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Activation of the Unfolded Protein Response Is Necessary and Sufficient for Reducing Topoisomerase IIα Protein Levels and Decreasing Sensitivity to Topoisomerase-Targeted Drugs

Miranda D. Gray, Melissa Mann, John L. Nitiss, and Linda M. Hendershot

Departments of Molecular Pharmacology (M.D.G., J.L.N.) and Genetics & Tumor Cell Biology (M.M., L.M.H.), St. Jude Children's Research Hospital, Memphis, Tennessee

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ABSTRACT

A wide range of chemotherapeutic agents has been identified that are active against solid tumors. However, resistance remains an important obstacle to the development of curative regimens. Whereas much attention has been paid to acquired drug resistance, a variety of physiological pathways also have been described that reduce the sensitivity of previously untreated tumors to cytotoxic antitumor agents. Treatment of cells with pharmacological agents that alter the environment of the endoplasmic reticulum (ER) and activate the unfolded protein response (UPR) can render cells resistant to topoisomerase II poisons. We describe experiments showing that activation of the mammalian ER stress response is both necessary and sufficient to decrease topoisomerase IIα protein levels and to render cells resistant to etoposide, a topoisomerase II-targeting drug. This is not caused by the elevated levels of BiP that are a hallmark of this response, because a cell line that has been engineered to overexpress BiP does not show increased resistance to etoposide. The UPR was shown to be required for altered drug sensitivity, because the BiP-overexpressing cell line, which is unable to activate the UPR, did not show decreased topoisomerase II levels or increased resistance to etoposide in response to stress conditions. The transient overexpression of an unfolded protein activated the UPR and led to the concomitant loss of topoisomerase IIα protein from the cells, demonstrating that UPR activation is sufficient for the changes in topoisomerase II levels that had been observed previously with pharmacological induction of the UPR.

Because of their rapid growth and inadequate vascularization, the microenvironment of tumor cells can become limiting (Ma and Hendershot, 2004). The decreased levels of oxygen and nutrients can alter the homeostasis of the endoplasmic reticulum (ER), causing unfolded proteins to accumulate in the ER, which activate the unfolded protein response (UPR) pathway. The initial phases of this response, which can occur in all cells, are designed to protect the cell until the stress subsides. Mammalian cells respond to ER stress by increasing the transcription of resident ER chaperones to prevent protein aggregation (Lee, 1987), decreasing protein translation to limit the accumulation of unfolded proteins (Brostrom et al., 1996), arresting cells in the G_1 phase of cell cycle (Melero and Fincham, 1978) to prevent the propagation of cells experiencing physiological stress and increasing the degradative capacity of the cell (Hampton, 2000) to decrease the load of unfolded proteins. If the ER stress is prolonged, apoptotic pathways are activated (Nakagawa et al., 2000; Hitomi et al., 2004) to destroy continually affected cells. It is assumed that cancer cells take advantage of the cytoprotective elements of the response and disable the cytotoxic ones, although this has not been carefully studied.

Changes in the ER environment are sensed by three ER-localized transmembrane proteins Ire1, PERK, and ATF6. The ER chaperone BiP binds to the luminal portions of these three proteins in the absence of stress and maintains them in an inactive state (Bertolotti et al., 2000; Shen et al., 2002). When unfolded proteins begin to accumulate, BiP is released from the transducers, leading to their dimerization and activation in the case of Ire1 and PERK or transport to the Golgi for processing in the case of ATF6. ATF6 is liberated from the ER membrane by the S1P and S2P proteases (Ye et al., 2000) and translocated to the nucleus, in which it activates the transcription of its targets (e.g., ER chaperones like BiP and

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ABBREVIATIONS: ER, endoplasmic reticulum; UPR, unfolded protein response; GFP, green fluorescent protein; CHO, Chinese hamster ovary; CHO-BiP^{OE}, Chinese hamster ovary cells overexpressing BiP; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); ATF6, activating transcription factor 6.

GRP94, and transcription factors liked XBP-1 and CHOP). Ire1 possesses an endoribonuclease activity that is activated during ER stress to remove 26 nucleotides from the XBP-1 transcript. This alters the reading frame of XBP-1, resulting in a remodeled transcription factor with both a DNA binding and transactivation domain (Yoshida et al., 2001; Calfon et al., 2002). The first target of spliced XBP-1 to be identified is EDEM, which is a component of the ER degradation machinery (Yoshida et al., 2003). PERK is an ER-localized member of the eIF-2α kinase family (Shi et al., 1998; Harding et al., 1999). Phosphorylation of eIF-2α prevents the formation of translation initiation complexes, thereby blocking protein synthesis. In addition to preventing the accumulation of proteins, this block in protein synthesis leads to the rapid loss of D1 cyclin from cells causing them to arrest in G_1 (Brewer and Diehl, 2000). Activation of eIF-2α kinases also leads to increased translation of ATF4 (Harding et al., 2000), which transactivates another group of stress-inducible genes (e.g., GADD34 and CHOP) (Ma and Hendershot, 2003).

DNA topoisomerases play essential roles in replication, transcription, and chromosome segregation (Wang, 1996; Nitiss, 1998). The two major families of topoisomerases—type I enzymes that introduce transient single-strand cuts in DNA, and type II enzymes that make double-stranded breaks—are both targets of clinically important anticancer agents (Wang, 1996; Osheroff, 1998). Mammalian cells have two topoisomerase II isozymes, topoisomerase IIα and IIβ, and both enzymes are targeted by most topoisomerase II-targeting agents (Walker and Nitiss, 2002). Drugs targeting either class of topoisomerase act by blocking the religation of the normally transient cleaved intermediate of the topoisomerase reaction (Chen and Liu, 1994), thus activating DNA-damage checkpoints, which can lead to apoptosis using the same pathways as other DNA-damaging agents (Kaufmann, 1998). Consequently, reduced levels of topoisomerases can lead to drug resistance, because they reduce the amount of topoisomerase/DNA complexes and, therefore, the amount of drug-induced DNA damage (Nitiss et al., 1993). The reduction in topoisomerase II enzyme levels is frequently accompanied by a reduction in their mRNA levels, suggesting alterations in the transcriptional regulation of the proteins (Nitiss and Beck, 1996). In addition, post-translational modifications, such as phosphorylation, ubiquitination, and sumoylation can lead to changes in protein stability or protein localization (Mao et al., 2000; Li and Liu, 2001; Chikamori et al., 2003), resulting in reduced levels of topoisomerase-mediated DNA damage.

The cellular levels of topoisomerase IIα can also be regulated by some stress conditions. Treatment of cells with UPR-inducing agents induces resistance to doxorubicin and other topoisomerase II-targeting agents (Shen et al., 1987; Hughes et al., 1989), because of a dramatic reduction in topoisomerase II levels (Shen et al., 1989; Yun et al., 1995). In these studies, pharmacological agents were used to induce ER stress, making it difficult to determine whether the resistance to these poisons is a specific response of the UPR pathway or an indirect effect of the drugs used to activate the response. We demonstrate here that activation of the mammalian UPR is both necessary and sufficient to reduce the sensitivity of cells to topoisomerase II poisons. This will allow determination of the arm(s) of the pathway responsible for changes in drug sensitivity and may allow a targeted intervention to increase the chemotherapeutic efficacy of drugs that target topoisomerase IIα.

Materials and Methods

Cell Lines. The derivation of Chinese hamster ovary (CHO) cells that overexpress BiP has been described previously (Dorner et al., 1992). The NIH3T3 murine fibroblast line, 293 human embryonic kidney cell line, COS-1 African Green monkey fibroblast line, CHO cell line, and CHO-BiP^{OE}, CHO cells that have been engineered to overexpress hamster BiP were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% Fungizone (Cambrex Bio Science Walkersville, Inc., Walkersville, MD).

Western Blotting. Equal numbers of cells were lysed directly in SDS sample buffer [62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromphenol blue], and proteins were separated on SDS-polyacrylamide gels under reducing conditions and transferred to membranes for Western analysis. The membranes were blocked with 0.1% gelatin, 0.1% Triton X-100, or 5% powdered instant milk, 0.1% Tween in Tris saline, and then probed with indicated antiserum. For rodent BiP, a rabbit polyclonal anti-BiP antiserum (Hendershot et al., 1995) was used at a concentration of 2 μl/ml. For the detection of primate BiP, a monoclonal rat anti-BiP antibody (Bole et al., 1986) was used at a concentration of 60 μl/ml, Ig heavy chains were detected with a polyclonal rabbit anti-human γ heavy chain antiserum (Southern Biotechnology Associates, Birmingham, AL) at a concentration of 2 μg/ml, and CHOP was observed with a polyclonal rabbit anti-CHOP antisera (Brewer et al., 1999) at a concentration of 4 μg/ml. Antisera directed against actin and Hsc70 were obtained from Abcam, Inc. (Cambridge, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. In all cases, the appropriate species-specific secondary antiserum (Southern Biotechnology Associates) was used, followed by incubation with protein A-conjugated horseradish peroxidase (E. Y. Labs, San Mateo, CA), and visualization with the enhanced chemiluminescence reagent (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Murine topoisomerase IIα was detected with either a commercial reagent specific for this isoform (TopoGEN, Columbus, OH) or with an affinity-purified rabbit anti-human topoisomerase IIα antiserum produced in our laboratory. The topoisomerase IIα antiserum was raised against three peptides (MEVSPLQPVNEN, VEAKEKQDEQVGLPG, and KRAAPKGTKRDPAL), which are conserved in human and mouse topoisomerase IIα but are not found in topoisomerase IIβ from either species. The serum was affinity-purified against the three peptides and was found to specifically recognize recombinant topoisomerase IIα but not topoisomerase IIβ by Western blot analysis. Human topoisomerase IIα (TopoGEN) and IIβ (BD Biosciences, San Jose, CA) were detected with commercial reagents specific for each isoform.

Northern Analysis. Total cellular RNA was isolated from approximately 10^7 cells using the RNAeasy kit (QIAGEN, Valencia, CA). Isolated RNA was separated by agarose gel electrophoresis and transferred for hybridization (Brewer et al., 1997). A 1.5-kb PstI-EcoRI fragment was isolated from a hamster BiP cDNA clone and used to detect BiP, a 1.7-kb polymerase chain reaction product amplified from mouse cDNA was used to detect topoisomerase IIα, a 600-base pair EcoRI-XbaI fragment isolated from murine CHOP cDNA was used to detect CHOP, and a 1.1-kb fragment purchased (BD Biosciences Clontech, Palo Alto, CA) to detect GAPDH, which served as a loading control.

Clonogenic Survival Assays. Cells were plated at 4×10^5 cells per 100-mm plate in 10 ml of media. After 16 h, the indicated ER stress-inducing drug was added to the plates at the specified concentration and times. Cells were then incubated with the indicated concentrations of etoposide for 2 h, washed with 10 ml of PBS, and trypsinized. Varying numbers of cells were plated in triplicate on 100-mm plates to ensure that between 30 and 300 colonies would be

present on the plate for each treatment regimen. Plates were incubated for 7 to 10 days to allow colony formation. The plates were then washed with PBS and stained with crystal violet. Colonies were counted, and survival was determined by multiplying the number of colonies by the dilution factor and expressing this number as the percentage of survival relative to surviving colonies that were not treated with etoposide.

For determining sensitivity to ionizing radiation, cells were plated and irradiated in 100-mm dishes in culture media. After irradiation, appropriate dilutions were plated, and cells were incubated for 7 to 10 days as above before determining surviving percentages. The total time required for irradiation (the time cells were not in an incubator) was approximately 10 min.

Expression of Ig Heavy Chain to Induce ER Stress Response. Stratagene's (La Jolla, CA) pSG5 vector was altered to create an expanded multicloning site into which a humanized γ heavy chain (Liu et al., 1987) was inserted. COS-1 cells were cotransfected with the pGreen Lantern vector, which contains a cDNA encoding the green fluorescent protein (GFP; Invitrogen, Carlsbad, CA), along with either an empty vector (pSG5) or the pSG5-γ vector using the FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN). Forty-eight hours after transfection, cells were trypsinized and GFP^+ cells were isolated using a FACSVantage/SE Cell Sorter (BD Biosciences). We consistently observed approximately 15% GFP^+ cells in the cells transfected with pGreen Lantern alone, and approximately 11% GFP^+ cells in the doubly transfected cells (data not shown). An aliquot of doubly transfected GFP^+ cells were costained with tetramethylrhodamine B isothiocyanate-conjugated anti-human γ and found to be ~95% positive for γ heavy chain (data not shown). The sorted cells were counted, and 5×10^5 cells of each were rinsed with PBS and immediately lysed in SDS sample buffer. The lysates were electrophoresed on reducing SDS-polyacrylamide gels, transferred to nitrocellulose paper, and analyzed by Western blotting as indicated.

Results

Induction of the UPR Results in Etoposide Resistance. Previous studies have shown that pharmacological induction of the UPR reduces cellular levels of topoisomerase IIα protein and decreases their sensitivity to topoisomerase II-targeting drugs. Because one aspect of the mammalian UPR is an arrest of cells in the G_1 phase of the cell cycle, which is known to lead to reduced levels of topoisomerase IIα transcripts, we first determined the kinetics of topoisomerase IIα loss in response to UPR activation. Thapsigargin inhibits the ER calcium ATPase, which leads to a depletion of ER calcium, thus altering the folding of proteins in this organelle and leading to a rapid induction of the UPR (Lee, 1992). NIH3T3 cells were treated with thapsigargin for the indicated times, and topoisomerase IIα protein and mRNA levels were determined. As early as 4 h after thapsigargin treatment, topoisomerase IIα protein levels were dramatically decreased (Fig. 1A), which corresponded to UPR induction, as judged by the increased transcription of the *BiP* gene (Fig. 1C). However, this was well before topoisomerase IIα transcript levels were affected (Fig. 1C) and too early for a significant number of the cells to arrest in G_1 (data not shown and Shen et al., 1989; Brewer et al., 1999). NIH3T3 cells were also treated with 2-deoxyglucose and tunicamycin (data not shown), both of which lead to inhibition of N-linked glycosylation and interfere with protein folding. Unlike thapsigargin, which can affect existing ER proteins, 2-deoxyglucose and tunicamycin only affect newly synthesized proteins, and therefore take somewhat longer to accumulate sufficient levels of unfolded proteins to activate the UPR. Therefore, the

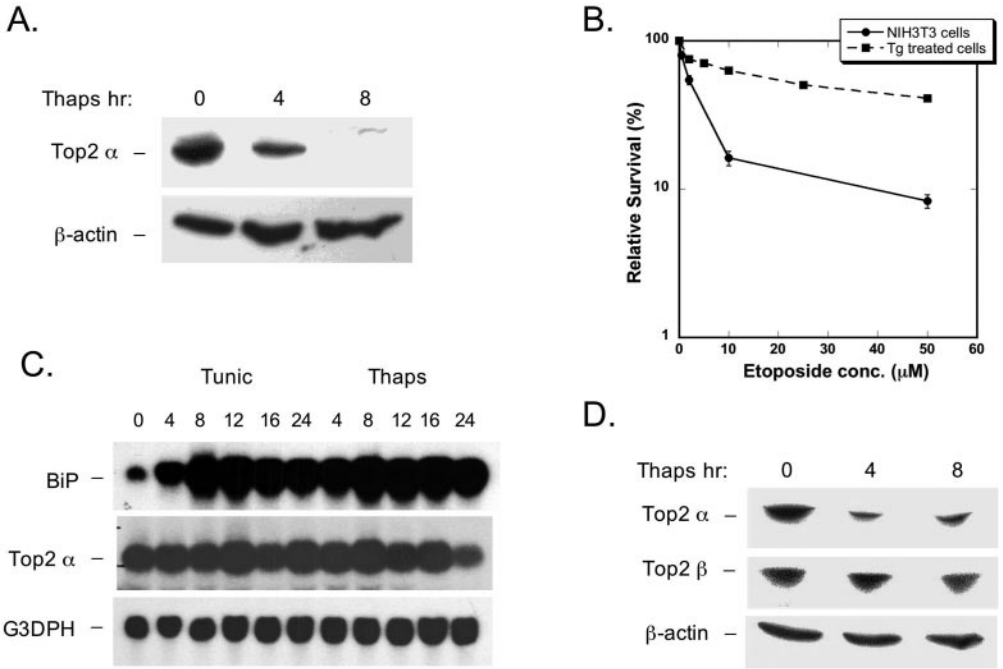

Fig. 1. Treatment of cells with thapsigargin induces resistance to etoposide and rapid depletion of topoisomerase IIα. A, NIH3T3 cells were treated with 1.0 μM thapsigargin for indicated times. The cell extracts were prepared, and topoisomerase II levels were assessed by Western blotting using an anti-topoisomerase IIα-specific antibody. β-Actin was probed as a loading control. B, NIH3T3 cells were pretreated with 2.0 μM thapsigargin for 6 h and then incubated with the indicated etoposide concentrations for 2 h. Cells were washed and recultured for 3 days, at which time the cell number was assessed and expressed as a percentage of cells surviving with no etoposide treatment. C, NIH3T3 cells were treated with either 2.5 μg/ml tunicamycin (Tunic) or 2.0 μM thapsigargin (Thaps) for the indicated number of hours. RNA was isolated, separated by gel electrophoresis, and probed for BiP and topoisomerase IIα transcripts. A probe directed against GAPDH mRNA was used as a loading control. D, 293 cells were treated with 2.0 μM thapsigargin for the indicated times. Cell extracts were prepared, and protein levels were assessed by Western blotting using an anti-human topoisomerase IIα antibody and an anti-human topoisomerase IIβ antibody. β-Actin was probed as a loading control.

loss of topoisomerase IIα protein from 2-deoxyglucose and tunicamycin-treated cells was slower than that observed with thapsigargin (data not shown).

To determine the effects of thapsigargin on the sensitivity of NIH3T3 cells to etoposide, cells were cultured in the presence or absence of thapsigargin for 6 h and then treated with the indicated amount of etoposide for 2 h before reculturing them in medium lacking thapsigargin and etoposide. Cell number was determined 72 h after etoposide treatment and was expressed as a percentage of cells remaining compared with cells that were not exposed to etoposide treatment (Fig. 1B). For NIH3T3 cells not pretreated with thapsigargin (control), 10 μM etoposide reduced cell number to 16% of that found in the culture without etoposide, and 50 μM etoposide reduced the relative cell number to 8%. Pretreatment of the NIH3T3 cells with thapsigargin increased the relative cell number to 63% with 10 μM and 40% with 50 μM etoposide. At 100 μM etoposide, a concentration that reduced the number of non–thapsigargin-treated cells to $<$5%, nearly 50% of the thapsigargin-treated cells persisted after 72 h. Thus, thapsigargin treatment led to the loss of topoisomerase IIα protein, but not transcripts, from NIH3T3 cells and afforded protection from etoposide-mediated killing. The loss of topoisomerase II protein is consistent with previous data obtained from various other cell lines with a variety of different agents known to induce ER stress (Shen et al., 1989; Yun et al., 1995), and our data suggest that this is mediated through post-transcriptional mechanisms, in agreement with an earlier study (Shen et al., 1989).

UPR-Induced Loss of Topoisomerase II from Cells Is Restricted to the α Form. Mammalian cells contain two different topoisomerase II isoforms, termed α and β (Drake et al., 1987). Both isoforms are sensitive to etoposide in vitro and confer etoposide sensitivity in vivo (Kaufmann et al., 1998). Thus, we wished to determine whether the effects of the UPR were specific to topoisomerase IIα or targeted both topoisomerase II isozymes. The sequence similarity between the two isoforms and the lack of antibodies uniquely specific for the murine β form made it necessary to perform this experiment in a human cell line. The human 293 embryonic kidney cell line was treated with thapsigargin for the indicated times, and cell lysates were prepared for Western blotting (Fig. 1D). Within 4 h, topoisomerase IIα levels had dropped dramatically, whereas topoisomerase IIβ levels remained unchanged even after 8 h of thapsigargin treatment. These data demonstrate that the loss of topoisomerase IIα during UPR activation is specific for the α isoform and is consistent with the possibility that the increased resistance of NIH3T3 cells to etoposide could be caused by changes in topoisomerase IIα.

Increased Levels of BiP Are Not Sufficient to Protect Cells from Etoposide or Other DNA-Damaging Agents. It has been suggested that the increased levels of BiP, a resident ER chaperone and the hallmark of the UPR, are responsible for protecting cells from etoposide during ER stress (Rao et al., 2002; Reddy et al., 2003). To examine this directly, we used a CHO cell line that had been engineered to overexpress hamster BiP transcripts at levels comparable with those obtained during ER stress (Dorner et al., 1992). Because these cells are somewhat unstable because of either the overexpression of BiP or their inability to activate the UPR, we characterized our clone to ensure that it was deficient in UPR induction after treatment with agents that induce ER stress. As anticipated, when the signals were normalized for loading, we found that the CHO-BiP^{OE} cells were expressing the BiP transgene mRNA at levels similar to those observed in the parental line after 6 h of thapsigargin treatment, and this level did not increase significantly after ER stress (Fig. 2A). When protein levels were similarly analyzed, we found the BiP overexpressing cells actually had slightly higher levels of BiP than those achieved in the parental cell line after UPR induction, and as expected, they were defective in their ability to induce CHOP protein (Fig. 2B).

To examine the effects of BiP overexpression on sensitivity to topoisomerase II-targeting drugs in the absence of ER stress, cells were exposed to etoposide briefly, and then cellular sensitivity was assessed by determining cell number or by clonogenic survival. In the first experiments, CHO cells and CHO-BiP^{OE} cells were treated with etoposide for 2 h. After etoposide exposure, cells were washed and recultured for 48 h. The number of cells present was determined by Coulter counter (Beckman Coulter, Fullerton, CA). For both CHO cells and CHO-BiP^{OE} cells, etoposide exposure caused a

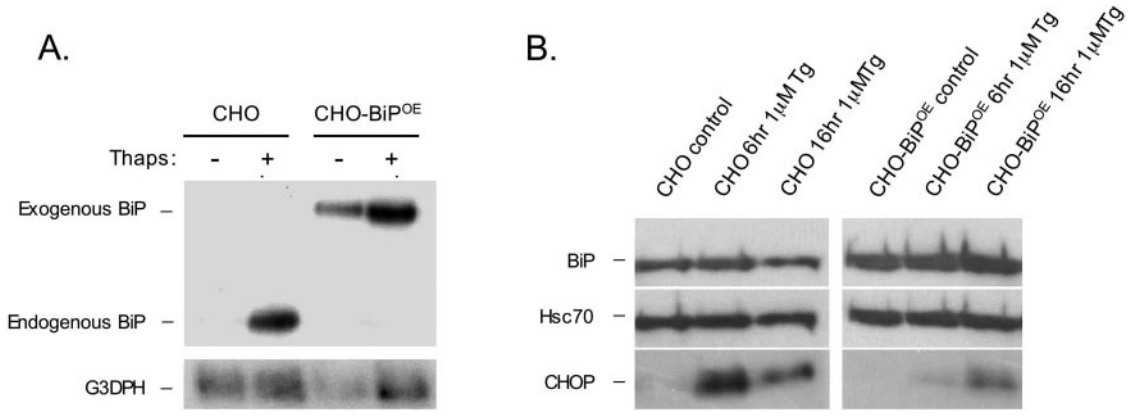

Fig. 2. Overexpression of BiP prevents UPR induction. A, CHO and CHO-BiP^{OE} cells were treated with 5.0 μM thapsigargin for 6 h. RNA was isolated, and a Northern blot was hybridized with a 1.5-kb PstI-EcoRI fragment from a hamster BiP cDNA clone. The positions of the endogenous BiP mRNA and the larger exogenous form are indicated to the right. GAPDH levels were hybridized as a loading control. B, CHO and CHO-BiP^{OE} cells were treated with 1.0 μM thapsigargin for 6 h. Cell extracts were prepared, and protein levels were determined by Western blotting using a rabbit polyclonal anti-BiP antiserum. β-Actin was probed as a loading control.

dose-dependent reduction in cell number, with no significant difference between the two cell lines (data not shown).

We also examined the sensitivity of CHO cells and CHO-BiP^{OE} cells to etoposide by clonogenic survival. Cells were treated with the indicated amounts of etoposide for 2 h, washed, and recultured in complete medium. After 7 to 10 days, the number of colonies was determined and plotted as a percentage of survival relative to untreated cells (Fig. 3A). At concentrations of less than 10 μM etoposide, BiP overexpressing cells seemed slightly more sensitive to etoposide than the parental cells, whereas at the highest concentration of etoposide, they were somewhat more resistant to etoposide sensitivity between the CHO and CHO-BiP^{OE} cells. These differences were not statistically significant (data not shown). Thus, increased levels of BiP that occur during UPR activation, and which are significantly less than that observed in the CHO-BiP^{OE} cell line, do not significantly protect against etoposide-mediated cell killing. Our results are the opposite of those reported previously, where significant protection against etoposide-mediated cell killing was observed in BiP overexpressing CHO cells (Reddy et al., 2003).

Because BiP overexpression was hypothesized to provide protection against a broad range of apoptotic stimuli, we decided to examine the effect of BiP overexpression on sensitivity to other types of DNA damage that result in apoptotic cell death. We chose ionizing radiation as a DNA-damaging agent because previous studies had suggested that UPR stress induction does not alter sensitivity to ionizing radiation (Hughes et al., 1989). CHO and CHO-BiP^{OE} cells were exposed to ionizing radiation, as described under *Materials and Methods*, and sensitivity was assessed by clonogenic survival assays (Fig. 3B). The sensitivity of the two cell lines to ionizing radiation was similar, with the CHO-BiP^{OE} cells actually showing slightly greater sensitivity to ionizing radiation. This is most apparent at higher doses, although after exposure to 10 Gy, there was less than a 3-fold difference in cell survival between the two cell lines. In results to be presented elsewhere, we also observed no difference in sensitivity between CHO and CHO-BiP^{OE} cells treated with the alkylating agents melphalan or methyl methane sulfonate. These results indicate that CHO-BiP^{OE} cells are not generally resistant to DNA-damaging agents.

UPR Activation Is Essential for Both Loss of Topoisomerase IIα and Resistance to Etoposide. The CHO cells that overexpress BiP do not activate the UPR cascade in response to ER stress, because there are sufficient amounts of BiP to keep the UPR signal transducers in an inactive state (Bertolotti et al., 2000; Shen et al., 2002). This provided us with an opportunity to determine whether UPR activation was required to reduce topoisomerase IIα levels during ER stress conditions and provide protection from etoposide. CHO and CHO-BiP^{OE} cells were treated with thapsigargin for 6 or 16 h, and cell lysates were prepared for Western blotting. Similar to other cell lines examined, the parental CHO cells demonstrated decreased expression of topoisomerase IIα in response to thapsigargin (Fig. 4A). When the CHO-BiP^{OE} cells that are unable to induce the ER stress response (Dorner et al., 1992) were similarly treated, topoisomerase IIα levels remained unchanged or slightly increased in response to thapsigargin. We also examined the effect of two other ER stress-inducing agents on topoisomerase IIα levels. Cells were treated with differing concentrations of either 2-deoxyglucose (Fig. 4B) or dithiothreitol (Fig. 4C) for 6 h, and topoisomerase IIα levels were examined. As observed with thapsigargin, topoisomerase IIα levels decreased in CHO cells after exposure to ER stress, whereas topoisomerase IIα levels remained unchanged in CHO-BiP^{OE} cells. Because ER stress was still occurring in both cell lines and unfolded proteins accumulated in both cell lines (Dorner et al., 1992), these experiments demonstrate that the loss of topoisomerase IIα from cells requires the activation of the UPR and is not occurring via a secondary effect of the drugs used to activate the UPR or to changes in the ER environment.

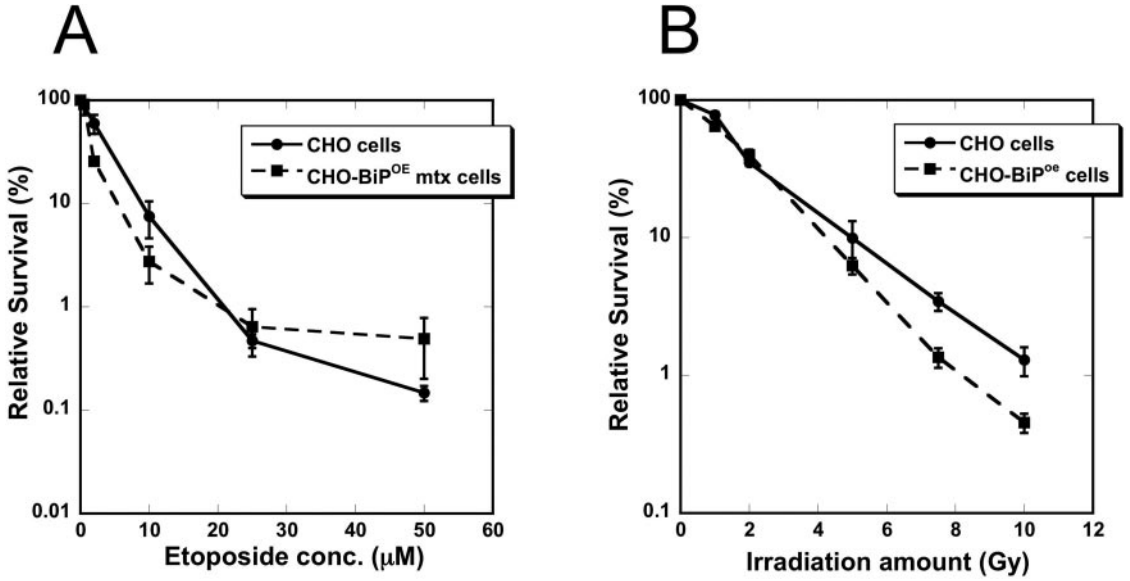

Fig. 3. Overexpression of BiP does not alter sensitivity to etoposide or ionizing radiation as determined by clonogenic survival. A, CHO and CHO-BiP^{OE} cells were treated with the indicated etoposide concentrations for 2 h, after which cells were washed, trypsinized, and recultured. Varying numbers of cells were plated in triplicate for each drug dosage and reincubated for 10 days. Survival is expressed as a percentage of colonies formed with no etoposide treatment. B, CHO and CHO-BiP^{OE} cells were exposed to increasing doses of ionizing radiation. Appropriate concentrations of cells depending on radiation dosage were plated in triplicate. Colonies were stained and counted after 10 days of incubation.

UPR Activation Is Required for Altered Sensitivity of Cells to Etoposide in Response to Agents that Cause ER Stress. To determine the requirements for UPR activation in protecting cells from etoposide-mediated killing, we induced ER stress in CHO and CHO-BiP^{OE} cells before treating with etoposide and performed clonogenic survival assays. Both cell lines were pretreated with 1.0 μM thapsigargin for 6 h before treating them with varying concentrations of etoposide for 2 h. After 7 to 10 days, surviving clones in each treatment group were counted and expressed as a percentage of cells surviving after the appropriate UPR induction alone. CHO cells showed enhanced resistance to etoposide after induction of the UPR with thapsigargin (Fig. 5A). In both cases, induction of the ER stress before etoposide treatment increased clonal survival from ~0.2 to nearly 10% at the maximum dose of etoposide used. Although pretreatment of CHO-BiP^{OE} cells with both UPR-inducing agents did increase resistance of these cells to etoposide (Fig. 5B), the level of protection was not as dramatic. The alterations in chemosensitivity were also examined by comparing IC_{50} and IC_{90} values for both cell lines (Table 1). Thapsigargin had little effect on the IC_{50} value of etoposide-treated CHO cells but led to a 2-fold increase in the IC_{90} value for etoposide. In contrast, there was no significant change seen in the IC_{50} or IC_{90} value for etoposide with the CHO-BiP^{OE} cells. In comparison, Table 1 also shows that the IC_{50} or IC_{90} value for ionizing radiation was unaffected by BiP overexpression. These experiments demonstrate that both loss of topoisomerase II and the decreased sensitivity of cells experiencing ER stress are dependent on activation of the UPR and are not an indirect effect of the drugs used to activate the UPR.

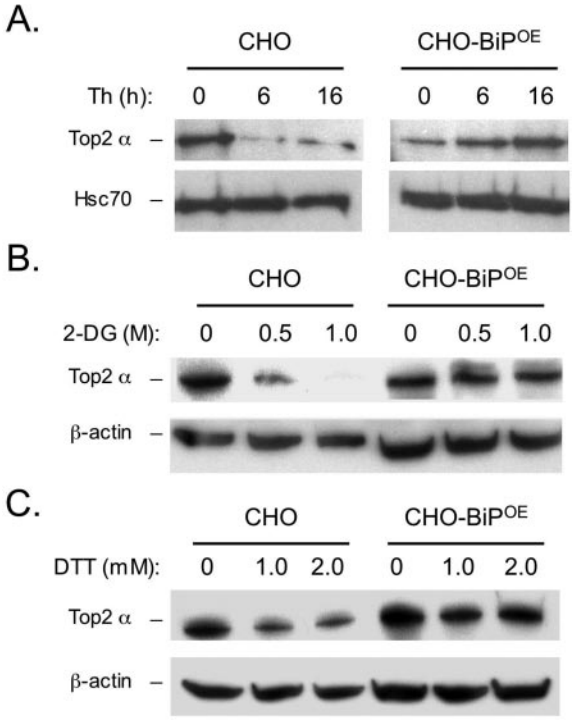

Fig. 4. Induction of the UPR pathway is necessary to induce the depletion of topoisomerase IIα. A, CHO and CHO-BiP^{OE} cells were treated with 1.0 μM thapsigargin for varying amounts of time. Cell extracts were prepared, separated by electrophoresis, and transferred for Western blotting. Topoisomerase II levels were assessed using a polyclonal anti-topoisomerase IIα antibody. Hsc70 protein levels were detected with a polyclonal anti-Hsc70 antibody, which served as a control for sample loading. These two cell lines were also treated with increasing concentrations of either 2-deoxyglucose (B) or dithiothreitol (C) for 6 h. Cell extracts were prepared, and topoisomerase II levels were assessed using an anti-topoisomerase IIα antibody. β-Actin levels were determined and served as a loading control.

Our experiments demonstrated that UPR activation is essential for alterations in chemosensitivity of cells to topoisomerase II-targeting agents, but they do not indicate whether UPR activation is sufficient. Instead of using drugs to activate the UPR, which can have additional effects in the cells, we chose to induce the ER stress response by overexpressing an incompletely folded and unassembled Ig heavy chain in cells. The heavy chain binds to BiP (Haas and Wabl, 1983), and like many other BiP binding proteins, it is able to induce the UPR (Lenny and Green, 1991), perhaps by triggering the release of BiP from the ER stress transducers (Bertolotti et al., 2000; Shen et al., 2002). COS-1 cells were mock-transfected (control), transfected with GFP alone, or cotransfected with GFP and a human Ig γ heavy chain. Forty hours after transfection, the latter two were harvested and sorted for GFP$^+$ cells. Equal cell numbers for all three experimental sets were lysed in SDS-sample buffer and separated onto SDS-polyacrylamide gels before transfer and Western blotting with the indicated antibodies (Fig. 6). The mock-transfected cells served as a control for BiP and topoisomerase II levels, and as expected, they did not express either γ heavy chains or CHOP, because they were not experiencing ER stress. The cells that were only expressing GFP were also negative for γ heavy chains but revealed a very minor induction of CHOP, perhaps because of the stress of transfection or cell sorting. The levels of BiP and topoisomerase II did not seem to be significantly affected. However, when cells expressing both GFP and γ heavy chains were examined, we found readily detectable levels of γ heavy chain, a concomitant induction of both BiP and CHOP, demonstrating that the heavy chains had activated the UPR in these cells, and finally a dramatic loss of topoisomerase IIα from the transfected cells. Thus, activation of the UPR without drugs is sufficient to induce the loss of topoisomerase IIα from cells.

Discussion

A number of studies have shown that treatment of cells with agents that induce ER stress renders cells more resistant to treatment with drugs targeting topoisomerase II (Shen et al., 1987; Hughes et al., 1989). These studies demonstrated that ER stress did not lead to resistance to all cytotoxic agents but were specific for drugs targeting topoisomerase II. In this work, we found that resistance to topoisomerase II-targeting agents is a direct effect of UPR activation because it fails to occur in cells in which UPR activation is genetically blocked. Cells overexpressing BiP are unable to activate the UPR in response to ER stress, and we observed that these cells remained sensitive to etoposide even though they had been preincubated with ER stress-inducing agents. Although UPR-inducing conditions do not alter drug accumulation (Shen et al., 1987), it remained possible that ER stress caused the loss of critical proteins that abrogated the effects of topoisomerase II-targeting drugs. By expressing an unfolded Ig heavy chain in the ER of cells, we obtained activation of the UPR without affecting the maturation of other secretory pathway proteins. These data

demonstrated that expression of an unfolded protein was sufficient to reduce topoisomerase II protein levels, linking the loss of topoisomerase II directly to UPR activation.

A critical aspect of drug resistance induced by UPR-inducing agents is that the resistance is relatively specific for topoisomerase II-targeting drugs. Resistance to DNA damage induced by ionizing radiation does not occur with UPR induction (Shen et al., 1987). Some resistance to camptothecins has been reported (Tomida et al., 1996); however, camptothecin resistance probably arises because of the G_1 arrest induced by UPR-inducing agents (Brewer et al., 1999). Camptothecin cytotoxicity requires ongoing DNA replication (Holm et al., 1989; D'Arpa et al., 1990; Nitiss and Wang, 1996); therefore G_1 arrest leads to camptothecin resistance. It is noteworthy that for drugs such as cisplatin and some other alkylating agents, UPR induction leads to enhanced drug sensitivity. These results suggest that UPR induction does not alter the ability of cells to commit to cell death; rather, induction of UPR provokes cellular responses that alter cell killing by specific classes of agents. Consistent with these results, we find that blocking UPR induction by BiP overexpression prevents the induction of resistance to topoisomerase II-targeting agents but does not affect sensitivity to ionizing radiation.

A simple hypothesis for resistance to topoisomerase II-targeting agents by UPR induction is the observed reduction in topoisomerase II (Hughes et al., 1989; Shen et al., 1989; Yun et al., 1995). Cell killing by topoisomerase-targeting drugs occurs through DNA damage arising from the trapping of the enzyme in a drug/enzyme/DNA ternary complex. When enzyme levels are reduced, DNA damage is diminished, resulting in reduced cell killing (Potmesil et al., 1988; Nitiss et al., 1992). We observed that BiP overexpressing cells failed to reduce topoisomerase IIα protein, and the cells remained sensitive to etoposide. Conversely, the induction of UPR by expression of an unfolded protein results in the loss of topoisomerase II (Fig. 6), demonstrating that UPR induction by an unfolded protein, as opposed to a drug, also alters topoisomerase II levels. Furthermore, we showed that UPR induction leads to the loss of topoisomerase IIα but not topoisomerase IIβ. This suggests that some sensitivity to topoisomerase II-targeting agents should persist, even when the UPR is induced. It may be possible to specifically target the β isoform of topoisomerase II (Gao et al., 1999), which could allow the targeting of cells undergoing ER stress.

Several effects of UPR activation could lead to decreases in topoisomerase II protein. Topoisomerase IIα is transcriptionally regulated during the cell cycle, with the highest mRNA levels occurring during G_2/M phase (Swedlow and Hirano, 2003). UPR activation initiates G_1 arrest in mammalian cells caused by a PERK-dependent loss of cyclin D1 protein translation (Brewer and Diehl, 2000). Thus, cell-cycle arrest could cause topoisomerase IIα loss by blocking its transcription. However, our data showed that topoisomerase IIα loss occurs before cells accumulate in G_1 and before topoisomerase II transcript levels are unaffected, in keeping with earlier studies (Shen et al., 1989). These results suggest that the loss of topoisomerase IIα during ER stress involves post-transcriptional mechanisms. One possibility is that the translational arrest occurring downstream of PERK could block topoisomerase II synthesis. Like cyclin D1, topoisomerase IIα translation could be affected longer than most proteins in cells experiencing ER stress. Another mechanism for depleting

TABLE 1
IC_{50} and IC_{90} values for CHO and CHO-BiP^{OE} clonogenic assays

Cell Lines and Treatments	IC_{50}	IC_{90}
CHO		
Etoposide 2 h	3.4 μM	9.5 μM
10 μM Thapsigargin 6 h + etoposide 2 h	4.1 μM	18.5 μM
Irradiation	1.6 Gy	5.0 Gy
CHO-BiP^{OE}		
Etoposide 2 h	1.5 μM	7.5 μM
10 μM Thapsigargin 6 h + etoposide 2 h	0.9 μM	7.5 μM
Irradiation	1.6 Gy	4.2 Gy

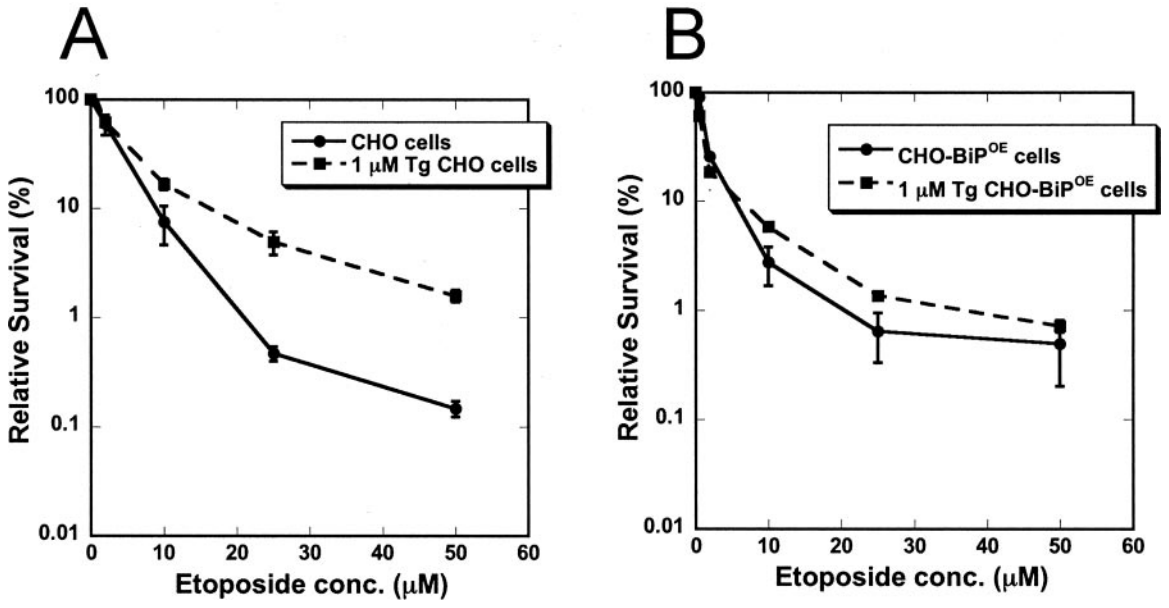

Fig. 5. Induction of the UPR is necessary for increased resistance to etoposide, as determined by clonogenic survival. CHO (A) and CHO-BiP^{OE} (B) cells were pretreated with 1.0 μM thapsigargin for 6 h, followed by the indicated etoposide concentrations for 2 h. Cells were washed and trypsinized, and varying numbers of cells were replated in triplicate. After reincubating for 10 days, colonies were stained, counted, and expressed as a percentage of cells surviving after treatment with thapsigargin alone.

topoisomerase IIα could be via enhanced protein turnover. UPR activation leads to nuclear accumulation of proteasomes (Ogiso et al., 2002), and proteasomal inhibitors attenuate both stress-induced resistance to etoposide and loss of topoisomerase IIα (Carlsson et al., 1978). A glucose-regulated destruction domain was recently identified on topoisomerase IIα that provides a binding site for Jab1/CNS5, which may mediate the degradation of topoisomerase II during ER stress (Yun et al., 2004). Jab1 binds to the cytosolic domain of inactive Ire1 in nonstressed cells, but it is released when Ire1 is activated (Oono et al., 2004). It is unclear why the loss of topoisomerase IIα should be a consequence of UPR activation. BiP overexpressing cells do not show a survival disadvantage when exposed to agents that lead to ER stress (Morris et al., 1997), indicating that topoisomerase IIα probably does not negatively affect cell survival under these stress conditions. Unfortunately, it has not been possible to ectopically express topoisomerase IIα in mammalian cells (Salmena et al., 2001) to determine whether enforced expression of this enzyme diminishes survival under UPR-inducing conditions.

It has also been suggested that elevated expression of the ER chaperone BiP plays a direct role in resistance to topoisomerase II-targeting agents (Rao et al., 2002; Gosky and Chatterjee, 2003; Reddy et al., 2003). Although BiP is normally localized in the ER, some studies have suggested that when high levels of BiP are synthesized, either because of transgene expression (Reddy et al., 2003) or during later phases of the ER stress response (Rao et al., 2002), BiP may also be found in the cytosol, in which it inhibits caspase cleavage and apoptosis in response to etoposide. When BiP overexpressing cells were compared with the parental cell line, we saw no resistance to etoposide, even though they express somewhat more BiP protein than cells in which the UPR has been induced. This result is in direct contradiction to a recent report showing that BiP overexpression was sufficient to confer resistance to topoisomerase II-targeting drugs and camptothecin (Reddy et al., 2003). Their experiments were carried out in CHO cells that were engineered to overexpress BiP and were derived from the same source as our lines. Although we cannot explain why our results differ from those reported, it is possible that the two BiP overexpressing cell lines do not express the same amount of BiP. We demonstrated that our BiP overexpressing cells remained impaired in UPR activation as expected (Morris et al., 1997). In the other study, it was suggested that BiP overexpression protected cells from etoposide treatment by inhibiting caspase cleavage. This model is difficult to reconcile with the patterns of drug sensitivity previously reported upon UPR induction. First, if BiP overexpression blocks caspase activation, it might be expected that BiP overexpression would also affect sensitivity to many other types of DNA damage. However, we found that BiP overexpression did not affect sensitivity to ionizing radiation, which is consistent with a previous study in which UPR induction did not affect the sensitivity of cells to this type of DNA damage (Hughes et al., 1989). Second, UPR induction actually increases cell killing by certain DNA-damaging agents like cisplatin (Chatterjee et al., 1997), which is also inconsistent with increased levels of BiP directly inhibiting apoptosis. Finally, it is clear from the results of Lock and colleagues that topoisomerase II-mediated DNA damage is correlated with topoisomerase II protein levels, which are reduced upon exposure to UPR-inducing conditions (Shen et al., 1989; Webb et al., 1991). Our results show that topoisomerase II levels are similar between the two cell lines. Topoisomerase II protein is reduced in wild-type CHO cells upon UPR induction but not in BiP overexpressing cells, which similarly show a continued sensitivity to etoposide during UPR activation. Together, our results are most consistent with some aspect of UPR induction other than BiP overexpression per se, causing resistance to topoisomerase II-targeting agents.

A resolution of the differences between our results and the results reported by Lee and colleagues will require a finer dissection of the UPR pathway and identification of the branch(es) that are directly responsible for UPR-induced loss of topoisomerase II. It should be possible to block steps in the UPR pathway that lead to the loss of topoisomerase II but leave other parts of the pathway intact and determine whether the loss of topoisomerase II is necessary and sufficient for UPR-mediated resistance to topoisomerase II agents. These studies should also be useful in identifying potential mechanisms of increasing the sensitivity of tumor cells to chemotherapeutic agents or minimizing their effects on normal cells.

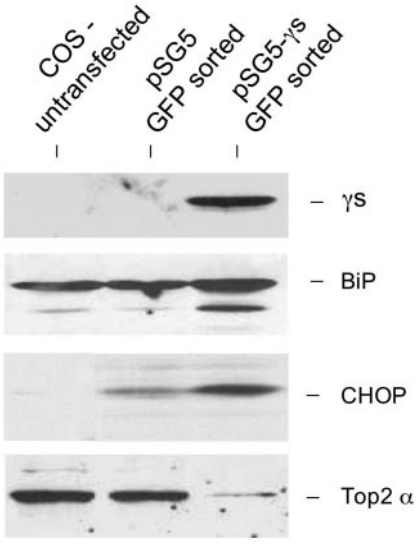

Fig. 6. UPR induction is sufficient to induce a reduction in topoisomerase IIα levels. COS-1 cells were cotransfected with an empty pSG5 vector and pGreen Lantern or with a pSG5 vector encoding the Ig γ heavy chain plus pGreen Lantern. Forty-eight hours after transfection, GFP-positive cells were isolated, and cell lysates were prepared and analyzed by Western blotting with the indicated antisera.

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Address correspondence to: Dr. John L. Nitiss, St. Jude Children's Research Hospital, Molecular Pharmacology Department, 332 N. Lauderdale, Memphis, TN 38105. E-mail: john.nitiss@stjude.org