Raf-1 and Bcl-2 Induce Distinct and Common Pathways That Contribute to Breast Cancer Drug Resistance[1]

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ABSTRACT

Overexpression of Bcl-2 plays a role in the development of drug resistance in leukemia and other apoptosis-prone tumors. Raf isoforms are serine/threonine kinases that act as signal transducers in cascades initiated by many growth factors and mitogens. Raf isoform activation has been linked to drug resistance in leukemia. In this study we investigated effects of Bcl-2 and Raf-1 on doxorubicin-induced growth inhibition of MCF-7 breast cancer cells. In the absence of doxorubicin, overexpression of Bcl-2 or a constitutively active form of Raf-1 in MCF-7 cells did not affect proliferation rate. Overexpression of Bcl-2 increased resistance of MCF-7 cells to doxorubicin in 2-day, 5-day, and 8-week assays. Analysis of doxorubicin sensitivity of individual MCF/Bcl-2 clones showed that doxorubicin resistance was positively correlated with level of Bcl-2 overexpression. Overexpression of constitutively active Raf-1 also increased resistance to doxorubicin. Induction of Raf-1 activity in MCF-7 cells overexpressing Bcl-2 resulted in greater doxorubicin resistance than induction of Raf-1 activity in MCF-7 cells lacking Bcl-2 overexpression. Furthermore, levels of P-glycoprotein mRNA were increased in MCF-7 cells overexpressing a constitutively active Raf-1. MCF-7 cells overexpressing constitutively active Raf-1 were also more resistant to paclitaxel, which, like doxorubicin, is a substrate of P-glycoprotein. These observations suggest both independent and overlapping roles for Raf-1 and Bcl-2 oncogenes in the resistance to growth inhibition by doxorubicin.

INTRODUCTION

The development of resistance by cancer cells to a wide variety of chemotherapeutic agents poses a major obstacle in the successful treatment of cancer. Drug resistance is observed in a broad range of cell types from breast and prostate to leukemic blasts (1). Many mechanisms contribute to drug resistance, including drug inactivation, extrusion of the drug by cell membrane pumps, mutations of drug targets, and failure to initiate apoptosis (2–5). Prevention of apoptosis can result from a variety of conditions, including retention of the mitochondrial membrane potential and cytokine stimulation (4, 6, 7). A more detailed understanding of drug resistance mechanisms in breast cancer may improve the success rate for many already developed chemotherapeutic agents by forming a basis for the design of adjuvant therapy.

The search for proteins responsible for drug-resistant phenotypes has implicated the antiapoptotic molecule Bcl-2. Bcl-2 belongs to a family of proteins regulating apoptosis that includes both proapoptotic and antiapoptotic members (8, 9). These proteins are believed to modulate apoptosis by forming homodimers or heterodimers with other Bcl-2 family members (10–15). Association of Bcl-2 family proteins with proteins outside the Bcl-2 family is another mechanism by which these proteins are regulated (16). Although a precise understanding of how Bcl-2 exerts its antiapoptotic effects remains elusive, it has been found to be overexpressed in many cancers including colorectal, prostate, and 70% of all breast cancers (12, 17, 18).

In addition to Bcl-2, other proteins have been linked with resistance to chemotherapeutic drugs. Raf isoforms are intermediates in signal transduction cascades initiated by growth factors (19–21). They exert their effects in part through the highly conserved Raf/MEK[3]/ERK pathway (22–25). A cascade of kinase activation occurs after the cognate receptor is ligated (19, 23, 25–27). Raf isoforms have been reported to increase expression of certain proteins, including $p21^{Cip1}$ (M_r 21,000 cyclin-dependent kinase-interacting protein) and c-Myc, when activated (28). Some reports have suggested that this signal transduction pathway may be involved in the regulation of

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[3] The abbreviations used are: MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; P-gp, P-glycoprotein; FBS, fetal bovine serum; AR, androgen receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TBST, 25 mM Tris (pH 8.0), 125 mM NaCl, and 0.025% Tween; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NCI/ADR-RES, National Cancer Institute/Adriamycin-resistant; PMA, phorbol 12-myristate 13-acetate.

several aspects of drug resistance (29–32). For instance, the Raf/MEK/ERK pathway regulates expression of the P-gp extrusion pump (33, 34). Moreover, a correlation between high Raf-1 activity and resistance to paclitaxel has been reported in early passage human cervical tumors (35). The Raf/MEK/ERK pathway has also been shown to synergize with Bcl-2 overexpression to increase the frequency of cytokine-independent hematopoietic cells (36, 37). This synergism may result in part from enhancement of Bcl-2 antiapoptotic function attributable to phosphorylation of serine 70 by ERK-1 and ERK-2 (38).

The purpose of this study was to examine the role of the Bcl-2 protein in the modulation of drug resistance and to assess the interaction between Bcl-2 and Raf-1 in conferring drug resistance. A correlation between increased Bcl-2 expression and resistance to doxorubicin was observed in MCF-7/Bcl-2 cells. Ectopic Raf-1 activity increased Bcl-2 expression and doxorubicin resistance. Raf-1 activation increased P-gp expression, whereas Bcl-2 overexpression did not. Thus, although Raf-1 and Bcl-2 overexpression both contribute to drug resistance, Raf-1 can induce additional signaling pathways to cause drug resistance.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells (American Type Culture Collection, Manassas, VA) and their retrovirally infected counterparts were grown in RPMI 1640 (Life Technologies, Inc., Bethesda, MD) supplemented with 5% FBS (Atlanta Biologicals, Atlanta, GA), 2 mM glutamine, 100 units/liter penicillin, and 100 μg/ml streptomycin. Cells were cultured in a humidified incubator with a 5% CO_2 atmosphere. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise indicated. Testosterone stock was 1 mM in ethanol, and doxorubicin stock was 5 mM in sterile water.

Retroviral Infection. Plasmid DNAs encoding recombinant retroviruses were transfected into the retroviral packaging cell line PA317 with Lipofectin (Life Technologies, Inc.), and retroviruses were passed sequentially from the retroviral packaging PA317 and φ2 cell lines to amplify their titers (39–41). MCF-7 cells were infected with viral stocks prepared from PA317 cells (42). The following retroviral constructs were used: (*a*) pN2-M-Bcl-2 contains a fusion between the SV40 early enhancer/promoter region and murine Bcl-2 cDNA inserted into retroviral vector pN2 encoding *neo*^{r} (neomycin resistance gene; Ref. 43); (*b*) pLNCΔRaf-1 contains human ΔRaf-1 cDNA encoding an amino terminally truncated form of Raf-1 that is constitutively active, which is inserted into retroviral vector pLNCX encoding *neo*^{r} (44); and (*c*) pLNTAR305ΔRC contains ΔRaf-1:AR cDNA encoding ΔRaf-1 fused to the steroid-binding domain of the human AR that can be used to conditionally activate Raf-1 with testosterone, which is inserted into the pLNSX3 retroviral vector encoding *puro*^{r} (puromycin resistance gene; Ref. 45). MCF-7 cells were infected with the pZipneo retrovirus, which is an empty retroviral vector encoding *neo*^{r}. MCF-7 cells with *neo*^{r} or *puro*^{r} were isolated by selection in medium containing 2 mg/ml G418 (Life Technologies, Inc.) or 2 μg/ml puromycin, respectively. Nomenclature of MCF-7 cells infected with various oncogenes is MCF/Bcl-2, MCF/ΔRaf-1:AR, MCF/pZipneo, and MCF/ΔRaf-1:AR+Bcl-2 for cells infected with Bcl-2, ΔRaf-1:AR, pZipneo, and ΔRaf-1:AR+Bcl-2 retroviruses, respectively.

IC_{50} Assay. Cells were seeded in 96-well flat-bottomed plates at 5000 cells/well and then grown for 2 days in medium containing various concentrations of doxorubicin. Each condition was assayed in quadruplicate. After doxorubicin exposure, medium containing doxorubicin was replaced with drug-free medium for 1 day to allow viable cells to proliferate. Next, cells were fixed with 60% tricloroacetic acid for 1 h and then extensively washed with water. Washed cells were stained with 0.4% sulforhodamine for 10 min and then washed with 1% acetic acid (46). Stained cells were dried, and then sulforhodamine was solubilized in 10 mM Tris (pH 10.5). Absorbance was measured with an Anthos (Anthos Labtec Instruments, Salzburg, Austria) plate reader at 540 nm. Whenever effects of testosterone were studied, charcoal-stripped FBS and phenol red-free RPMI 1640 were used to minimize possible androgenic effects of either steroids present in FBS or phenol red in medium.

Cell Proliferation Assay. Cells were seeded in 24-well plates at a density of 10^5 cells/well in the absence or presence of 50, 500, or 5000 nM doxorubicin. Cells were recovered with trypsin and EDTA (Life Technologies, Inc.) and washed with 5 ml of PBS. Cells were stained with trypan blue and counted with a hemacytometer. Cells were counted for 5 days in quadruplicate, and SEs were calculated.

Long-term Cell Viability Assay. MCF-7 or MCF/Bcl-2[pool] cells were seeded in 96-well plates at a density of 100 cells/well. Cells were cultured in the absence or presence of 1, 10, 100, or 1000 nM doxorubicin for 8 weeks. Cell culture medium was replaced every 3 days. The presence or absence of viable cells in each well was determined by light microscopy.

RNA Isolation. Total RNA was isolated with TRIzol (Life Technologies, Inc.). Pelleted cells were resuspendended in TRIzol and then incubated at room temperature for 5 min to permit disassociation of nucleoprotein complexes. Cellular debris was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Samples were extracted with chloroform and then precipitated for 10 min at 4°C with isopropanol. Samples were centrifuged at 12,000 × *g* for 10 min at 4°C. Pellets were washed with 75% ethanol and then recovered by centrifugation at 7,500 × *g* for 5 min at 4°C. RNA samples were air dried for 10 min and then resuspended in RNase-free water. RNA concentration and purity were determined using an Ultrospec III spectrophotometer (Pharmacia LKB Biochrom Ltd., Cambridge, United Kingdom). RNA samples were stored in aliquots at −70°C until used in RT-PCR experiments.

RT-PCR. For cDNA synthesis, 1 μg of total RNA was used. RNA was reverse transcribed in 10 μl of reverse transcriptase buffer containing 20 mM deoxynucleoside triphosphates, 20 μg/ml oligo-2′-deoxythymidine 5′-triphosphate, and 20 units of Moloney murine leukemia virus reverse transcriptase. After incubation at 42°C for 35 min, 40 μl of PCR mix were added, containing PCR buffer, deoxynucleoside triphosphates, 1 unit of Taq polymerase, and 5 pmol of each oligonucleotide primer. Oligonucleotide sequences for each primer are as follows: Bcl-2, 5′-CGACGACTTCTCCCGCCGCTACCGC-3′ (sense) and 5′-CCGCATGCTGGGGCCGTACAGTTCC-3′ (antisense); Bax, 5′-ATGGACGGGTCCGGGGAGCAGCCC-3′ (sense) and 5′-GGTGAGCACTCC-

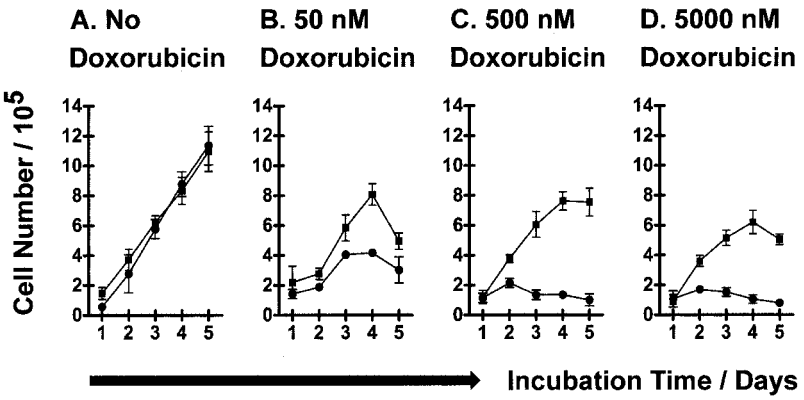

Fig. 1 Overexpression of Bcl-2 in MCF-7 cells increases resistance to doxorubicin. Proliferation of MCF/pZipneo (●) and MCF/Bcl-2[pool] (■) cells in the absence or presence of the indicated concentrations of doxorubicin: *A*, proliferation in the absence of doxorubicin; and *B–D*, proliferation in the presence of 50, 500, and 5000 nM doxorubicin, respectively. Cells were incubated in 24-well plates with RPMI 1640 containing 5% FBS. Initially, there were 10^5 cells/well. Cells were collected after treatment with trypsin and counted with a hemocytometer daily for 5 days in quadruplicate. *Error bars*, SE.

CGCCACAAAGAT-3′ (antisense); P-gp, 5′-CCCATCATTGCAATAGCAGG-3′ (sense) and 5′-GTTCAAACTTCTGCTCCTGA-3′ (antisense); and GAPDH, 5′-CGATGCTGGCGCTGAGTAC-3′ (sense) and 5′-CGTTCAGCTCAGGGATGAC-3′ (antisense).

PCR conditions were 28 cycles of denaturation at 94°C for 60 s, annealing at 60°C for 90 s, extension at 72°C for 90 s, and final extension at 72°C for 10 min. PCR products were electrophoresed in a 1% agarose gel containing 500 μg/liter ethidium bromide and visualized with UV light.

Western Blot Analysis. Cells were trypsinized, washed with RPMI 1640, and then centrifuged at 500 × *g* for 10 min at 4°C. Pellets containing 10^6 cells were resuspended in 100 μl of gold lysis buffer [25 mM Tris (pH 7.4), 50 mM NaCl, 0.5% sodium deoxycholate, 2% NP40, 0.2% SDS, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 50 μM leupeptin, and 500 μM sodium orthovanadate] and then shaken at 4°C for 15 min. Lysates were centrifuged at 16,000 × *g* for 15 min at 4°C to pellet cellular debris. Supernatant aliquots of 98 μl were mixed with 42 μl of sample buffer [200 mM Tris (pH 6.8), 33% glycerol, 6.6% SDS, 16.6% 2-mercaptoethanol, and 0.04% bromphenol blue]. Protein samples were boiled for 5 min then stored at −70°C. Protein samples were subjected to SDS-PAGE with 10% polyacrylamide. Proteins were transferred to polyvinylidene difluoride membranes (Micron Separations, Westbrough, MA), which were then incubated overnight in blocking buffer [25 mM Tris (pH 8.0), 125 mM NaCl, 0.1% sodium azide, 0.1% Tween, and 1% BSA] at 4°C. The next day, membranes were incubated for 2 h with anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA), antiactin (Santa Cruz Biotechnology), anti-phospho-ERK-1/2 (Cell Signaling Technology, Beverly, MA), anti-ERK-2 (Santa Cruz Biotechnology), or anti-$p21^{Cip1}$ (Oncogene Research, San Diego, CA) antibodies diluted 1:100, 1:15,000, 1:1,000, 1:200, or 1:100 in blocking buffer, respectively. Actin is a housekeeping protein used as a control. After 2 h, the membranes were washed three times with TBST and then incubated for 1 h with alkaline phosphatase-conjugated goat antirabbit IgG (Promega, Madison, WI) diluted 1:10,000 in TBST. Membranes were then washed three times in TBST and developed using the Promega ProtoBlot alkaline phosphatase system.

MTT Assay. MCF-7 cells were seeded in 96-well flat-bottomed plates at 2000 cells/well. Cells were incubated for 3 days in the absence or presence of the indicated concentrations of paclitaxel in 200 μl of medium. Cells were incubated for 4 h with 20 μl of 5 mg/ml MTT in PBS. After removal of the medium, 170 μl of DMSO were added to each well to dissolve formazan crystals. Absorbance at 540 nm was measured with a Biokinetics plate reader (Bio-Tek Instruments, Inc., Winooski, VT). Triplicate wells were assayed for each condition, and SDs were determined.

RESULTS

To determine the role of Bcl-2 in modulation of drug resistance, first proliferation was compared in MCF/pZipneo and MCF/Bcl-2[pool] cells, which were infected with retroviruses containing either an empty vector or a vector encoding Bcl-2, respectively. Fig. 1*A* shows that both MCF/pZipneo and MCF/Bcl-2[pool] cells had similar growth rates, thus, Bcl-2 overexpression had no significant effects on proliferation over a 5-day period.

Effects of doxorubicin were examined to determine whether Bcl-2 overexpression enabled MCF-7 cells to proliferate in the presence of doxorubicin. For Fig. 1, MCF/pZipneo and MCF/Bcl-2[pool] cell growth was compared in the absence or presence of 5000, 500, or 50 nM doxorubicin. Treatment with the highest concentration of doxorubicin (5000 nM; Fig. 1*D*) decreased growth of both MCF/pZipneo and MCF/Bcl-2[pool] cells; however, whereas MCF/pZipneo cell growth was completely blocked, MCF/Bcl-2[pool] cell growth was decreased by only 37%. Fig. 1*C* shows growth after treatment with a 10-fold lower concentration of doxorubicin (500 nM). At this concentration, MCF/pZipneo cells still did not grow, whereas MCF/Bcl-2[pool] cell growth decreased only 21%. Fig. 1*B* shows the data for cells treated with a 100-fold lower concentration (50 nM) of doxorubicin than that of the cells illustrated in Fig. 1*D*. At this low dosage, MCF/pZipneo cells still demonstrated a 56% decrease in proliferation, whereas MCF/Bcl-2[pool] cell growth decreased only 27%. These percentages were calculated from average growth over 5 days. Thus, Bcl-2 overexpression increases the ability of MCF-7 cells to grow in the presence of doxorubicin.

To further examine whether overexpression of Bcl-2 in MCF-7 cells has a protective effect, clones were isolated from the MCF/Bcl-2[pool] cells by limiting dilution, and their sensi-

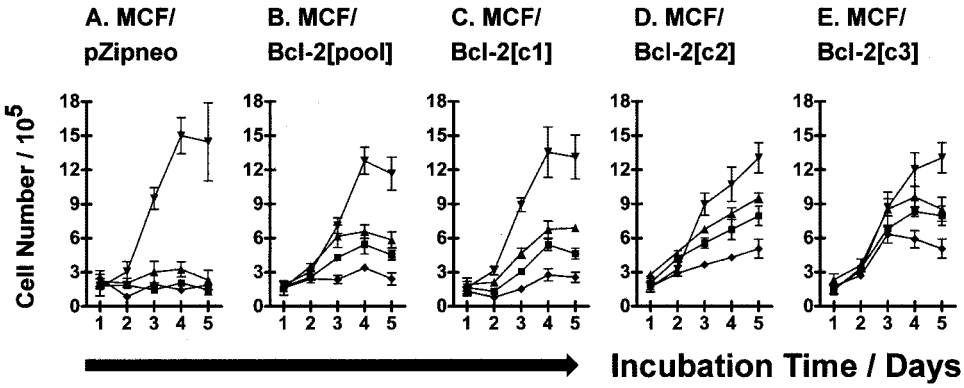

Fig. 2 Effect of Bcl-2 overexpression on doxorubicin sensitivity of individual Bcl-2-overexpressing clones. *A–E*, proliferation of MCF/pZipneo, MCF/Bcl-2[pool], MCF/Bcl-2[c1], MCF/Bcl-2[c2], and MCF/Bcl-2[c3] cells, respectively, in the absence or presence of the indicated concentrations of doxorubicin. Cells were cultured in the absence of doxorubicin (▼) or presence of 50 (▲), 500 (■), or 5000 nM (♦) doxorubicin. Cells were incubated in 24-well plates with RPMI 1640 containing 5% FBS. Initially there were 10^5 cells/well. Cells were collected after treatment with trypsin and counted with a hemocytometer daily for 5 days in quadruplicate. *Error bars*, SE.

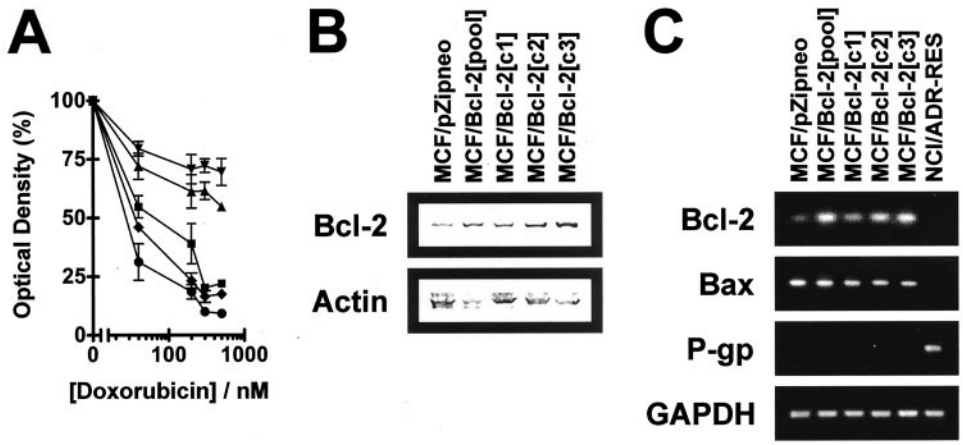

Fig. 3 Doxorubicin IC_{50} determination and Bcl-2 expression in individual Bcl-2-overexpressing clones. *A*, cell proliferation was assayed in quadruplicate with sulforhodamine for MCF/pZipneo (●), MCF/Bcl-2[pool] (■), MCF/Bcl-2[c1] (♦), MCF/Bcl-2[c2] (▲), and MCF/Bcl-2[c3] (▼) cells cultured for 2 days in the absence or presence of the indicated concentrations of doxorubicin. Mean absorbance for cells treated with each concentration of doxorubicin is plotted as a percentage of mean absorbance for cells not treated with doxorubicin. Doxorubicin concentration is plotted on a $\log_{10}$ scale. *Error bars*, SD. *B*, Western blot analysis of Bcl-2 and actin protein levels in MCF/pZipneo, MCF/Bcl-2[pool], MCF/Bcl-2[c1], MCF/Bcl-2[c2], and MCF/Bcl-2[c3] cells. *C*, RT-PCR analysis of Bcl-2, Bax, P-gp, and GAPDH mRNA levels in MCF/pZipneo, MCF/Bcl-2[pool], MCF/Bcl-2[c1], MCF/Bcl-2[c2], MCF/Bcl-2[c3], and NCI/ADR-RES cells.

tivities to doxorubicin were examined. Growth assays were performed in the absence and presence of 50, 500, or 5000 nM doxorubicin for MCF/pZipneo (Fig. 2*A*), MCF/Bcl-2[pool] (Fig. 2*B*), MCF/Bcl-2[c1] (Fig. 2*C*), MCF/Bcl-2[c2] (Fig. 2*D*), and MCF/Bcl-2[c3] cells (Fig. 2*E*). Treatment with the highest concentration of doxorubicin resulted in a decrease in growth by all MCF-7 cell lines; however, MCF/pZipneo cells demonstrated the most growth inhibition, with a proliferation decrease of 6.0-fold (Fig. 2*A*). MCF/Bcl-2[c3] exhibited the least amount of growth inhibition in 5000 nM doxorubicin, with only a 1.8-fold decrease in growth (Fig. 2*E*). After treatment with 500 or 50 nM doxorubicin, similar results were observed. In comparing the MCF/Bcl-2 clones, MCF/Bcl-2[c1] was the most sensitive to doxorubicin, whereas MCF/Bcl-2[c2] and MCF/Bcl-2[c3] were found to be more drug resistant.

Next, the doxorubicin IC_{50} was determined in MCF/pZipneo and MCF/Bcl-2[pool] cells (Fig. 3). Fig. 3*A* shows the dose-response effect of doxorubicin on growth inhibition of MCF/pZipneo and MCF/Bcl-2[pool] cells. Doxorubicin concentrations ranged from 40 to 500 nM. MCF/Bcl-2[pool] cells had a doxorubicin IC_{50} of 100 nM, whereas MCF/pZipneo cells had a doxorubicin IC_{50} that was 5-fold lower at 20 nM.

Dose-response effects of doxorubicin treatment on survival were also determined for MCF/Bcl-2[c1], MCF/Bcl-2[c2], and MCF/Bcl-2[c3] cells to assess their sensitivity to this drug (Fig. 3*A*). When comparing doxorubicin IC_{50} values relative to MCF/pZipneo, MCF/Bcl-2[pool] cells had a 5.0-fold increase, whereas MCF/Bcl-2[c1] cells had only a 2.5-fold increase. Growth inhibition percentages of MCF/Bcl-2[c2] and MCF/Bcl-2[c3] cells never decreased below 50%, even at the highest

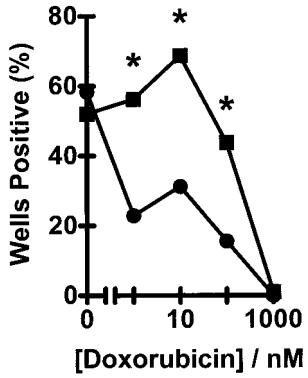

Fig. 4 Overexpression of Bcl-2 induces long-lasting resistance to doxorubicin. MCF-7 (●) and MCF/Bcl-2 (■) cells were seeded in 96-well plates at a density of 100 cells/well. Cells were cultured in the absence or presence of the indicated concentrations of doxorubicin. After 8 weeks of culture, the presence or absence of viable cells in each well was determined by light microscopy. At each concentration of doxorubicin, 96 wells containing MCF-7 or MCF/Bcl-2[pool] cells were assayed. The percentage of wells positive for viable cells is plotted for each concentration of doxorubicin. *Asterisks* denote statistically significant differences between proportion of wells positive for viable MCF/Bcl-2[pool] cells and proportion of wells positive for viable MCF-7 cells ($P < 0.001$). Doxorubicin concentration is plotted on a $\log_{10}$ scale.

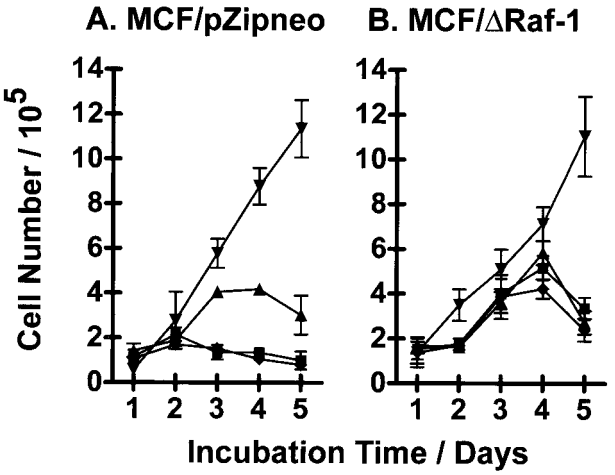

Fig. 5 Proliferation of MCF/pZipneo and MCF/ΔRaf-1 cells. *A* and *B*, proliferation of MCF/pZipneo and MCF/ΔRaf-1 cells, respectively. MCF-7 transfectants were grown in the absence of doxorubicin (▼) or in the presence of 50 (▲), 500 (■), or 5000 nM (♦) doxorubicin. Cells were cultured in 24-well plates with RPMI 1640 containing 5% FBS. Initially, there were 10^5 cells/well. Cells were collected and counted with a hemocytometer daily for 5 days. Each MCF-7 transfectant was tested in quadruplicate. *Error bars*, SE.

concentration of doxorubicin assayed (500 nM). Taken together, these results indicate that MCF/Bcl-2[c2] and MCF/Bcl-2[c3] cells are more resistant to doxorubicin than MCF/Bcl-2[pool] cells.

To determine whether Bcl-2 protein expression differed among MCF/pZipneo, MCF/Bcl-2[pool], MCF/Bcl-2[c1], MCF/Bcl-2[c2], and MCF/Bcl-2[c3] cells, Western blot analysis was performed (Fig. 3*B*). Densitometry analysis normalized to actin levels showed that Bcl-2 protein expression was 1.6-fold higher in MCF/Bcl-2[pool] cells than in MCF/pZipneo cells. Both MCF/Bcl-2[c2] and MCF/Bcl-2[c3] clones had higher Bcl-2 protein levels than MCF/pZipneo cells. Conversely, MCF/Bcl-2[c1] cells had a higher Bcl-2 protein level than MCF/pZipneo cells and a lower Bcl-2 protein level than MCF/Bcl-2[pool] cells. Thus, higher levels of Bcl-2 expression were correlated with increased capacity of these cells to grow in the presence of doxorubicin.

Expression of Bcl-2, Bax, and P-gp mRNAs was also investigated by RT-PCR to compare their levels among each MCF-7 cell line (Fig. 3*C*). NCI/ADR-RES cells were used as a control because these cells express P-gp mRNA. All lanes had relatively equal amplification of the housekeeping gene GAPDH, implying that equal amounts of each mRNA were used in these experiments. Bcl-2 mRNA expression was increased in MCF/Bcl-2[pool] and in both MCF/Bcl-2[c2] and MCF/Bcl-2[c3] clones. In lanes containing MCF/pZipneo and MCF/Bcl-2[c1] RT-PCR products, less Bcl-2 mRNA was detected. Bcl-2 mRNA was not detected in NCI/ADR-RES cells.

In contrast to the variation in Bcl-2 mRNA levels detected, relatively equal levels of Bax mRNA were detected in all MCF-7 transfectants, but not in NCI/ADR-RES cells, in which no Bax mRNA was detected. The levels of P-gp mRNA were examined in these cells to assess whether P-gp was expressed in response to Bcl-2 overexpression. P-gp transcripts were not detected in any MCF-7 transfectant but were detected in the NCI/ADR-RES cell line.

Proliferation assays lasting 5 days demonstrated induction of doxorubicin resistance by Bcl-2 overexpression (Figs. 1 and 2), but these assays did not show whether such drug resistance was long-lasting or, instead, whether Bcl-2 merely delayed cellular responses to doxorubicin. To examine this question, effects of Bcl-2 overexpression on long-term viability of MCF-7 cells in the presence and absence of doxorubicin were determined. MCF-7 and MCF/Bcl-2[pool] cells seeded at a density of 100 cells/well were cultured in the presence or absence of 1, 10, 100, or 1000 nM doxorubicin for 8 weeks in 96-well plates.

In the absence of doxorubicin, approximately 55% of wells seeded with either MCF-7 or MCF/Bcl-2[pool] cells were positive for viable cells after 8 weeks (Fig. 4). However, in the presence of 1, 10, or 100 nM doxorubicin, wells seeded with MCF/Bcl-2[pool] cells had a higher proportion of wells positive for viable cells than wells seeded with MCF-7 cells. At these three concentrations, wells seeded with MCF/Bcl-2[pool] cells had a likelihood of containing viable cells after 8 weeks that ranged from approximately 45% to 65%. In contrast, wells seeded with MCF-7 cells had a likelihood of containing viable cells after 8 weeks that ranged from approximately 15% to 30% at these three concentrations. These differences were determined to be statistically significant ($P < 0.001$). Only 1 of 96 wells seeded with MCF/Bcl-2[pool] cells was positive for viable cells after 8 weeks of treatment with 1000 nM doxorubicin. In contrast, none of 96 wells seeded with MCF-7 cells were positive for viable cells under these conditions. These data suggest that overexpression of Bcl-2 induces long-lasting doxorubicin resistance in MCF-7 cells as opposed to a short-term delay in cellular drug response.

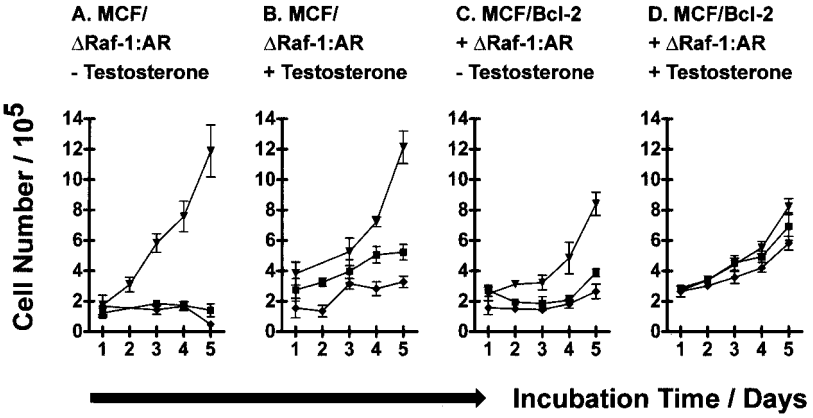

Fig. 6 Induction of Raf-1 activity with testosterone in MCF/ΔRaf-1:AR cells induces doxorubicin resistance that can be increased by overexpression of Bcl-2. *A* and *B*, proliferation of MCF/ΔRaf-1:AR cells in the absence or presence of 500 nM testosterone, respectively, in the absence or presence of the indicated concentrations of doxorubicin. *C* and *D*, proliferation of MCF/ΔRaf-1:AR+Bcl-2 cells in the absence or presence of 500 nM testosterone, respectively, in the absence or presence of the indicated concentrations of doxorubicin. Cells were cultured in the absence of doxorubicin (▼) or in the presence of 500 (■), or 5000 nM (◆) doxorubicin. Cells were seeded in 24-well plates at a density of 10^5 cells/well. Cells were collected after treatment with trypsin and counted daily with a hemocytometer for 5 days in quadruplicate. *Error bars*, SE.

We have shown previously that expression of Raf-1 can increase the resistance of MCF-7 cells to doxorubicin (47). Hence, we investigated effects of Raf-1 on growth of MCF-7 cells in the presence of doxorubicin. To determine effects of Raf-1 on cell proliferation, growth assays were performed on MCF/pZipneo (Fig. 5*A*) and MCF/ΔRaf-1 (Fig. 5*B*) cells. Both MCF-7 transfectants were incubated in the absence or presence of 50, 500, or 5000 nM doxorubicin. When doxorubicin was absent, all MCF-7 transfectants had similar proliferation rates over a period of 5 days. However, with increasing concentrations of doxorubicin, growth of MCF/pZipneo cells decreased dramatically. Both 5000 and 500 nM doxorubicin reduced MCF/pZipneo cell number 4.5-fold after 5 days. The lowest concentration of doxorubicin, 50 nM, decreased MCF/pZipneo cell growth an average of only 2.0-fold.

MCF/ΔRaf-1 cells demonstrated similar decreases in growth in response to all three doxorubicin concentrations. When treated with 5000 nM doxorubicin, average growth decreased by 2.0-fold after 5 days *versus* growth in the absence of doxorubicin. Treatment with either 500 or 50 nM doxorubicin resulted in decreases of less than 1.5-fold.

To determine whether interactions between Raf-1 and Bcl-2 had an effect on the ability of cells to grow in the presence of doxorubicin, growth assays were performed on the MCF/ΔRaf-1:AR and MCF/ΔRaf-1:AR+Bcl-2 cells in the absence (Fig. 6, *A* and *C*) or presence (Fig. 6, *B* and *D*) of testosterone, which activates ΔRaf-1:AR. Both cell lines were incubated with different concentrations of doxorubicin. When no drugs were added, all cell lines had approximately the same amount of growth over a period of 5 days. However, with increasing concentrations of doxorubicin, growth of both MCF/ΔRaf-1:AR and MCF/ΔRaf-1:AR+Bcl-2 cells decreased.

In response to treatment with 5000 nM doxorubicin, MCF/ΔRaf-1:AR cells, in the absence of testosterone, demonstrated a >4.5-fold decrease in growth compared with no doxorubicin, whereas MCF/ΔRaf-1:AR+Bcl-2 cell number decreased by only 2.4-fold, illustrating the protective effects of Bcl-2. In response to treatment with 500 nM doxorubicin, growth of MCF/ΔRaf-1:AR+Bcl-2 and MCF/ΔRaf-1:AR cells was reduced by 1.7-fold and >4.5-fold, respectively, in the absence of testosterone. Thus, Bcl-2 had a protective effect in the presence of doxorubicin on the MCF/ΔRaf-1:AR+Bcl-2 cells in the absence of Raf-1 activation.

Table 1 Inhibition of MCF/ΔRaf-1:AR and MCF/ΔRaf-1:AR + Bcl-2 cell growth by doxorubicin in the presence or absence of testosterone

Cell line	± 500 μM Testosterone	Doxorubicin 500 nM	Doxorubicin 5000 nM
MCF/ΔRaf-1:AR	+	1.7-fold	3.1-fold
MCF/ΔRaf-1:AR	−	>4.5-fold	>4.5-fold
MCF/ΔRaf-1:AR + Bcl-2	+	0.0-fold	1.3-fold
MCF/ΔRaf-1:AR + Bcl-2	−	1.7-fold	2.4-fold

When MCF/ΔRaf-1:AR and MCF/ΔRaf-1:AR+Bcl-2 were cultured in the presence of testosterone, which activates the ΔRaf-1:AR (47), cells were more resistant to doxorubicin. When treated with 5000 nM doxorubicin and incubated with testosterone, MCF/ΔRaf-1:AR cell growth decreased 3.1-fold, whereas MCF/ΔRaf-1:AR+Bcl-2 cell growth only decreased 1.3-fold. Treatment with the lower concentration of doxorubicin yielded a 1.7-fold decrease in MCF/ΔRaf-1:AR cell growth, and in MCF/ΔRaf-1:AR+Bcl-2 cells there was no significant change in growth rate over a 5-day period. Decrease in growth of each MCF-7 transfectant in response to different treatments was calculated as described previously and is presented in Table 1. Thus, Bcl-2 and Raf-1 overexpression each increased the ability of MCF-7 cells to grow in the presence of doxorubicin.

Levels of Bcl-2, Bax, and P-gp mRNAs were analyzed by RT-PCR for MCF/pZipneo, MCF/Bcl-2[pool], NCI/ADR-RES, and MCF/ΔRaf-1 cells (Fig. 7*A*). NCI/ADR-RES cells and the housekeeping gene GAPDH were again used as controls (as in the data shown in Fig. 3). An increased level of Bcl-2 mRNA was detected in MCF/Bcl-2[pool] cells (Fig. 7*A*, *Lane 2*), as expected. An increased level of Bcl-2 mRNA was observed in MCF/ΔRaf-1 cells (Fig. 7*A*, *Lane 4*). It is likely that a portion of doxorubicin resistance elicited by Raf-1 (Figs. 5 and 6) is attributable to induction of Bcl-2 expression by Raf-1. Levels of proapoptotic Bax mRNA were approximately equal in MCF-7 transfectants, but no Bax mRNA was detected in NCI/ADR-RES cells (Fig. 7*A*, *Lane 3*). P-gp mRNA expression was

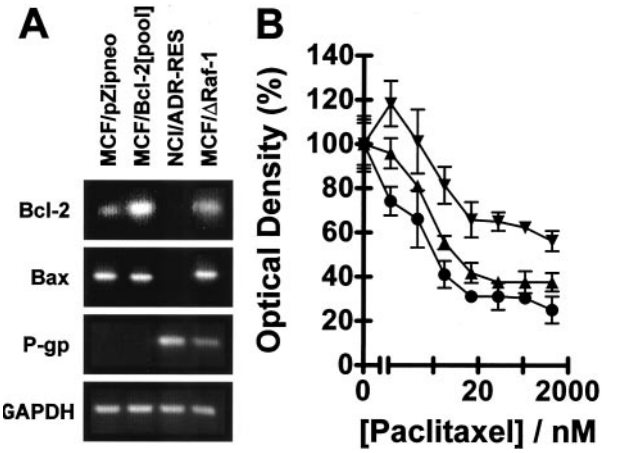

Fig. 7 RT-PCR analysis of Bcl-2, Bax, P-gp, and GAPDH mRNA levels and effect of paclitaxel on cell growth in the presence and absence of Raf-1 activation. *A*, P-gp mRNA is increased in MCF-7 cells overexpressing ΔRaf-1. Levels of Bcl-2, Bax, P-gp, and GAPDH mRNAs were examined by RT-PCR analysis for MCF/pZipneo, MCF/Bcl-2[pool], NCI/ADR-RES, and MCF/ΔRaf-1 cells. *B*, effect of Raf-1 activation on the growth-inhibitory effects of paclitaxel. Cell proliferation was assayed in triplicate with MTT for MCF-7 and MCF/ΔRaf-1:AR cells cultured for 3 days in the absence or presence of the indicated concentrations of paclitaxel. MCF/ΔRaf-1:AR cells were cultured in either the presence (▼) or absence (▲) of 500 nM testosterone. MCF-7 cells (●) were cultured without testosterone. Mean absorbance for cells treated with various concentrations of paclitaxel is plotted as a percentage of mean absorbance for cells not treated with paclitaxel. Paclitaxel concentration is plotted on a $\log_{10}$ scale. *Error bars*, SD.

observed only in NCI/ADR-RES cells (Fig. 7*A*, *Lane 3*), as expected, and in MCF/ΔRaf-1 cells (Fig. 7*A*, *Lane 4*).

If P-gp affects the sensitivity of these cells to doxorubicin, we would expect to find cross-resistance to paclitaxel, another P-gp substrate (48). Treatment of MCF/ΔRaf-1:AR cells with testosterone attenuated the growth-inhibitory effects of paclitaxel (Fig. 7*B*). The paclitaxel IC_{50} of MCF/ΔRaf-1:AR cells in the presence of 500 nM testosterone was at least 10-fold higher than that of MCF/ΔRaf-1:AR cells and parental MCF-7 cells in the absence of testosterone. In contrast, no effect of Bcl-2 on paclitaxel sensitivity was detected (data not shown).

To verify that treatment of MCF/ΔRaf-1:AR cells with testosterone induced ΔRaf-1 activity, Western blot analysis was performed with an antibody directed toward phospho-ERK-1/2. ERK-1 and ERK-2 become phosphorylated in response to activation of the Raf/MEK/ERK pathway by Raf-1. MCF/ΔRaf-1:AR cells were left untreated or treated for 30 min, 1 h, 4 h, or 24 h with PMA, testosterone, serum, or both serum and testosterone. Treatment with PMA or serum caused rapid phosphorylation of ERK-1/2 within 30 min that continued until at least 1 h of treatment had been completed (Fig. 8). This effect observed with PMA or serum treatment was short-lived, with levels of phosphorylated ERK-1/2 dropping from their peak after 4 h of treatment and even further after 24 h of treatment.

In contrast, treatment with testosterone yielded sustained levels of phosphorylated ERK-1/2 that increased slightly as the duration of treatment increased. Treatment with both testosterone and serum was similar to serum alone in that phosphorylation of ERK-1/2 was short-lived. However, levels of phosphorylated ERK-1/2 after 4 h of treatment were significantly greater after treatment with testosterone plus serum than after treatment with serum alone. ERK phosphorylation after treatment of MCF/ΔRaf-1:AR cells with testosterone is most relevant in the presence of serum because MCF/ΔRaf-1:AR cells were cultured in the presence of serum for experiments described above (Figs. 6 and 7). These changes in levels of phosphorylated ERK-1/2 were not attributable to changes in total ERK-1/2 protein levels because probing with an antibody to ERK-2 showed that total ERK-1/2 protein levels remained constant throughout all treatment conditions.

Levels of $p21^{Cip1}$ protein have been reported to increase after induction of Raf activity with steroid treatment of hematopoietic cells retrovirally infected with conditionally active A-Raf:ER, B-Raf:ER, and Raf-1:ER vectors (28). To explore whether induction of Raf-1 activity with testosterone has a similar effect in MCF/ΔRaf-1-AR cells, Western blot analysis was also performed with an antibody directed toward $p21^{Cip1}$ (Fig. 8). Levels of $p21^{Cip1}$ protein did not correlate with levels of ERK-1/2 phosphorylation. Instead, $p21^{Cip1}$ protein levels gradually decreased as time progressed, regardless of treatment.

DISCUSSION

Our results indicate that increased doxorubicin resistance is observed in MCF/Bcl-2 cells than the parental cell line. To determine whether this drug resistance can be attributed to expression of P-gp, P-gp mRNA levels were assayed and found to be undetectable in MCF/pZipneo cells, MCF/Bcl-2[pool] cells, and isolated clones of MCF/Bcl-2[pool] cells. However, it was detected in NCI/ADR-RES and MCF/ΔRaf-1 cells. We conclude from these data that overexpression of Bcl-2 protein is responsible for the drug-resistant phenotype observed and that there is no up-regulation of P-gp. In contrast, cells overexpressing constitutively active ΔRaf-1 express P-gp mRNA (Fig. 7*A*) and have higher levels of P-gp activity (47) and doxorubicin resistance (Fig. 5). Moreover, MCF-7 cells transfected with conditionally active Raf-1:AR demonstrated increased P-gp mRNA levels (data not shown) and resistance to doxorubicin (Fig. 6) and paclitaxel (Fig. 7*B*) when treated with testosterone.

Results of experiments performed with MCF/Bcl-2 clones further support the finding that overexpression of Bcl-2 is associated with increased drug resistance. It was found that MCF/Bcl-2[c2] and MCF/Bcl-2[c3] cells had substantially increased Bcl-2 protein and mRNA compared with control MCF/pZipneo or MCF/Bcl-2[pool] cells. Both MCF/Bcl-2[c2] and MCF/Bcl-2[c3] also had a >25-fold increase in doxorubicin IC_{50} values relative to MCF/pZipneo cells. Moreover, in response to treatment with the highest concentration of doxorubicin (5000 nM), both MCF/Bcl-2[c2] and MCF/Bcl-2[c3] cells demonstrated greater proliferation than MCF/Bcl-2[c1] cells. MCF/Bcl-2[c1] cells, which had an even lower doxorubicin IC_{50} than MCF/Bcl-2[pool] cells, also had lower Bcl-2 mRNA and protein levels. This correlation between doxorubicin IC_{50} and levels of Bcl-2 mRNA and protein suggests that the ability to survive in the presence of chemotherapeutic agents is directly related to the amount of Bcl-2 expressed by the cell.

Mechanisms by which Bcl-2 causes resistance to doxoru-

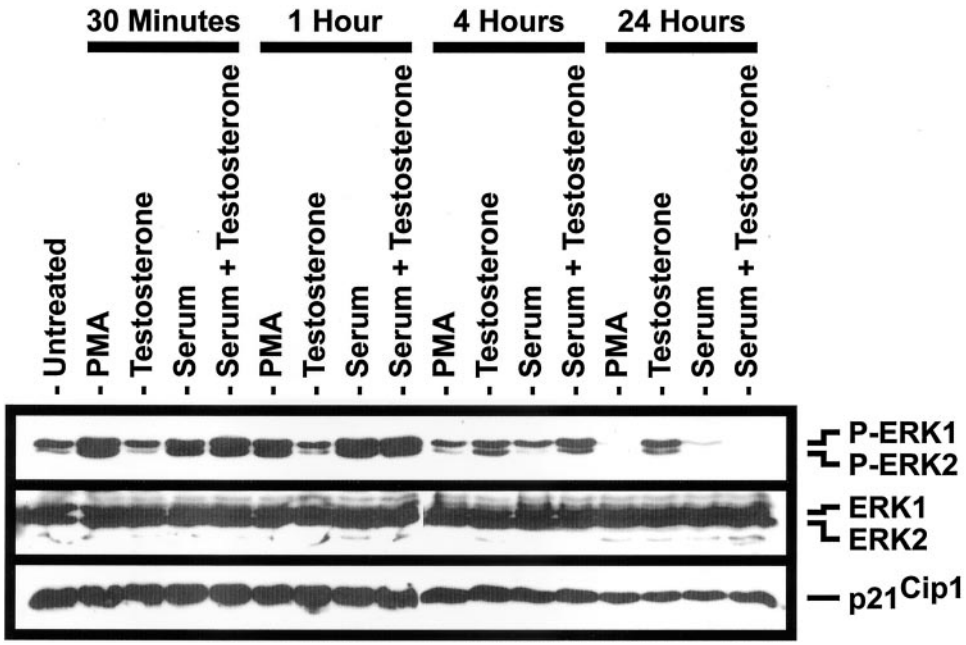

Fig. 8 Testosterone induces sustained phosphorylation of ERK-1/2 in MCF/ΔRaf-1:AR cells. MCF/ΔRaf-1:AR cells were treated with 1 μM PMA, 500 nM testosterone, 5% serum, or 5% serum + 500 nM testosterone for the durations indicated. Western blot analysis was performed with antibodies directed toward phospho-ERK-1/2, ERK-2, or $p21^{Cip1}$.

bicin remain unclear. Doxorubicin treatment was observed to increase protein levels of $p21^{Cip1}$ and its transcriptional activator p53 in both MCF/pZipneo and MCF/Bcl-2 clones (data not shown). Levels of these proteins, which have tumor-suppressing and growth-arresting functions, respectively, increase in response to DNA damage. Because doxorubicin treatment increased levels of $p21^{Cip1}$ and p53 in both the presence and absence of Bcl-2 overexpression, abrogation of DNA damage sensing can be excluded as a possible mechanism of doxorubicin resistance mediated by Bcl-2. Additionally, Bcl-2 overexpression induced long-term resistance to doxorubicin that lasted at least 8 weeks. These data suggest that Bcl-2 induces drug resistance by mechanisms that do not merely delay cell death but instead are long-lasting in nature.

Because it has been hypothesized that Raf-1 or a downstream kinase in this pathway may phosphorylate Bcl-2, MCF-7 cells overexpressing ΔRaf-1 were also examined (30, 49, 50). A report that high Raf-1 activity was associated with paclitaxel resistance in early passage human cervical tumors suggests that Raf-1 may regulate expression of Bcl-2. It has been observed that both MCF/ΔRaf-1:AR cells cultured in the presence of testosterone and MCF/ΔRaf-1 cells demonstrate reduced levels of apoptosis in response to treatment with chemotherapeutic agents (47) that are comparable with levels observed in MCF/Bcl-2 cells reported here. Moreover, there was only a 0.5-fold difference in growth observed between MCF/Bcl-2[pool] and MCF/ΔRaf-1 cells over a 5-day period in the presence of 500 nM doxorubicin.

The increased survival of cells possessing an activated Raf-1 kinase in response to treatment with chemotherapeutic agents led to attempts to uncover mechanisms downstream of Raf-1 activation. As mentioned previously, there were no detectable levels of P-gp in cells overexpressing Bcl-2. However, MCF-7 cells expressing an activated Raf-1 kinase were found to have up-regulated P-gp levels (47) in comparison with parental or MCF/Bcl-2 cells.

Additionally, growth assays were performed on MCF/ΔRaf-1:AR+Bcl-2 cells in the presence of doxorubicin. Because both MCF/ΔRaf-1-AR cells cultured in the presence of testosterone and MCF/Bcl-2 cells demonstrated drug-resistant properties, we examined whether induction of Raf-1 activity in MCF/ΔRaf-1:AR+Bcl-2 cells would yield even more doxorubicin resistance than induction of Raf-1 activity in MCF/ΔRaf-1:AR cells. In the presence of MCF/ΔRaf-1:AR+Bcl-2 cells demonstrated an average growth rate 1.5-fold higher than that of MCF/ΔRaf-1:AR cells. Therefore, induction of drug resistance by Raf-1 was further increased by overexpression of Bcl-2.

In conclusion, MCF-7 breast cells overexpressing Bcl-2 protein continued to proliferate in the presence of doxorubicin in both short- and long-term assays. Mechanisms by which Bcl-2 prevents apoptosis in these cells remain unclear, but do not include abrogation of DNA damage sensing. Activation of ΔRaf-1 in MCF-7 cells also increased resistance to doxorubicin. The finding that Bcl-2 mRNA levels are increased in cells expressing activated Raf-1 suggests that the Raf/MEK/ERK pathway may regulate Bcl-2 expression, perhaps by ERK-mediated phosphorylation of $p90^{RSK}$ isoforms that in turn activate CREB. Activated CREB has been proposed to induce Bcl-2 transcription by binding to the human *bcl-2* promoter (51–54).

These studies provide a model for the study of drug resistance in breast cancer cells. Through these and future developments, an increased understanding of tumor progression may be obtained. Moreover, mutations such as amplification of the c-*erb*B-2 oncogene, which is frequently detected in breast cancer patients, may cause Raf isoform activation, which would result in resistance of cells to certain chemotherapeutic drugs. Our studies indicate that Raf-1 and Bcl-2 induce distinct and overlapping pathways, which can contribute to drug resistance.

Raf-1 may increase expression of P-gp. However, increased Bcl-2 expression may also collaborate with Raf-1 to contribute to further drug resistance.

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