 1986) and efflux (Kamimoto et al., 1989), and the ability to bind compounds that reverse MDR, such as verapamil and cyclosporin A (Cornwell et al., 1987a; Foxwell et al., 1989).

Regulation of P-gp expression has been studied extensively in normal and malignant tissues, and many factors are now known to increase the expression of *MDR*1. For example, Chaudhary et al. showed that activation of PKC by the diacylglycerol analog, 12-*O*-tetradecanoylphorbol-13-acetate, increased MDR1 mRNA and P-gp expression (Chaudhary and Roninson, 1992). Heat shock (Chin et al., 1990), UV irradiation (Hu et al., 2000), certain chemotherapeutic drugs (Chaudhary and Roninson, 1993), heavy metals (Chin et al., 1990), hormones (Altuvia et al., 1993), oncogenes and tumor suppressor genes (Chin et al., 1992), and growth factors such as EGF (Rohlff and Glazer, 1995), were also shown to increase the expression of *MDR*1. However, it is not known how these diverse agents lead to *MDR*1 activation.

Several of the stimuli known to increase *MDR*1 expression utilize signal transduction pathways initiated through PLC. Activation of PLC is one of the most common transmembrane signal transduction mechanisms used by a wide array of extracellular ligands (Rana and Hokin, 1990). PLC produces two second messengers by catalyzing the conversion of phosphatidylinositol 4,5-bisphosphate to Ins(1,4,5)P_3 and diacylglycerol. Ins(1,4,5)P_3 stimulates the release of Ca^{2+} from

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistant or multidrug resistance; EGF, epidermal growth factor; PLC, phospholipase C; Ins(1,4,5)P_3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; MEK, MAP/ERK kinase (mitogen-activated protein kinase kinase); PKC, protein kinase C; CAT, chloramphenicol acetyl transferase.

intracellular stores and diacylglycerol activates PKC. PLC has been implicated in regulation of many cellular activities, including cell growth and transformation (Nebigil, 1997). In addition, the activity of PLC is increased in many human tumors (Noh et al., 1994). However, little is known about the role of PLC in drug resistance. Therefore, in this study, we postulated that PLC activation is linked to downstream signaling events that can regulate *MDR*1 expression in response to diverse stimuli. Our results implicate this pathway in the regulation of *MDR*1 expression and suggest the possibility of targeting signaling components as a mean to inhibit the expression of this drug resistance gene.

Materials and Methods

Cell Cultures, Expression Vectors, and Reagents. PLC-γ/3T3 cells were provided by Dr. Mark R. Smith (National Institutes of Health, Bethesda, MD). PLC-γ/3T3 and NIH 3T3 cell lines were maintained in Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2/95% air. The human renal carcinoma line HTB-46 was purchased from American Type Culture Collection (Manassas, VA) and grown in McCoy's 5A modified medium containing 10% fetal bovine serum under conditions identical to those described above. Cells were checked routinely and found to be free of contamination by mycoplasma or fungi.

The human *MDR*1 promoter construct, MDRCAT, and deletion constructs, were kindly supplied by Dr. K. V. Chin (The Cancer Institute of New Jersey, New Brunswick, NJ). MDRCAT contains 1.8 kilobase pairs of genomic sequences upstream from the initiation codon (ATG) of the human *MDR*1 gene cloned directly in front of the CAT gene in pSV00CAT (Chin et al., 1992). The pMJ30-PLC-γ1 and its control vector pMJ30 were provided by Dr. S.-G. Rhee (National Heart, Lung, and Blood Institute, Bethesda, MD). v-Raf and c-Raf-C4 constructs were gifts from Dr. Marilyn M. Cornwell (Fred Hutchinson Cancer Research Center, Seattle, WA).

[^{14}C]Chloramphenicol was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Luciferase Assay System was purchased from Promega Corp. (Madison, WI). Additional reagents were purchased from the following sources: PDGF and EGF (Invitrogen, Carlsbad, CA); PD98059 and U73122 (Biomol, Plymouth Meeting, PA); U0126 (Promega Corp., Madison, WI); doxorubicin (Sigma Chemical Co., St. Louis, MO); LipofectAMINE and TRIzol Reagent (Invitrogen). Antibodies were obtained from the following sources: monoclonal anti-MAPK and anti-β-actin antibodies, Sigma (Saint Louis, MO); monoclonal anti-phospho-MAPK (New England BioLabs, Beverly, MA).

PLC Activity. PLC activity was measured by assaying the generation of Ins(1,4,5)P_3 from phosphatidylinositol 4,5-bisphosphate. Briefly, Ins(1,4,5)P_3 was extracted from cell lysates using perchloric acid. Acid extracts were neutralized to pH 7.5 by titration with ice-cold KOH (10 M). Ins(1,4,5)P_3 was measured by competitive binding to a bovine adrenal Ins(1,4,5)P_3-binding protein using the Amersham assay kit (TRK1000; Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Activation of PKC. Activation of PKC was determined by measuring the activity and subcellular redistribution of the enzyme. Briefly, 1×10^7 cells were collected and washed twice with PBS/1.0 M sucrose and pelleted in a Microfuge. The cellular pellets were resuspended within 20 s in 50 μl of double-distilled water by passage through a narrow-gauge needle and immediately reconstituted with 1.0 ml of ice-cold buffer A (20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 100,000*g* to obtain the soluble and particulate fractions. Particulate fractions were solubilized with buffer A containing 1% Nonidet P40, and both cytosolic and solubilized membrane fractions were separated by DEAE-cellulose chromatography. Aliquots (50 μl) of the DEAE-purified material were assayed for PKC activity in the presence of 10 mM Mg acetate, 0.75 mM $CaCl_2$, 100 μM [γ-^{32}P]ATP, and 25 μg of histone H1. Conditions were adjusted to ensure linearity of the reaction with respect to the time of incubation and concentrations of samples.

Transient Transfections and Reporter-Gene Assays. NIH 3T3 cells (3×10^5) were plated in 60-mm cell culture dishes then cotransfected with MDRCAT (1 μg) and pMJ30-PLC-γ1 (4 μg) using LipofectAMINE. Forty-eight hours after transfection, CAT activity was measured using equivalent amounts of total protein and quantified by scintillation counting of the acetylated ^{14}C-labeled chloramphenicol. PLC-γ/3T3 cells were used for cotransfection with MDRCAT, v-Raf, or c-Raf-C4. pGL3, a luciferase expression vector, was used as an internal control for transfection efficiency by normalizing CAT activity to the luciferase activity.

Northern Blot Analysis. RNA was prepared from treated cells using TRIzol Reagent according to the manufacturer's protocol. Twenty micrograms of total RNA from each sample were electrophoresed, blotted onto nitrocellulose, and probed for *MDR*1 using cDNA 5A probe. An α-^{32}P-labeled β-actin probe was used to determine loading of RNA.

Western Blot Analysis. Cells were lysed on ice for 30 min with radioimmunoprecipitation assay buffer (10 mM sodium phosphate, pH 7.2, 1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 2 mM EDTA) supplemented with fresh 1% aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin and centrifuged at 14,000*g* at 4°C for 10 min. Proteins were resolved by 7 to 10% SDS-PAGE and transferred to nitrocellulose membranes. The blots were incubated in blocking solution consisting of 5% milk in Tris-buffered saline/Tween 20 (0.1% Tween 20) for 1 h at 25°C, then immunoblotted with monoclonal anti-MAPK, anti-phospho-MAPK, or anti-β-actin antibodies. Detection by enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Experiments were done under conditions of linearity with respect to protein contents.

Results

Effects of PLC-γ1 Transfection on the Activity of PLC, PKC, and the *MDR*1 Promoter. To create a model in which to study the effect of PLC activation on *MDR*1 expression, we transfected NIH 3T3 cells with a PLC-γ1 expression vector. Figures 1, A and B, demonstrate that PLC-γ1 transfection activated PLC-mediated signaling as measured by increased production of Ins(1,4,5)P_3 (Fig. 1A) and redistribution of PKC activity from the cytosol to the particulate fraction (Fig. 1B). To determine the effect of PLC on the expression of *MDR*1, we next cotransfected NIH 3T3 cells with MDRCAT and PLC-γ1 expression vectors then measured the activity of the *MDR*1 promoter. Figure 1C demonstrates that PLC-γ1 increased *MDR*1 promoter activity up to 5-fold in a dose-dependent manner compared with empty vector controls, which had no effect on the expression of MDRCAT (data not shown).

To define the region of the *MDR*1 promoter responsible for the effect of PLC on *MDR*1 expression, we studied the effect of PLC-γ1 transfection on a series of cotransfected *MDR*1 promoter deletion constructs. As shown in Fig. 2, deletion of sequences from −1073 to −106 or deletion of intron 1 and exon 2 had no effect on PLC-induced activity of *MDR*1 promoter as well as basal activity. Deletion of GC-rich sequences from −106 to −99 did not affect basal activity of the promoter but abolished the activation by PLC (Fig. 2). These results

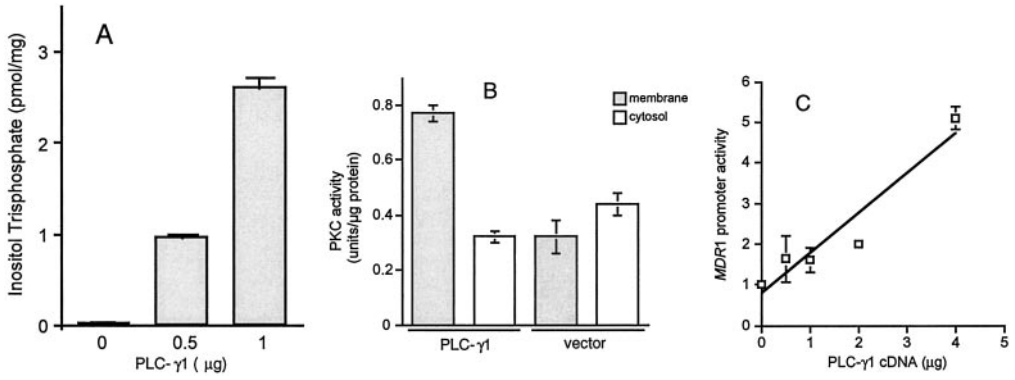

Fig. 1. Effect of PLC-γ1 transfection on the activity of PLC, PKC, and *MDR*1 Promoter. A and B, NIH 3T3 cells were transfected with pMJ30-PLC-γ1 expression vector. PLC activity was measured by assaying the generation of Ins(1,4,5)P3 using Amersham's assay system. Activation of PKC was determined by examining the subcellular redistribution of PKC activity. C, NIH 3T3 cells were plated in 100-mm cell culture dishes, then cotransfected with 1 μg of MDRCAT and various amounts of pMJ30-PLC-γ1 using LipofectAMINE. Forty hours after transfection, the CAT activity was measured with equivalent amounts of protein extracts and quantified by scintillation counting of the percentage of acetylated ^{14}C-labeled chloramphenicol. Each bar or point represents the mean ± S.E. from one of three similar experiments.

suggest that the GC-rich region between −106 and −99 was required for PLC-induced activation of the *MDR*1 promoter. Deletion of regulatory sequences between −99 and −11 resulted in loss of basal activity (greater than 90% decrease; Fig. 2), which is consistent with previously reported results (Cornwell and Smith, 1993b; Goldsmith et al., 1993).

Effect of Raf on PLC-Induced Activation of the *MDR*1 Promoter. PKC can phosphorylate and activate Raf kinase (Kolch et al., 1993). Therefore, we determined whether the activation of the *MDR*1 promoter by PLC involved Raf kinase activity. We undertook transient cotransfection experiments using a PLC-γ1 stable transfectant, PLC-γ1/3T3 and constructs of v-Raf or c-Raf-C4. *MDR*1 promoter activity was 2- to 5-fold greater in PLC-γ1/3T3 cells than in control cells (Fig. 3). Cotransfection of PLC-γ1/3T3 cells with v-Raf, a constitutively activated Raf-1, further increased *MDR*1 promoter activity up to 15-fold over that of the control cell line and 3-fold over the PLC-γ1 transfectants (Fig. 3A). Raf-C4, a dominant-negative mutant of Raf-1, markedly reduced the activation of the *MDR*1 promoter induced by PLC (60 to 70% inhibition) (Fig. 3B).

Involvement of MAPK in PLC Activation of *MDR*1. MAPK is downstream of Raf-1 and is responsible for transcriptional regulation of several genes (Treisman, 1996). We found that the constitutively activated Raf increased the activation of MAPK by 3-fold, and dominant-negative mutant

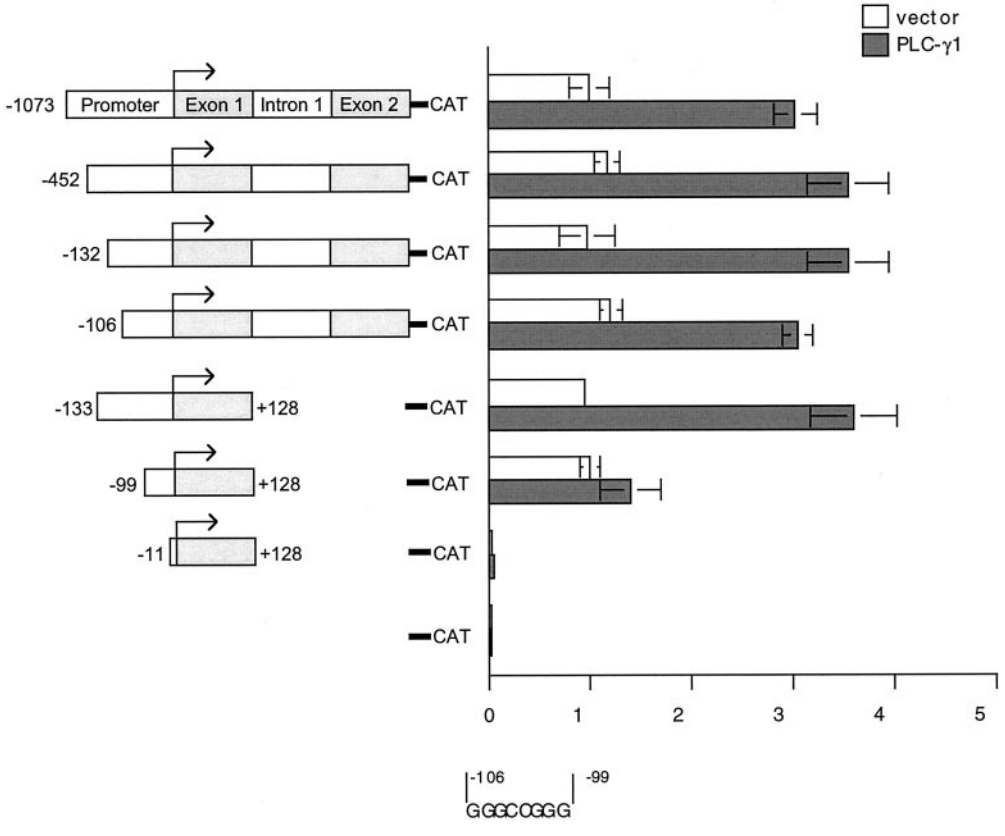

Fig. 2. Effect of PLC-γ1 transfection on the activity of *MDR*1 promoter deletion constructs. NIH 3T3 cells were plated in 100-mm cell culture dishes, then cotransfected with 1 μg of the *MDR*1 promoter deletion constructs and 4 μg of pMJ30-PLC-γ1 using LipofectAMINE. Forty hours after transfection, the CAT activity was measured with equivalent amounts of protein extracts and quantified by scintillation counting of the percentage of acetylated ^{14}C-labeled chloramphenicol. Each point represents the mean ± S.E. from one of three similar experiments.

of Raf inhibited the activation of this enzyme to a similar extent as the inhibition of *MDR*1 promoter activity (60 to 70% inhibition) (data not shown). To assess the role of MAPK in PLC-induced activation of *MDR*1 expression, we measured the phosphorylation of p42 and p44 MAPK in cells transfected with PLC-γ1. As shown in Fig. 4A, the phosphorylation of MAPK, as measured by the ratio of the phosphorylated p42 band (Fig. 4, top) normalized to the total p42 band (Fig. 4, bottom), was increased by 2.3-fold in PLC-γ1-transfected cells compared with cells transfected with empty vector, despite a slight decrease in MAPK protein as measured by Western blot of the total enzyme (Fig. 4A, bottom). These results suggested that increased PLC activity resulted in activation of MAPK.

Doxorubicin is a chemotherapeutic agent that activates PLC (Yang et al., 1995) and induces the expression of *MDR*1 (Abolhoda et al., 1999). Therefore, we investigated whether doxorubicin also activated the MAPK pathway. Figure 4B demonstrates that treatment of NIH 3T3 cells with doxorubicin-activated MAPK as measured by the increased phosphorylation of p42 (top). U0126, a MEK inhibitor, blocked basal MAPK activity and the effect of doxorubicin on MAPK phosphorylation (Fig. 4B, top). Figure 4B, bottom, demonstrates that these treatments had little effects on total MAPK protein.

Effects of Activators and Inhibitors of PLC and MAPK on the Expression of *MDR*1. To further delineate the role of PLC-mediated signaling in *MDR*1 expression, we studied the effects of activators and inhibitors of PLC and MAPK. Figure 5A shows that PDGF, an activator of PLC-γ1, increased *MDR*1 promoter activity in a dose-dependent manner in both control and PLC-γ1 transfected cells. The MEK inhibitor, PD98059, decreased the activity of the *MDR*1 promoter in NIH 3T3 cells (Fig. 5B). Doxorubicin also increased the activity of the *MDR*1 promoter (Fig. 6), and this effect was blocked by a MEK inhibitor (Fig. 6).

To determine the biological relevance of these observations, we next assessed the effects of activators and inhibitors of the PLC signaling pathway on the expression of endogenous MDR1 mRNA using a human renal carcinoma cell line. Figure 7 demonstrates that heat shock, PDGF, and EGF increased the expression of MDR1 mRNA by 1.9- to 4.3-fold in HTB-46 cells. Treatment of HTB-46 cells with the PLC inhibitor, U-73122, completely abolished basal and PDGF-stimulated expression of MDR1 mRNA (Fig. 7). The MEK inhibitor, U0126, had little effect on basal *MDR*1 expression but diminished the heat shock-induced expression of endogenous MDR1 mRNA (Fig. 7).

Discussion

Several lines of evidence suggest that PLC-mediated signaling represents one mechanism by which diverse stimuli could activate *MDR*1 gene expression. For example, heat shock (Chin et al., 1990), growth factors (Rohlff and Glazer, 1995), protein kinase C agonists (Chaudhary and Roninson, 1992), heavy metals (Chin et al., 1990), and certain chemotherapeutic drugs (Chaudhary and Roninson, 1993; Abolhoda et al., 1999) induced *MDR*1 expression and were also shown to converge on signal transduction pathways downstream of PLC. Yet, the critical role that PLC might play in this regard had not been investigated. In the current studies, we present evidence that supports a central role for PLC in the regulation of *MDR*1 expression.

Transient transfection of NIH 3T3 cells with a PLC-γ1 expression vector increases intracellular signaling as measured by the production of $Ins(1,4,5)P_3$ (Fig. 1A) and activation of PKC (Fig. 1B). Augmentation of PLC-mediated signaling activates the *MDR*1 promoter (Fig. 1C). These data are consistent with the role of PLC signaling in the transcriptional regulation of other genes. For example, Schalasta and Doppler (1990) demonstrated that PLC activity is necessary for transcriptional activation of c-*fos* and inhibitor of PLC inhibited c-*fos* transcription.

Transcriptional regulation of *MDR*1 expression has been under intense investigation. Numerous transcription factors including NF-IL6 (Combates et al., 1997), YB-1 (Bargou et al., 1997), p53 (Chin et al., 1992; Goldsmith et al., 1995), NF-Y (Jin and Scotto, 1998), Sp1 (Cornwell and Smith, 1993b; Rohlff and Glazer, 1998), and EGR-1 (McCoy et al., 1995) are known to bind to several canonical sequences within the *MDR*1 promoter. For example, the sequences involved in stimulation of the *MDR*1 promoter by wild-type p53 were contained within the region between −39 and +53 (Goldsmith et al., 1995), and the −50 G-box is involved in the activation of basal promoter activity by Sp1 (Goldsmith et al., 1995). In addition, the sequences between −134 and +286 and the Y-box that is located between −82 and −73 are reported to be required for basal promoter activity (Goldsmith et al., 1993; Madden et al., 1993). Using a series of nested deletion constructs, we found that a GC-rich region between −99 and −106 is essential for *MDR*1 promoter ac-

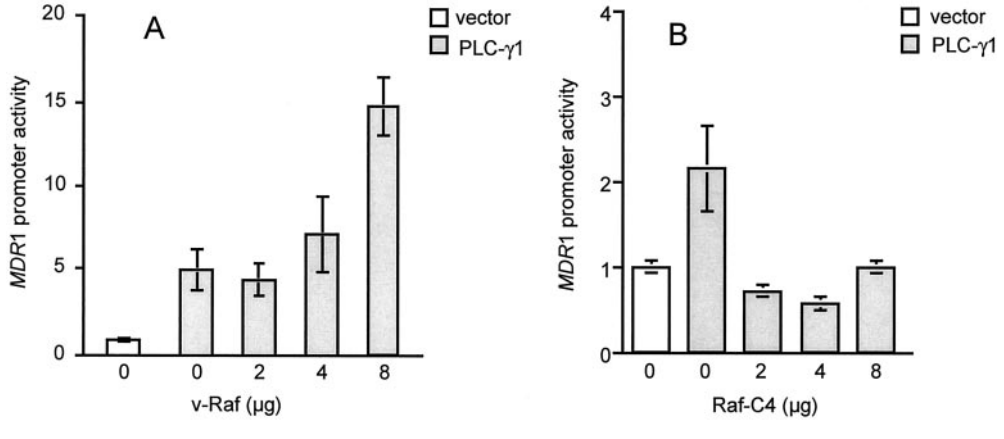

Fig. 3. Effect of wild-type and dominant-negative Raf on PLC-induced activation of the *MDR*1 promoter. NIH 3T3/PLC-γ1 cells were plated in 100-mm cell culture dishes then cotransfected with MDRCAT (1 μg) and v-Raf (A) or Raf-C4 (B) using LipofectAMINE. Forty hours after transfection, CAT activity was measured using equivalent amounts of protein extracts and quantified by scintillation counting of the percentage of acetylated ^{14}C-labeled chloramphenicol. Each bar represents the mean ± S.E. from one of three similar experiments.

tivation after PLC transfection (Fig. 2). This region was shown by Miyazaki et al. (1992) to produce *MDR*1 activation by heat-shock, an effect mediated by the heat-shock factor (Kim et al., 1997). We previously demonstrated that heat shock activates PLC in MDR cell lines (Yang et al., 1995), and Chin et al. (1990) demonstrated that heat-shock activates *MDR*1 gene expression. These results suggest that the effect of heat shock on *MDR*1 expression is mediated through PLC. In contrast, Cornwell and Smith (1993a) found that the transcriptional response to v-Raf required the *MDR*1 promoter region between −69 and −41. Because there are multiple responsive elements in the *MDR*1 promoter, these data suggest that v-Raf might act on more than one site depending on the cellular context and that sequences from −99 to −106 may provide an additional required signal for PLC activation. Deletion of other GC-rich regions of the promoter had no effect on PLC-stimulated activation of *MDR*1 (Fig. 2). Several sites between the basal promoter and −99 to −106 contain elements that control basal transcription but not stimulated transcription of the *MDR*1 promoter. These include a consensus Inr sequence extending from −13 to +23 that was shown to be required for accurate initiation of transcription from this promoter; a NF-R1 site between −56 and −45 believed to contain a repressor binding site, and an inverted Y box at −82 to −73. However, these elements are believed to affect basal rather than stimulated transcription. (Scotto and Egan, 1998).

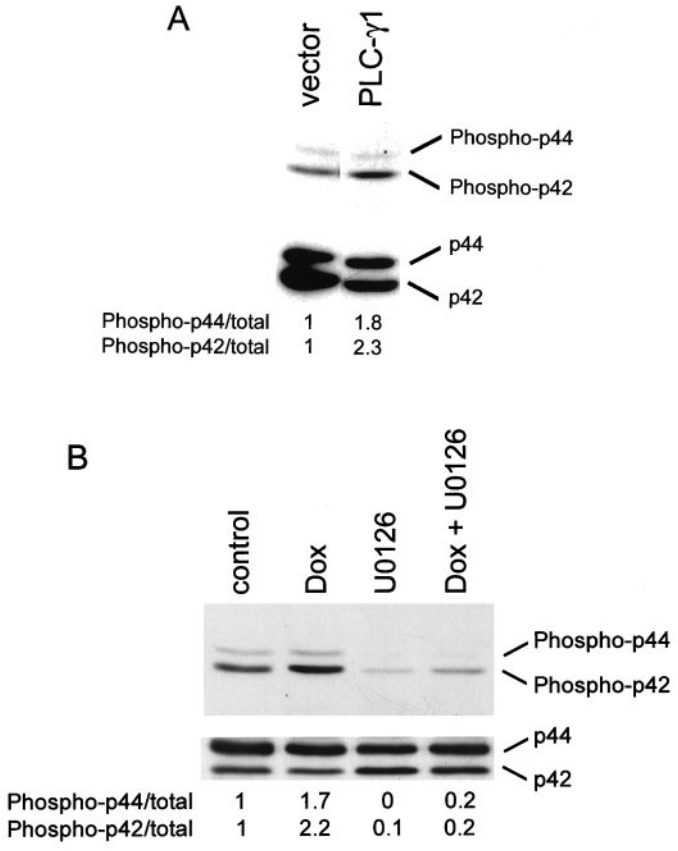

Fig. 4. Effect of PLC, doxorubicin, and MAPK inhibition on the activation of MAPK. NIH 3T3 cells were transfected with the PLC-γ1 expression vector (A) or treated with Dox (100 nM), U0126 (50 μM), or the combination for 48 h (B), then analyzed for phosphorylation of MAPK by Western blot using an anti-phospho-p44/42 MAPK antibody. Results are representative of three similar experiments.

PDGF, an activator of PLC-mediated signaling (Kim et al., 1991), increased *MDR*1 promoter activity in both control and PLC-transfected NIH 3T3 cells (Fig. 5A). At low PDGF concentrations, we did not see augmentation of this response in the PLC-γ1 transfectant. This may not be unexpected because NIH 3T3 cells express abundant PDGF receptors (data not shown) and at low ligand concentrations PLC is unlikely to be important. At high PDGF concentrations, we find a significant augmentation of *MDR*1 promoter activity in the PLC-γ1 transfectant, suggesting that during ligand-receptor occupancy PLC becomes important. In addition, the activation of *MDR*1 is inhibited by PD98059 (Fig. 5B), an inhibitor of MEK activity.

The activation of PLC initiates signals that converge on Raf-dependent pathways. We found that activation of Raf signaling by v-raf, a constitutively active form of the enzyme (Cornwell and Smith, 1993a), increases PLC induced-*MDR*1 promoter activity (Fig. 3A) and that Raf-C4, a dominant-negative raf mutant (Cornwell and Smith, 1993a), blocks this effect (Fig. 3B). These data support those of Cornwell and Smith (1993a) who found that Raf kinase is involved in the activation of the *MDR*1 promoter by serum and growth factors. Furthermore, these data indicate that the effect of certain growth factors on *MDR*1 expression is mediated by PLC, and that Raf is an important downstream component of this PLC-mediated signal transduction pathway.

Activation of Raf stimulates the MEK-MAPK (ERK1/ERK2) cascade, which phosphorylates many transcriptional factors and regulates transcription of a variety of genes (Treisman, 1996). Therefore, we studied the effects of PLC-γ1 on

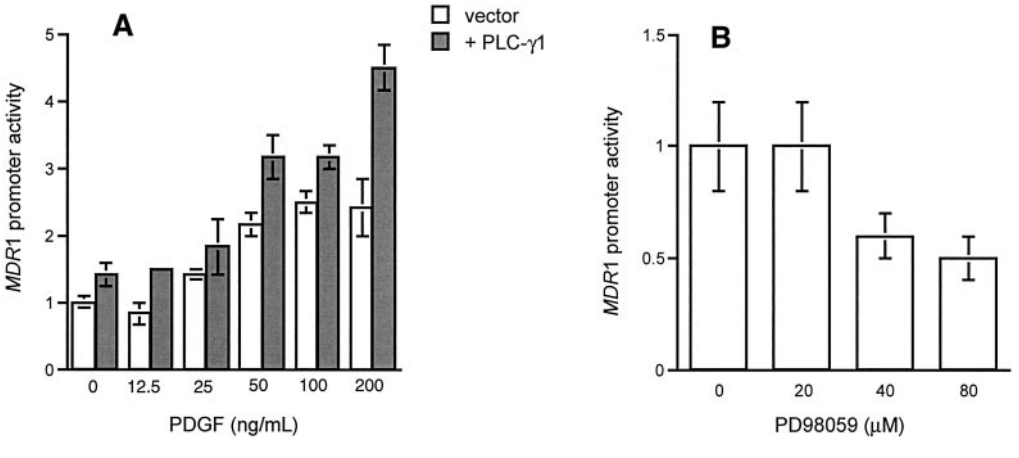

Fig. 5. Effect of PDGF and PD98059 on the activity of the *MDR*1 promoter. A, NIH 3T3 cells were cotransfected with MDRCAT (1 μg) and pMJ30-PLC-γ1 (4 μg), then treated with PDGF (50 ng/ml) for 24 h. B, NIH 3T3 cells were transfected with MDRCAT (1 μg), then treated with different concentrations of PD98059 for 24 h. CAT activity was measured with equivalent amounts of protein extracts and quantified by scintillation counting of acetylated ^{14}C-labeled chloramphenicol. Each point represents the mean ± S.E. from one of three similar experiments.

MAPK activity. As shown in Fig. 4, transient transfection of NIH 3T3 cells with PLC-γ1 increases MAPK activity as measured by phosphorylation of p42 and p44 (Fig. 4A). The MEK inhibitor, U0126, inhibits the phosphorylation of p42 and p44 (Fig. 4B). Our results demonstrate that MAPK (p42/p44) is an important component of PLC-mediated regulation of *MDR*1 expression. These data provide a previously missing link between extracellular stimuli and transcription factors that are known to be involved in the regulation of *MDR*1. Although the roles of those factors in *MDR*1 expression and their binding sites on the *MDR*1 promoter have been studied extensively, the upstream regulators of these factors was poorly understood. Our studies demonstrate that activation of PLC provides a route to *MDR*1 transcription through the PKC-Raf-MAPK and may shed light on how transcription factors are activated by various stimuli. Because PLC is activated by a variety of environmental stresses and the *MDR*1 gene product is required for cell survival, the activation of *MDR*1 by PLC may represent a cellular adaptation mechanism. In fact, a role of Ras-MAPK signaling pathway in promoting cell survival was recently reported by Bonni et al. (1999), who demonstrated a dual mechanism comprising post-translational modification and inactivation of a component of the cell death machinery and increased transcription of prosurvival genes. In addition, Osborn et al. previously reported that stress-activated/c-Jun NH_2-terminal protein kinase, a member of the MAPK family, was activated in response to several chemotherapeutic agents such as doxorubicin, vinblastine, and VP-16 (Osborn and Chambers, 1996). However, they also found that induction of *MDR*1 by phorbol ester occurred independently of the MAPK pathway (Osborn et al., 1999). This difference may reflect the complexity of the signal transduction pathways involved in regulation of *MDR*1 expression and/or the cellular context under investigation.

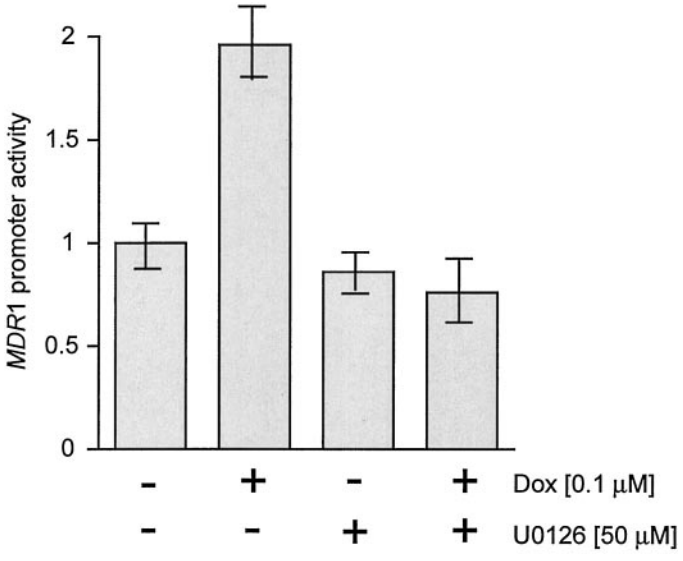

Fig. 6. Effect of doxorubicin and U0126 on the activity of the *MDR*1 promoter. NIH 3T3 cells were transfected with MDRCAT (1 μg), then treated with Dox (100 nM) or U0126 (50 μM), or the combination for 48 h. CAT activity was measured with equivalent amounts of protein extracts and quantified by scintillation counting of acetylated ^{14}C-labeled chloramphenicol. Each bar represents the mean ± S.E. from one of three similar experiments.

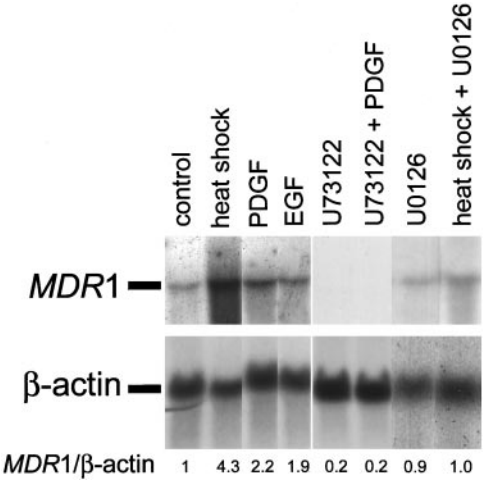

Fig. 7. Effect of activators and inhibitors of PLC-signaling on the expression of the endogenous *MDR*1 gene. HTB-46 cells were heat shocked for 30 min or treated with PDGF (200 ng/ml) or EGF (500 ng/ml) for 2 h in the presence or absence of U73122 (2.5 μM) or U0126 (5 μM). MDR1 mRNA was analyzed by Northern blot. Results are representative of three similar experiments.

The effects of cellular manipulations on promoter-reporter constructs do not necessarily reflect an effect on the endogenous gene. However, we found evidence that supports the role of PLC in expression of MDR1 mRNA in a human renal carcinoma cell line (Fig. 7). Heat-shock, a potent inducer of both *MDR*1 (Chin et al., 1990; Miyazaki et al., 1992) and PLC (Calderwood and Stevenson, 1993), increases MDR1 mRNA in HTB-46 cells; this effect is abrogated by a MEK inhibitor, U0126 (Fig. 7). Similarly, activators of PLC such as PDGF and EGF increase MDR1 mRNA and the PLC inhibitor U73122 blocks this effect (Fig. 7). We also observed that U73122 completely abolished basal expression of MDR1 mRNA (Fig. 7); however, we cannot conclude with certainty that this is fully attributable to inhibition of PLC, because the PLC inhibitor may not be totally selective.

Attempts to overcome MDR with P-gp modulators have had limited success. Several groups have turned to alternative approaches. For example, Futscher et al. found that cotreatment of P-gp(+) cells with P-gp substrates and modulators can prevent the emergence of P-gp(+) cells in the surviving population, suggesting that earlier use of modulators may produce a meaningful therapeutic gain (Futscher et al., 1996). Others have concentrated on the transcriptional regulation of *MDR*1 and on post-transcriptional modification of P-gp as alternative approaches to overcoming drug resistance (Glazer and Rohlff, 1994; Rohlff and Glazer, 1995). It seems now that signal transduction pathways central to cell growth and differentiation can regulate the expression and post-transcriptional modification of *MDR*1 and its gene product, P-gp. The current studies help elucidate the signaling mechanisms that regulate the expression of *MDR*1 gene and identify several targets to potentially prevent emergence of P-gp-expressing cells in tumor populations. In fact, Jin et al. (2000) demonstrated that pharmacologic inhibition of *MDR*1 expression through targeting transcription might be possible. They demonstrated that ecteinascidin 743, a transcription-targeted chemotherapeutic, could abrogate transcriptional activation of both the endogenous *MDR*1 gene and *MDR*1 promoter (Jin et al., 2000). Similarly, one could envision the application of PLC inhibitors to block *MDR*1 activation in response to chemotherapeutic agents.

In summary, our results suggest that a variety of extracellular factors modulate the expression of *MDR*1 through a common PLC-mediated signaling pathway. Inhibition of the component(s) of this pathway might represent an approach to preventing P-gp-mediated drug resistance.

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References

Abolhoda A, Wilson AE, Ross H, Danenberg PV, Burt M, and Scotto KW (1999) Rapid activation of MDR1 gene expression in human metastatic sarcoma after in vivo exposure to doxorubicin. *Clin Cancer Res* **5:**3352–3356.

Altuvia S, Stein WD, Goldenberg S, Kane SE, Pastan I, and Gottesman MM (1993) Targeted disruption of the mouse mdr1b gene reveals that steroid hormones enhance mdr gene expression. *J Biol Chem* **268:**27127–27132.

Bargou RC, Jurchott K, Wagener C, Bergmann S, Metzner S, Bommert K, Mapara MY, Winzer KJ, Dietel M, Dorken B, et al (1997) Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic MDR1 gene expression. *Nat Med* **3:**447–450.

Bonni A, Brunet A, West AE, Datta SR, Takasu MA, and Greenberg ME (1999) Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science (Wash DC)* **286:**1358–1362.

Calderwood SK and Stevenson MA (1993) Inducers of the heat shock response stimulate phospholipase C and phospholipase A2 activity in mammalian cells. *J Cell Physiol* **155:**248–256.

Chaudhary PM and Roninson IB (1992) Activation of *MDR*-1 (P-glycoprotein) gene expression in human cells by protein kinase C agonists. *Oncol Res* **4:**281–290.

Chaudhary PM and Roninson IB (1993) Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J Natl Cancer Inst* **85:**632–639.

Chen CJ, Chin E, Ueda K, Clark CP, Pastan I, Gottesman MM, and Roninson IB (1986) Internal duplication and homology with bacterial transport proteins in the *mdr*1 (P-glycoprotein) gene from multidrug resistant human cells. *Cell* **47:**381–389.

Chin KV, Tanaka S, Darlington G, Pastan I, and Gottesman MM (1990) Heat shock and arsenite increase expression of the multidrug resistance (MDR1) gene in human renal carcinoma cells. *J Biol Chem* **265:**221–226.

Chin KV, Ueda K, Pastan I, and Gottesman MM (1992) Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. *Science (Wash DC)* **255:**459–462.

Combates NJ, Kwon PO, Rzepka RW, and Cohen D (1997) Involvement of the transcription factor NF-IL6 in phorbol ester induction of P-glycoprotein in U937 cells. *Cell Growth Differ* **8:**213–219.

Cornwell MM, Gottesman MM, and Pastan I (1986) Increased vinblastine binding to membrane vesicles from multidrug-resistant KB cells. *J Biol Chem* **261:**7921–7928.

Cornwell MM, Pastan I, and Gottesman MM (1987a) Certain calcium channel blockers bind specifically to multidrug resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. *J Biol Chem* **262:**2166–2170.

Cornwell MM and Smith DE (1993a) A signal transduction pathway for activation of the mdr1 promoter involves the proto-oncogene c-raf kinase. *J Biol Chem* **268:**15347–15350.

Cornwell MM and Smith DE (1993b) SP1 activates the MDR1 promoter through one of two distinct G-rich regions that modulate promoter activity. *J Biol Chem* **268:**19505–19511.

Cornwell MM, Tsuruo T, Gottesman MM, and Pastan I (1987b) ATP-binding properties of P glycoprotein from multidrug-resistant KB cells. *FASEB J* **1:**51–54.

Foxwell BMJ, Mackie A, Ling V, and Ryffel B (1989) Identification of the multidrug resistance-related P-glycoprotein as a cyclosporin binding protein. *Mol Pharmacol* **36:**543–546.

Futscher BW, Foley NE, Gleason-Guzman MC, Meltzer PS, Sullivan DM, and Dalton WS (1996) Verapamil suppresses the emergence of P-glycoprotein-mediated multidrug resistance. *Int J Cancer* **66:**520–525.

Glazer RI and Rohlff C (1994) Transcriptional regulation of multidrug resistance in breast cancer. *Breast Cancer Res Treat* **31:**263–271.

Goldsmith ME, Gudas JM, Schneider E, and Cowan KH (1995) Wild type p53 stimulates expression from the human multidrug resistance promoter in a p53-negative cell line. *J Biol Chem* **270:**1894–1898.

Goldsmith ME, Madden MJ, Morrow CS, and Cowan KH (1993) A Y-box consensus sequence is required for basal expression of the human multidrug resistance (mdr1) gene. *J Biol Chem* **268:**5856–5860.

Gottesman MM, and Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* **62:**385–427.

Hamada H and Tsuruo T (1988) Characterization of the ATPase activity of the M_r 170,000 to 180,000 membrane glycoprotein (P-glycoprotein) associated with multidrug resistance in K562/ADM cells. *Cancer Res* **48:**4926–4932.

Hu Z, Jin S, and Scotto KW (2000) Transcriptional activation of the MDR1 gene by UV irradiation. Role of NF-Y and Sp1. *J Biol Chem* **275:**2979–2985.

Jin S, Gorfajn B, Faircloth G, and Scotto KW (2000) Ecteinascidin 743, a transcription-targeted chemotherapeutic that inhibits MDR1 activation. *Proc Natl Acad Sci USA* **97:**6775–6779.

Jin S, and Scotto KW (1998) Transcriptional regulation of the MDR1 gene by histone acetyltransferase and deacetylase is mediated by NF-Y. *Mol Cell Biol* **18:**4377–4384.

Kamimoto Y, Gatmaitan Z, Hsu J, and Arias IM (1989) The function of Gp170, the multidrug resistance gene product, in rat liver canalicular membrane vesicles. *J Biol Chem* **264:**11693–11698.

Kim HK, Kim JW, Zilberstein A, Margolis B, Kim JG, Schlessinger J, and Rhee SG (1991) PDGF stimulation of inositol phospholipid hydrolysis requires PLC-gamma 1 phosphorylation on tyrosine residues 783 and 1254. *Cell* **65:**435–441.

Kim SH, Hur WY, Kang CD, Lim YS, Kim DW, and Chung BS (1997) Involvement of heat shock factor in regulating transcriptional activation of MDR1 gene in multidrug-resistant cells. *Cancer Lett* **115:**9–14.

Kolch W, Heldecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, and Rapp UR (1993) Protein kinase C alfa activates RAF-1 by direct phosphorylation. *Nature (Lond)* **364:**249–252.

Madden MJ, Morrow CS, Nakagawa M, Goldsmith ME, Fairchild CR, and Cowan KH (1993) Identification of 5′ and 3′ sequences involved in the regulation of transcription of the human mdr1 gene in vivo. *J Biol Chem* **268:**8290–8297.

McCoy C, Smith DE, and Cornwell MM (1995) 12-*O*-tetradecanoylphorbol-13-acetate activation of the MDR1 promoter is mediated by EGR1. *Mol Cell Biol* **15:**6100–6108.

Miyazaki M, Kohno K, Uchiumi T, Tanimura H, Matsuo K, Nasu M, and Kuwano M (1992) Activation of human multidrug resistance-1 gene promoter in response to heat shock stress. *Biochem Biophys Res Commun* **187:**677–684.

Nebigil CG (1997) Suppression of phospholipase C beta, gamma, and delta families alters cell growth and phosphatidylinositol 4,5-bisphosphate levels. *Biochemistry* **36:**15949–15958.

Noh DY, Lee YH, Kim SS, Kim YI, Ryu SH, Suh PG, and Park JG (1994) Elevated content of phospholipase C-gamma 1 in colorectal cancer tissues. *Cancer* **73:**36–41.

Osborn MT, Berry A, Ruberu MS, Ning B, Bell LM, and Chambers TC (1999) Phorbol ester induced MDR1 expression in K562 cells occurs independently of mitogen-activated protein kinase signaling pathways. *Oncogene* **18:**5756–5764.

Osborn MT and Chambers TC (1996) Role of the stress-activated/c-Jun NH2-terminal protein kinase pathway in the cellular response to Adriamycin and other chemotherapeutic drugs. *J Biol Chem* **271:**30950–30955.

Rana RS, and Hokin LE (1990) Role of phosphoinositides in transmembrane signaling. *Physiol Rev* **70:**115–164.

Rohlff C, and Glazer RI (1995) Regulation of multidrug resistance through the cAMP and EGF signalling pathways [published erratum appears in *Cell Signal* 1996;**8:**151]. *Cell Signal* **7:**431–443.

Rohlff C and Glazer RI (1998) Regulation of the MDR1 promoter by cyclic AMP-dependent protein kinase and transcription factor Sp1. *Int J Oncol* **12:**383–386.

Schalasta G and Doppler C (1990) Inhibition of c-fos transcription and phosphorylation of the serum response factor by an inhibitor of phospholipase C-type reactions. *Mol Cell Biol* **10:**5558–5561.

Scotto K, and Egan D (1998) Transcriptional regulation of MDR1 genes. *Cytotechnol* **27:**257–269.

Treisman R (1996) Regulation of transcription by MAP kinase cascades. *Curr Opin Cell Biol* **8:**205–215.

Yang JM, Chin KV, and Hait WN (1995) Involvement of phospholipase C in heat-shock-induced phosphorylation of P-glycoprotein in multidrug resistant human breast cancer cells. *Biochem Biophys Res Commun* **210:**21–30.

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