Endoplasmic Reticulum Chaperone Protein GRP78 Protects Cells from Apoptosis Induced by Topoisomerase Inhibitors

ROLE OF ATP BINDING SITE IN SUPPRESSION OF CASPASE-7 ACTIVATION*

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A large number of correlative studies have established that the activation of the unfolded protein response (UPR) alters the cell's sensitivity to chemotherapeutic agents. Although the induction of the glucose-regulated proteins (GRPs) is commonly used as an indicator for the UPR, the direct role of the GRPs in conferring resistance to DNA damaging agents has not been proven. We report here that without the use of endoplasmic reticulum (ER) stress inducers, specific overexpression of GRP78 results in reduced apoptosis and higher colony survival when challenged with topoisomerase II inhibitors, etoposide and doxorubicin, and topoisomerase I inhibitor, camptothecin. While investigating the mechanism for the GRP78 protective effect against etoposide-induced cell death, we discovered that in contrast to the UPR, GRP78 overexpression does not result in G1 arrest or depletion of topoisomerase II. Caspase-7, an executor caspase that is associated with the ER, is activated by etoposide. We show here that specific expression of GRP78 blocks caspase-7 activation by etoposide both in vivo and in vitro, and this effect can be reversed by addition of dATP in a cell-free system. Recently, it was reported that ectopically expressed GRP78 and caspases-7 and -12 form a complex, thus coupling ER stress with procaspase-7 but not with procaspase-3. Lastly, a GRP78 mutant deleted of its ATP binding domain fails to bind procaspase-7 and loses its protective effect against etoposide-induced apoptosis.

Resistance to chemotherapy remains a major obstacle for the treatment of cancer. The complexity of drug resistance in human cancer strongly suggests the involvement of multiple pathways. One mechanism, both intrinsic and acquired, is the result of genetic alterations within cancer cells. Another mechanism may result from environmental conditions that occur naturally in solid tumors. Because of poor vascularization, solid tumors usually contain regions undergoing glucose starvation and hypoxia, resulting in acidosis and alterations in cell metabolism (1). These pockets of hypoxia and nutrient deprivation occur in well differentiated, slow growing, non-metastatic tumors, as well as in rapidly growing, aggressive anaplastic malignancies.

Stress conditions in cell culture, such as glucose starvation, commonly cause the glucose-regulated stress response (2), which is part of a general cellular defense mechanism referred to as the unfolded protein response (UPR) (3). One characteristic of the UPR is the induction of the endoplasmic reticulum (ER) resident stress proteins referred to as the glucose-regulated proteins (GRPs) (4). The GRPs are Ca2+-binding chaperone proteins with protective properties. The best characterized GRP is GRP78, a 78-kDa protein also referred to as BiP. As a protein chaperone, GRP78 is known to form complexes with heterologous proteins that are processed through the ER (5).

Overexpression and antisense approaches in cell systems show that GRP78 can protect cells against cell death caused by disturbance of ER homeostasis (6–11). Whereas GRP78 overexpression could limit damage in normal tissues and organs exposed to ER stress, the anti-apoptotic function of GRP78 also predicts that its natural induction in neoplastic cells could lead to cancer progression and drug resistance (12). In a variety of cancer cell lines, solid tumors, and human cancer biopsies, the level of GRP78 is elevated, correlating with malignancy (13–20).

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Concentration of 30 mM and stored at 4°C. The C.1 and AD-1 cells were maintained in the above 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin. 

16. Using human cancer and other cell lines, a large number of stress induction studies show that a glucose-regulated stress response results in the induction of GRP78 and other coordinately regulated GRP genes correlating with cellular drug resistance (17–22). Nonetheless, the direct role of GRPs in conferring drug resistance has not been proven. This is because of the inherent problems associated with using stress inducers or deficiencies in certain cell functions to induce the GRPs, because the inducing conditions can exert other unknown pleiotropic effects, possibly affecting multiple cellular pathways. Furthermore, the mechanisms for the protective function of the ER localized GRPs in drug resistance are not understood.

Many of the cytotoxic drugs, including topoisomerase inhibitors such as etoposide, initiate programmed cell death (23). DNA damaging agents such as etoposide can trigger cell death through the p53-mediated caspase cell death signaling cascade, resulting in cytochrome c release and the activation of caspase-3 (24). Caspases-3, -6, and -7 are members of the apoptotic executing group of caspases with caspase-7 structurally and functionally most similar to caspase-3 (25). Active caspase-7 has been shown to be associated with the mitochondria and the ER membranes, whereas caspase-3 remains cytosolic (26, 27). Although these observations suggest that similar apoptotic executioner functions in different cellular compartments and act on distinct substrates, there is limited information on the contribution of organelles such as the ER in the apoptotic process.

Through specific overexpression of GRP78 in Chinese hamster ovary (CHO), human leukemia, and bladder carcinoma cell lines, we show that GRP78 overexpression directly confers resistance to cytotoxic drug-induced apoptosis, correlating with a higher survival rate in clonogenic assays. We discover that GRP78 overexpression differs from the UPR inducers in that it does not affect topoisomerase II level or arrest cells in G1. Upon etoposide treatment, caspase-7 is activated, and GRP78 overexpression suppresses its activation. However, the question remains as to how a protein such as GRP78 that is presumed to be an ER lumenal protein can interfere with caspase activation in the cytoplasm. Based on our discovery that a subpopulation of GRP78 can exist as an ER transmembrane protein and that its physical and functional interaction with procaspase-7 is dependent on the GRP78 ATP binding domain, a mechanism for GRP78 to confer resistance to etoposide-induced apoptosis is presented.

**EXPERIMENTAL PROCEDURES**

**Cell Culture Conditions**—The establishment of C.1 and AD-1 cell lines that are derivatives of CHO overexpressing wild-type or mutated hamster GRP78 has been described (9). The CHO cells were maintained in α-minimum Eagle’s medium with nucleosides supplemented with 10% dialyzed fetal calf serum and 1% penicillin/streptomycin/neomycin antibiotics. The C.1 and AD-1 cells were maintained in the above conditions in the presence of 0.1 μg/ml methotrexate but without added nucleosides. The establishment of stable T24/83 human transitional bladder carcinoma cell lines overexpressing human GRP78 or transfected with the empty expression vector (pcDNA3.1) has been described (28). The T24/83 cell lines were maintained in M199 medium supplemented with 10% fetal calf serum containing 1% penicillin/streptomycin/neomycin antibiotics and 200 μg/ml G418. The human T cell leukemia Jurkat cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum containing 1% penicillin/streptomycin/neomycin antibiotics. All the cells were maintained at 37 °C in a humidified atmosphere of 5% CO2/95% air.

**Reagents**—Etoposide (Calbiochem) was dissolved in Me2SO at a concentration of 30 mM and stored at -20 °C. Methotrexate (Sigma) was dissolved in a minimum amount of 1 N NaOH, diluted with water to 1 mM, and stored at -20 °C. Doxorubicin (Bedford Laboratories, Bedford, OH) at 2 mg/ml and camptothecin (Amersham Biosciences) at 20 mg/ml were supplied as isostatic solutions.

**Cell Cycle Analysis**—Following seeding, exponentially growing cells were trypsinized at different days and fixed in 70% ethanol. The fixed cells were treated with PBS containing 0.1% (v/v) Triton X-100, 0.2 mg/ml DNase-free RNase, and 20 μg/ml propidium iodide (PI) for 30 min at room temperature. The cell cycle distributions were analyzed by fluorescence-activated cell sorting (FACS) analysis (FACScan; BD Biosciences). The cell cycle distribution measurements were repeated three to four times.

**Clonogenic Survival Assays**—Four thousand cells were seeded into 10-cm-diameter dishes. Two days after seeding, cells were treated with etoposide for 6 h, doxorubicin for 1 h, or camptothecin for 24 h at different concentrations as indicated. After drug treatment, the cells were grown in fresh medium for 10 to 14 days. The colonies were washed with ice-cold PBS, fixed with methanol, and stained with 10% Giemsa to obtain the clonogenic survival fractions, which were determined by dividing the number of surviving colonies in the drug-treated cells by the number of colonies in the non-treated control groups. Each assay was repeated three to four times.

**Annexin V Staining and FACS Analysis**—CHO, C.1, and T24/83 cells were trypsinized, washed twice with ice-cold PBS, pH 7.4, and resuspended in 1× binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of 1 × 106 cells/ml. One hundred μl of cell suspension was transferred to 5-ml plastic tubes, and 5 μl of annexin V-fluorescein isothiocyanate (PharMingen) and 4 μl of 0.5 mg/ml PI were added. The cells were gently vortexed and incubated in the dark at room temperature for 20 min. Four hundred μl of binding buffer was added to each tube, and annexin V staining was evaluated by flow cytometry within 1 h. Cells negative for both PI and annexin V staining are live cells, annexin V positive staining cells are early apoptotic cells, and PI positive and annexin V positive staining cells are primarily cells in late stages of apoptosis. The experiments were repeated two to three times.

**Caspase-7 Activation Assays**—The cells were either non-treated or treated with 100 μM etoposide for 6 h and harvested after 24 h. The cells were suspended in 5 volumes of a hypotonic buffer (5 mM Tris-HCl, pH 7.4, 5 mM KCl, 1.5 mM MgCl2, 0.1 mM EGTA, pH 8.0, and 1 mM dithiothreitol) in the presence of 2 μg/ml leupeptin, pepstatin, and aprotinin protease inhibitors. After incubation on ice for 20 min, surose was added to a final concentration of 250 mM, and the cells were disrupted by douncing eight times in a 1-mL Wheaton Dounce homogenizer. The homogenate was centrifuged twice at 750 × g for 10 min. The supernatant was clarified again at 16,000 × g for 15 min at 4 °C and designated as the cytoplasmic fraction. For in vitro caspase-7 activation assay, 150 μg of cell-free extract was incubated with various amounts of cytochrome c and dATP at 37 °C for 1 h. Equal amounts of total proteins were separated, and Western blotting was performed for caspase-7.

**Western Blotting**—The cell lysate was prepared in radioimmune precipitation assay buffer and subjected to immunoblot with antibodies against GRP78, GRP94, topoisomerase II, caspase-7, and β-actin as described (29). Nitrocellulose membranes containing the transferred Gels were blocked in TBS-T containing 5% non-fat dry milk and 0.1% Tween 20 for 1 h at room temperature and were probed with the respective primary antibodies. For GRP78, an anti-KDEL mouse monoclonal antibody (SPA-827), an anti-GRP78 rabbit polyclonal antibody directed against the carboxyl ten amino acids of rat GRP78 (SPA-820) (StressGen, Victoria, Canada), or an anti-hamster GRP78 rabbit polyclonal antibody (gift from Dr. Linda Hendershot) at 1:3000, 1:2000, and 1:5000 dilution, respectively, was used. Dilutions for the other primary antibodies were as follows: anti-calnexin rabbit polyclonal antibody (SPA-865) (StressGen) at 1:2000, anti-cathepsin B rabbit polyclonal antibody (SPA-800) (StressGen) at 1:3000, anti-β-actin mouse monoclonal antibody (Sigma) at 1:5000, anti-caspase-7 mouse monoclonal antibody (BD Biosciences) at 1:1000, anti-caspase-3 rabbit polyclonal antibody (Cell Signaling, Beverly, MA) at 1:1000, and anti-topoisomerase II mouse monoclonal antibody (SWTS71) (Oncogene, San Diego, CA) at 1:1000. Respective horseradish peroxidase-conjugated secondary antibodies were used, and the protein bands were visualized by the ECL method (Amersham Biosciences).

**Chloramphenicol Transfection Death Assay**—The cytotoxicity assays have been described (31). Briefly, Jurkat cells were transiently transfected with either CMV-neo-Bcl2 (gift of C. M. Zacharchuk, National Institutes of Health) (32) or expression vectors for wild-type hamster GRP78 or a GRP78 ATP-binding site mutant G227D (gift of L. M. Henderson, St. Jude Hospital, Memphis, TN) (30). After drug treatment, cell lysates were prepared and assayed for caspase-7 activity in the surviving cells. The percent cytotoxicity was calculated as described previously (32).

**DNA Fragmentation Assays**—The cells were either non-treated or treated with 100 μM etoposide for 12 h and harvested after 48 h. The
DNA fragmentation assays were performed using an apoptosis DNA ladder kit (Roche Molecular Biochemicals) according to manufacturer’s instructions (31).

**Immunofluorescence Staining and Image Analysis**—CHO and C.1 cells were grown to 60% confluence in chamber slides (Nalge Nunc International, Naperville, IL), washed twice with PBS, and fixed with 4% paraformaldehyde in PBS for 10 min. The cells were then washed with PBS and permeabilized in PBS containing 0.1% Triton X-100 and 5% bovine serum albumin for 30 min. For detection of GRP78, the cells were stained with a 1:1,000 dilution of anti-GRP78 (C-20) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a 1:50 dilution of anti-goat Texas red-conjugated secondary antibody (Vector Labs, Burlingame, CA). For detection of caspase-7, the cells were treated with antisera against caspase-7 followed by Texas red-conjugated secondary antibody and viewed by confocal microscopy. Subcellular distribution of GRP78 is primarily perinuclear, indicative of ER localization.

**RESULTS**

**Specific Overexpression of GRP78 Confers Resistance to Topoisomerase Inhibitors through Protection against Drug-induced Apoptosis**—A CHO cell line derivative (C.1) has been established that has stably integrated high copy numbers of a diphospholase reductase plasmid expressing the hamster GRP78 protein (9). Quantitation of the immunoblots of whole cell extracts showed 5-fold higher GRP78 level in C.1 cells compared with the parental CHO cells, whereas the level of GRP94, also an ER-localized chaperone protein, and a 45-kDa unidentified protein (X) recognizable by the anti-KDEL antibody was relatively constant in both cell lines (Fig. 1A). In situ immunofluorescence imaging using anti-GRP78 antibody further revealed that in both CHO and C.1 cells, the majority of GRP78 was concentrated in the perinuclear region, consistent with its location in the ER (Fig. 1B). In agreement with the immunoblot analysis, the intensity of the immunofluorescent signal for GRP78 was greater in the majority of C.1 cells compared with CHO cells.

To examine directly whether specific overexpression of GRP78 can lead to the development of drug resistance, CHO and C.1 cells were exposed to various drugs, and cell survival was measured using clonogenic survival assays. Various dosages of etoposide (also referred to as VP16), adriamycin (also referred to as doxorubicin), and camptothecin were tested. Both etoposide and adriamycin are inhibitors of topoisomerase II, and camptothecin is a topoisomerase I inhibitor. Typical re-
For 1 h (A), adriamycin for 1 h (B), or camptothecin for 24 h (C) as indicated. After 2 weeks of growth, the surviving colonies were stained with Giemsa and counted. The surviving fractions were plotted against the drug concentration as indicated. The S.D. for the etoposide-treated cells performed in triplicate are shown.

Fig. 2. Clonogenic survival assays for CHO and C.1 cells. An equal number of cells were seeded, and after 2 days of culture they were subjected to various concentrations of etoposide for 6 h (A), adriamycin for 1 h (B), or camptothecin for 24 h (C) as indicated. After 2 weeks of growth, the surviving colonies were stained with Giemsa and counted. The surviving fractions were plotted against the drug concentration as indicated. The S.D. for the etoposide-treated cells performed in triplicate are shown.

Results for each of the drugs tested were shown in Fig. 2. The surviving fractions for the cell lines were calculated based on the surviving colony number compared with the non-treated control. With all three drugs, C.1 cells overexpressing GRP78 conferred higher resistance than CHO cells. These experiments establish that specific overexpression of GRP78, in the absence of the UPR, is sufficient to render CHO cells more resistant to topoisomerase I and II inhibitors.

To determine whether GRP78 protects the cells from etoposide-induced apoptosis, CHO and C.1 cells were either non-treated or treated with etoposide and labeled with annexin V and PI. The apoptotic cells were identified by annexin V labeling. For CHO cells, the percentage of apoptotic cells increased 10-fold (from 9 to 90%) upon etoposide treatment; for C.1 cells, the increase was 4.7-fold (from 15 to 70%) (Fig. 3A). More extensive DNA fragmentation was also detected in etoposide-treated CHO but not C.1 cells (Fig. 3B). These results support that GRP78 overexpression suppresses etoposide-induced apoptosis.

Overexpression of GRP78 in a Human Bladder Carcinoma Cell Line Suppresses Etoposide-induced Apoptosis—To extend the observations of the CHO cell derivatives to human cancer cell lines, we utilized a pair of stably transfected human transitional bladder carcinoma T24/83 cell lines selected and cultured under identical conditions. The cell line, referred to as T24/83-GRP78, overexpressed human GRP78, and the other line, referred to as T24/83-pcDNA, was stably transfected with the empty expression vector pcDNA (28). Immunoblot analysis followed by normalization against β-actin revealed a 3-fold increase in the level of GRP78 expression in the T24/83-GRP78 cells as compared with T24/83-pcDNA cells (Fig. 4A, inset). Overexpression of GRP78 in T24/83-GRP78 cells did not affect the expression level of ER chaperone proteins GRP94, protein disulfide isomerase and calreticulin, or heat shock protein HSP47 (Fig. 4A) (data not shown). Whole cell imaging revealed much greater GRP78 immunofluorescence for the T24/83-GRP78 cells, confirming the results of the immunoblots (Fig. 4B). In the same cells, it is evident that the majority of GRP78 was concentrated in the perinuclear region, consistent with ER localization. Non-specific staining was not observed in either T24/83 cell lines immunostained with normal goat IgG (data not shown).

In agreement with the CHO cell lines, T24/83 cells overexpressing GRP78 exhibited more resistance to etoposide in clonogenic survival assays (Fig. 4A). Similar protection was observed for adriamycin and camptothecin (data not shown). For T24/83-cDNA cells, etoposide treatment increased the percentage of annexin V-labeled cells 2.7-fold (from 7 to 19%), as compared with an increase of 1.4-fold (from 8 and 11%) for T24/83-GRP78 cells (Fig. 4C). Thus, there were less apoptotic cells in etoposide-treated T24/83 cells overexpressing GRP78 than the cells stably transfected with the empty vector. This is consistent with a higher sensitivity of the T24/83-pcDNA cells to etoposide in clonogenic survival assays.

GRP78 Overexpression Does Not Affect Topoisomerase II Levels or Cell Cycle Distribution—Previously, it has been reported that glucose starvation, commonly used as an inducer for the UPR, can result in the depletion of topoisomerase II (35). This could explain the development of resistance against topoisomerase II inhibitors such as etoposide and adriamycin following glucose starvation. With the availability of the GRP78 overexpressing cell lines, we can determine the effect of GRP78 overexpression on topoisomerase II level in the absence of an UPR. To test this, CHO and C.1 cells were either non-treated or treated with etoposide, and the level of topoisomerase II was determined by immunoblotting (Fig. 5A). We observed that specific GRP78 overexpression has no effect on the topoisomerase II protein level. In both CHO and C.1 cells, the level of topoisomerase II remained constant before and after treatment with etoposide.

The majority of UPR inducers arrest cells in G1 (36). This could contribute to resistance against topoisomerase inhibitors, because the cytotoxic effect of these drugs requires DNA replication (37). Analysis of the cell cycle distribution of exponentially growing cells showed CHO and C.1 cells with similar G1, S, and G2 distribution profiles (Table I). In contrast, CHO cells treated with tunicamycin or thapsigargin, both standard UPR inducers, showed more cells in G1 and a dramatic reduction in S phase cells. A similar pattern was observed for exponentially growing T24/83 cells. In both the vector-transfected and GRP78 overexpressing cells, the percentage of G1, S, and G2 cells is similar. Cells treated with tunicamycin or thapsigargin showed a higher percentage of G2 cells and a lower percentage in S phase (Table I). Collectively, these results show that in contrast to the UPR, specific overexpression of GRP78 does not alter the cell cycle distribution.

GRP78 Overexpression Suppresses Etoposide-mediated Activation of Caspase-7—Among the executor caspases, caspase-7 has been reported to be associated with the ER. Upon induction of apoptosis, procaspase-7 (35 kDa) is first converted into a 32-kDa intermediate, which is further processed into active 20- and 11-kDa subunits. First, we determined whether caspase-7 is affected by etoposide treatment in our experimental system. The CHO and C.1 cells were either non-treated or treated with etoposide, and cytoplasmic extracts were prepared. Caspase-7 processing was monitored by Western blotting with specific antibody that recognizes both procaspase-7 and active caspase-7. We observed that treating CHO cells with etoposide resulted in activation of caspase-7, giving rise to the 32-kDa intermediate form (Fig. 5A). Upon longer exposure of the autoradiogram, the active 20- and 11-kDa forms were evident in the etoposide-treated cells. For C.1 cells overexpressing GRP78, we detected a low level of caspase-7 activation in both the non-treated and etoposide-treated cells. In contrast to the CHO cells, the levels of the intermediate and activated form of caspase-7 were not increased following etoposide treatment (Fig. 5A). Thus, GRP78 overexpression is able to suppress caspase-7 activation in vivo.
Next, we used cell-free systems to test the effect of GRP78 overexpression on cytochrome c-mediated activation of caspase-7. Cytoplasmic extracts were prepared from CHO and C.1 cells and incubated with increasing amounts of cytochrome c. For both cell lines, there was a low level of basal activation of procaspase-7 in the absence of cytochrome c. Upon addition of cytochrome c, caspase-7 activation was higher in CHO cells than C.1 cells, as evidenced by the increase in the active 20- and 11-kDa forms in the CHO samples compared with the C.1 samples (Fig. 5B). In the presence of both cytochrome c and dATP, the suppressive effect of the C.1 samples was reversed (Fig. 5C). At 1 mM dATP, both cell lines showed equivalent amounts of the 32- and 20-kDa forms, suggesting that dATP releases procaspase-7 from GRP78, resulting in its activation.

**Complex Formation between Endogenous GRP78 and Caspase-7 in the ER**—The functional interaction between GRP78 and caspase-7 suggests that the two proteins may be in close proximity in the ER. In examining the distribution of GRP78 and caspase-7 in *situ* using immunofluorescence, we observed that in C.1 cells, caspase-7 exhibits a perinuclear pattern indicative of ER localization (Fig. 1C). Confocal microscopy further revealed caspase-7 (green) in close proximity with a subfraction of GRP78 (red), as indicated by discrete specks of yellow immunofluorescence in the merged image. The colocalization of GRP78 and caspase-7 was primarily detected in the perinuclear/ER region. Despite the presence of a low amount of GRP78 in the nucleus as reported previously (9), caspase-7 was not detected inside the nucleus, and co-staining was not observed.

To confirm the physical interaction of endogenous caspase-7 with GRP78, whole cell extracts were prepared from CHO and C.1 cells. Following immunoprecipitation with anti-caspase-7 antibody, Western blots were performed using antibodies against GRP78 and caspase-7. In agreement with the co-localization results obtained from confocal microscopy, procaspase-7 forms a complex with a high level of GRP78 in C.1 cells (Fig. 6A, lane 2). For CHO cells, GRP78 was detected as a faint band in the anti-caspase-7 immunoprecipitate using an anti-KDEL antibody (Fig. 6A, lane 1), and the signal for GRP78 was very enhanced when an anti-hamster GRP78 antibody was used for the Western blots (Fig. 6B, lane 1). Using anti-caspase-3 as the immunoprecipitating antibody, we did not detect GRP78 association with procaspase-3 in Western blots (Fig. 6A, lanes 3 and 4). Thus, endogenous GRP78 constitutively associates with procaspase-7 but not with procaspase-3.

**A Subfraction of GRP78 Exhibits Properties of an ER Transmembrane Protein**—To understand how GRP78, presumably an ER lumen protein, can interact with caspase-7, a cytosolic caspase, we investigated the topology of GRP78. An examination of the hydrophaticity profile of GRP78 reveals that whereas it is highly hydrophilic, it contains four evolutionarily conserved hydrophobic domains (I through IV) that can form possible transmembrane helices (Fig. 7A) (38). The TMped program scored domains I and IV as significant, and domain I matches with the hydrophobic leader peptide that targets GRP78 to the ER. If GRP78 can exist as an ER transmembrane protein, it should exhibit sensitivity to limited trypsin digestion and co-purify with the ER membrane.

To test this, we first performed limited trypsin digestion of microsomes isolated from both CHO and C.1 cells, followed by Western blot analysis using an antibody directed against the carboxyl ten amino acids of rat GRP78. An example of the digestion pattern for the C.1 cells was shown in Fig. 7B. At low dose of trypsin digestion, a resistant carboxyl band of about 35 kDa was detected. At the higher dose of trypsin, the intensity of the 35-kDa band became stronger, and a minor band of around 50-kDa was also visible. The digestion pattern for the CHO cells was the same, with the resistant bands more prominent for C.1 cells correlating with GRP78 overexpression (data not shown). To test that the trypsin treatment did not digest ER proteins localized inside the ER lumen, we confirmed that calreticulin, which is a known ER luminal protein, was not

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**Fig. 3.** GRP78 overexpression protects cells from etoposide-induced apoptosis. A, measurement of apoptotic cells after etoposide treatment. C.1 and CHO cells were either non-treated (Ctrl) or treated with 30 μM etoposide (Etop) for 6 h. Forty-eight h after drug treatment, the cells were labeled with annexin V-fluorescein isothiocyanate and PI. The distribution pattern of live and apoptotic cells was determined by FACS analysis. Viable cells are those with low annexin or no annexin and PI staining (lower left panel). Early stage apoptotic cells are represented by high annexin and low PI staining (lower right panel), later stage apoptotic cells represented by are high annexin and high PI staining (upper right panel), and necrosis is represented by cells with high PI and low annexin staining (upper left panel). B, DNA fragmentation pattern of CHO and C.1 cells following etoposide treatment. Five μg of genomic DNA from non-treated cells (Ctrl) or cells treated with etoposide (Etop) or treated with 30 μM etoposide (Etop) for 6 h. Forty-eight h after drug treatment, the cells were labeled with annexin V-fluorescein isothiocyanate and PI. The distribution pattern of live and apoptotic cells was determined by FACS analysis. Viable cells are those with low annexin or no annexin and PI staining (lower left panel). Early stage apoptotic cells are represented by high annexin and low PI staining (lower right panel), later stage apoptotic cells represented by are high annexin and high PI staining (upper right panel), and necrosis is represented by cells with high PI and low annexin staining (upper left panel). B, DNA fragmentation pattern of CHO and C.1 cells following etoposide treatment. Five μg of genomic DNA from non-treated cells (Ctrl) or cells treated with etoposide (Etop) were electrophoresed on 1.8% agarose gel in parallel with DNA size markers as indicated on the left. The DNA pattern was visualized by ethidium bromide staining.
affected (Fig. 7B). Under the same conditions, calnexin, a known ER transmembrane protein, was partially digested at the low trypsin dosage and fully digested at the high dose of trypsin, yielding a resistant amino-terminal band as expected (Fig. 7B).

Second, sodium carbonate extraction of the microsome membrane fractions was performed to directly prove that GRP78 could exist as an integral transmembrane protein (Fig. 7C). As a control, calnexin, an ER transmembrane protein, was exclu-
FACS analysis was performed to determine the percentage of cells in the cell cycle. The cells were either nontreated or treated with Tuni or Tg, and FACs analysis was performed to determine the percentage of cells in G1, S, and G2 phase. The cells were the either nontreated or treated with Tuni or Tg, and FACs analysis was performed to determine the percentage of cells in G1, G2, and S phase. The experiments were repeated two to five times. The S.D. are shown.

|       | G1     | S    | G2     |
|-------|--------|------|--------|
| CHO   | 40 ± 5 | 18 ± 3 | 30 ± 2 |
| CHO + Tuni | 62 ± 3 | 5 ± 1 | 24 ± 3 |
| CHO + Tg | 55 ± 4 | 9 ± 2 | 26 ± 2 |
| C.1   | 44 ± 4 | 20 ± 2 | 28 ± 5 |
| T24/83-pcDNA | 52 ± 7 | 20 ± 8 | 30 ± 6 |
| T24/83-pcDNA + Tuni | 63 ± 3 | 3 ± 2 | 23 ± 2 |
| T24/83-pcDNA + Tg | 77 ± 4 | 6 ± 2 | 15 ± 2 |
| T24/83-GRP78 | 50 ± 4 | 21 ± 9 | 28 ± 4 |
| T24/83-GRP78 + Tuni | 75 ± 8 | 6 ± 2 | 22 ± 3 |
| T24/83-GRP78 + Tg | 72 ± 4 | 9 ± 3 | 22 ± 2 |

**DISCUSSION**

Despite a large number of studies linking the UPR to alteration of drug sensitivity, specific targets that are induced by the UPR responsible for this effect have not been established. The induction of GRP78 has been commonly used as an indicator for the UPR (4, 12); however, the question remains whether GRP78 is a contributing factor for drug resistance or a consequence of altered cell metabolism. In this study, we provide several lines of evidence that specific overexpression of GRP78 is sufficient to confer drug resistance in at least two cell types, and the protective effect of GRP78 is dependent on a functional ATP binding domain. Further, in this study, we uncovered characteristics of the UPR that are distinct from overexpression of GRP78.

To examine GRP78 function without the interference of a general stress response, we utilized two sets of independently derived cell lines that specifically overexpress GRP78 without concomitant increase of other ER chaperone proteins or stress proteins. This also allowed us to dissect the specific contribution of GRP78 and its protective mechanism apart from the other UPR targets. First, we showed by clonogenic survival assays that overexpression of GRP78 in both CHO and T24/83 human bladder carcinoma cell lines renders the cells more resistant to etoposide, doxorubicin, and camptothecin. This provides direct evidence that GRP78 overexpression contributes to the development of drug resistance. Although etoposide treatment of cells does not lead to induction of GRP78 within the first few hours, we and others (20) have observed that cells surviving long exposure to etoposide overexpress GRP78. In support of the clinical importance of these observations, overexpression of GRP78 was reported in drug-resistant primary human lung cancer and ovarian cancer cell lines (21).

One of the most interesting questions arising from these studies is how does GRP78, an ER lumen protein, protect cells from DNA damage imposed by the topoisiomerase inhibitors? Topoisiomerase inhibitors such as etoposide interact with the enzyme to stabilize topoisiomerase-DNA complex, blocking strand-passing activity, thereby resulting in DNA breakage (37). Because these drugs target S phase cells, it has been proposed that the chaperone function of GRP78 could affect growth factor processing, creating a cell proliferation block to escape drug killing that only occurs in cycling cells (22). Here we confirm that classic inducers of the UPR such as tunicamycin dramatically reduces the percentage of S phase cells (36). However, for both CHO and T24/83 cells overexpressing GRP78, there is minimal change in the cell cycle distribution compared with the parental cells or cells transfected with the vector alone. Thus, although the UPR inducers may alter drug sensitivity because of G1 arrest, specific GRP78 overexpression does not reduce the portion of S phase cells, and thus this mechanism cannot account for the protective effect of GRP78.
Glucose starvation induces GRP78 and can result in the depletion of topoisomerase II (35). We show here that specific GRP78 overexpression in the absence of the UPR has no effect on the topoisomerase II level, so this mechanism is also excluded. Using both CHO and T24/83 cells, we demonstrate here by annexin V labeling, DNA fragmentation, and transient cell

**FIG. 6. Co-immunoprecipitation of procaspase-7 with GRP78.** A, cell lysates from CHO and C.1 cells were immunoprecipitated with anti-caspase-7 (lanes 1 and 2) or anti-caspase-3 (lanes 3 and 4) antibodies. The immunoprecipitates were applied to 12% SDS-PAGE and Western blotted with antibodies against the KDEL epitope, caspase-7, and caspase-3. The positions of GRP78, caspase-7, and caspase-3 and protein size markers are indicated. B, cell lysates in extraction buffer from CHO and AD-1 cells were immunoprecipitated with anti-caspase-7 antibody (lanes 1 and 2). The immunoprecipitates were applied to 8% SDS-PAGE and Western blotted with anti-GRP78 (hamster GRP78 antibody) and anti-caspase-7 antibodies. Whole cell extracts (WCE) from CHO and AD-1 cells were immunoblotted in parallel (lanes 3 and 4). The positions of GRP78, procaspase-7, and the deletion mutant form of GRP78 (Δ78) are indicated. C, schematic drawing of wild-type (WT) GRP78 and AD-1 showing the location of the signal sequence (S), the ATP-binding domain, and the AD-1 deletion spanning amino acids 175 to 201.

**FIG. 7.** A subfraction of GRP78 exhibits properties of a transmembrane protein. A, the hydrophobicity plot of GRP78 was generated using the Kyte-Doolittle method with a window size of 17. The transmembrane domains were predicted using TMPred program (48). Four putative hydrophobic domains (I–IV) are identified. Represented below is a schematic drawing of the mature GRP78 protein with the hydrophobic domains IV and III as putative transmembrane domains generating carboxyl 35- and 50-kDa trypsin resistant fragments. B, limited trypsin digestion. Isolated microsomes from C.1 cells were either non-treated (lane 1) or subjected to trypsin digestion at the concentration of 0.01% (lane 2) or 0.05% (lane 2). At the end of the reaction, the amount of GRP78 was detected by Western blotting using the rabbit polyclonal anti-GRP78 antibody recognizing the carboxyl terminus (StressGen, Victoria, Canada) (left panel). The full-length GRP78 band is indicated by a closed arrow, and the 35- and 50-kDa proteolytic products are indicated by open arrows highlighted with a star. The same membrane was stripped and re-probed with a rabbit polyclonal anti-calreticulin (CRT) antibody (middle panel) or a rabbit polyclonal anti-calnexin antibody recognizing the amino terminus of calnexin (right panel). The full-length CRT and calnexin are indicated by closed arrows, and the 70-kDa proteolytic product of calnexin is indicated by an open arrow highlighted by a star. C, sodium carbonate extraction. The microsome (M) fraction was either non-treated (lane 1) or treated with 100 mM sodium carbonate and separated into pellet (P) and supernatant (S) fractions (lanes 2 and 3, respectively). The protein samples from each fraction were separated by 10% SDS-PAGE and subjected to Western blotting with rabbit anti-GRP78 antibody (left panel), rabbit anti-calreticulin antibody (middle panel), and rabbit anti-calnexin antibody (right panel).
death assays that the protective effect of GRP78 acts through interference of drug-induced apoptosis. Using the CHO cells as a model, we confirmed that caspase-7, a caspase associated with the ER, is activated by etoposide (23). We further demonstrated that GRP78 overexpression suppresses the activation of caspase-7 both \textit{in vivo} and \textit{in vitro}. Substrates of caspase-7 include poly(ADP-ribose) polymerase, as well as the ER transmembrane protein sterol regulatory element binding protein-1 (39). As executor caspases, caspase-7, caspase-3, and caspase-6 can act in concert to facilitate the apoptotic process. Thus, one mechanism for the protective effect of GRP78 is that its overexpression can suppress the full activation of the multiple caspase-mediated cell death pathways in drug-treated cells, contributing to the development of drug resistance.

How might GRP78, an ER resident protein, interfere with cytosolic caspase-7 function? Upon ER stress, it was reported recently (40) that in 293T cells GRP78 was found in the cytosolic, nuclear, and ER fractions, and GRP78 from microsomes of ER-stressed 293T cells was partially digested by trypsin. This same study showed that ectopically expressed GRP78 and caspase-7 and -12 form a complex and suppress cell death mediated by combined ectopic expression of these caspases. We observed that overexpression of cytosolic GRP78 did not result in protection against ER stress.\(^2\) Further, whether endogenous GRP78 constitutively forms a complex with procaspase-7 under physiologic conditions is not known, and the significance of GRP78 and procaspase-7 interaction in stress conditions other than those targeted to the ER remain to be determined. Using a DNA damaging agent such as etoposide as the apoptotic inducer in several different cell lines specifically overexpressing GRP78, we made several new observations that address some of the unresolved issues.

Through \textit{in situ} immunofluorescence studies, we showed that under normal culture conditions GRP78 and caspase-7 can constitutively co-localize in the perinuclear/ER region. \textit{In vivo} association of endogenous GRP78 with procaspase-7 can be detected prior to and following drug treatment (see Figs. 1 and 6) (data not shown). Through limited trypsin digestion and biochemical extractions of microsomes, we provide direct evidence that a subpopulation of GRP78 exists constitutively as a transmembrane protein, and this subpopulation of GRP78 increases with overexpression. Further, because the ER is a membrane source, the fact that GRP78 can exist as a transmembrane protein provides a plausible explanation for the recent unexpected findings that GRP78 can be found constitutively on the cell surface membrane of different cell types (41–43). Although future studies are required to determine whether GRP78 directly or indirectly interacts with procaspase-7, the transmembrane topology of GRP78 suggests that it is able to directly interact with cytosolic components mediating the apoptotic pathway. Our observation that GRP78 overexpression blocks caspase-7 activation both \textit{in vivo} and \textit{in vitro} supports the hypothesis that GRP78 facilitates the formation of an inhibitory complex suppressing the activation of caspase-7 (40). When GRP78 is overexpressed, it can form more inhibitory complexes, as well as preserving the integrity of the existing inhibitory complex. It is interesting to note that GRP78 resembles the X-linked inhibitor of apoptosis (XIAP) such that both genes contain an internal ribosome entry site element that allows efficient translation under physiological stress conditions (44, 45).

Through the use of several cell types overexpressing wild-type and mutant forms of GRP78, we demonstrated the requirement of a functional ATP binding domain for GRP78 interaction with procaspase-7 and its anti-apoptotic effects against DNA damaging agents. As in the case for protection against ER stress (9), mutation of a single amino acid destroying the ability of GRP78 to bind ATP abolishes its protection against etoposide-induced apoptosis. Members of the HSP70 family of chaperone proteins such as GRP78 contain ATP binding/hydrolisis activities that mediate their ability to aid in protein folding by coordinating the sequential binding and release of the protein substrate. Furthermore, a GRP78 ATPase mutant can act in a dominant negative manner suppressing endogenous GRP78 function (30). The ATP binding site may be required for the association and dissociation of the inhibitory complex. We further note that the mechanism of GRP78 protection may be a more complex process. In addition to acting as a protein chaperone, GRP78 may also regulate the activity of ER kinases that signal stress (46). Further dissection of the functional domains of GRP78 required for drug resistance will address these issues.

Because human cancer cells are inherently heterologous, different types of cancer cells can use diverse signaling and defense mechanisms to acquire resistance to specific drugs. Thus, the dependence on GRP78 for the development of drug resistance is likely to vary among cancer cells. For example, activation of NF-\(\kappa\)B may be the primary pathway leading to resistance against the topoisomerase II-directed agent teniposide in EMT6 mouse mammary tumor cells (47). In these same cells, the induction of GRP78 mRNA by castanospermine, which blocks oligonucleotide modification reactions by inhibiting glycosidase I and II, did not confer survival advantage against teniposide. Nonetheless, the effectiveness of castano-

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\(^2\) R. Kaufman, unpublished results.
In our previous studies, we found that spermine is able to raise GRP78 protein level in these cells. The mechanism by which spermine exerts this effect is under investigation.

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Endoplasmic Reticulum Chaperone Protein GRP78 Protects Cells from Apoptosis Induced by Topoisomerase Inhibitors: ROLE OF ATP BINDING SITE IN SUPPRESSION OF CASPASE-7 ACTIVATION
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