Increased Calcium Influx and Ribosomal Content Correlate with Resistance to Endoplasmic Reticulum Stress-induced Cell Death in Mutant Leukemia Cell Lines*

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Cell clones were derived by treatment of HL-60 cells with stepwise increasing concentrations of econazole (Ec), an imidazole antifungal that blocks Ca\(^{2+}\) influx and induces endoplasmic reticulum (ER) stress-related cell death in multiple mammalian cell types. Clones exhibit 20- to more than 300-fold greater resistance to Ec. Unexpectedly, they also display stable cross-resistance to tunicamycin, thapsigargin, dithiothreitol, and cycloheximide but not doxorubicin, etoposide, or Fas ligand. Phenotypic analysis indicates that the cells display increased store-operated calcium influx and resistance to ER Ca\(^{2+}\) store depletion by Ec. E2R2, the most resistant clone, was observed to maintain protein synthesis levels after treatment with Ec or thapsigargin. Expression of GRP78, an ER-based chaperone, was induced by these ER stress treatments but to equal degrees in HL-60 and E2R2 cells. By using microarray analysis, at least 15 ribosomal protein genes were found to be overexpressed in E2R2 compared with HL-60 cells. We also found that ribosomal protein content was increased by 30% in E2R2 as well as other clones. The resistance phenotype was partially reversed by the ribosome-inactivating protein saporin. Therefore, increased store-operated calcium influx, resistance to ER Ca\(^{2+}\) store depletion, and overexpression of ribosomal proteins define a novel phenotype of ER stress-associated multidrug resistance.

Calcium signals play a central role in many cellular activities including cell movement, secretion, proliferation, gene transcription, and cell death (1, 2). In non-excitable cells, the endoplasmic reticulum (ER)1 plays a key role in calcium signaling. Receptor-mediated activation of phospholipase C generates the second messenger inositol 1,4,5-triphosphate that diffuses rapidly through the cytosol to interact with inositol 1,4,5-triphosphate receptor on the ER membrane releasing calcium stored in the ER lumen. The resulting depletion of calcium within the ER triggers calcium entry from the extracellular milieu through the plasma membrane. This capacitive or store-operated calcium (SOC) influx (3) serves to both expand the initial calcium signal and replenish the emptied ER store (4, 5). Although the nature of SOC channel remains unclear, the importance of SOC influx in maintaining cell viability has been demonstrated in different studies (6–13).

In addition to its key role in Ca\(^{2+}\) signaling, the ER is a site for protein synthesis, and these two functions intersect under conditions of ER stress. For instance, treatment of cells with thapsigargin (Tg), the inhibitor of the sarcoplasmic/endoplasmic Ca\(^{2+}\)-ATPase responsible for transporting Ca\(^{2+}\) into the ER, rapidly depletes ER Ca\(^{2+}\)-stores (14), inhibits the function of Ca\(^{2+}\)-dependent chaperones, and triggers the unfolded protein response (15). Econazole (Ec) is an imidazole antifungal that depletes Ca\(^{2+}\) from the ER of mammalian cells and blocks Ca\(^{2+}\) influx (16, 17). These effects result in sustained depletion of Ca\(^{2+}\) from ER stores and profound inhibition of protein synthesis leading to cell death (11). We have shown that some transformed cells exhibit several orders of magnitude greater sensitivity to Ec than normal cells (12, 13). This enhanced sensitivity may be due to the activated nature of tumor cells since we have shown that concurrent stimulation of cells with growth factors such as steel factor (9, 11), epidermal growth factor, or bombesin (13) enhances cell death; this is a process we refer to as activation-enhanced cell death. However, the nature of the differential sensitivity is not fully understood.

To investigate further the molecular events associated with resistance and sensitivity to Ca\(^{2+}\) and ER stress-induced cell death, we developed mutant cell clones by selection of HL-60 leukemia cells with stepwise increasing concentrations of econazole. The resultant clones exhibit resistance to Ec and cross-resistance to other ER stress-inducing agents including Tg, tunicamycin (inhibitor of N-linked glycosylation), dithiothreitol (DTT; disulfide bond reducing agent), and cycloheximide (protein synthesis inhibitor). Phenotypic analysis indicates that these cells also exhibit resistance to Ec-induced ER Ca\(^{2+}\) depletion, display increased SOC influx, increased ribosomal protein content, and sustained protein synthesis levels in the presence of Ec and Tg. These studies therefore define a novel phenotype of multidrug resistance associated with inducers of ER stress.

MATERIALS AND METHODS

Culture and Selection Conditions—Human HL-60 promyelocytic leukemia cells were grown in RPMI 1640 containing 10% fetal bovine serum (FBS) and antibiotics. In order to generate resistant cells, the cells were exposed to stepwise increasing concentrations of Ec that caused a reduction in the proportion of viable cells by at least 90% followed by a recovery phase. At the end of a series of such steps, cells were cloned by limiting dilution and expanded for further study. To maintain the phenotype of resistance, the Ec-resistant clones designated E2R2, E2R4, E2R6 were challenged with econazole once a month to ensure stability of the resistant phenotype. All the phenotypic char-

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1 The abbreviations used are: ER, endoplasmic reticulum; Ec, econazole; SOC, store-operated calcium; Tg, thapsigargin; DTT, dithiothreitol; FBS, fetal bovine serum; PBS, phosphate-buffered saline; WT, wild type; TRP, transient receptor potential.
KCl (5 mM), CaCl<sub>2</sub> (1.4 mM), MgCl<sub>2</sub> (1 mM), glucose (5.6 mM), bovine serum albumin (0.05%). Cells were incubated in loading buffer (30 min; closed circles, HL-60; open circles, E2R2; closed squares, E2R4; open squares, E2R6). Data were expressed as mean ± S.E.

**Apoptosis Assay**—HL-60 and E2R2 cells were treated as for the clonogenic assay for 24 h. At the end of the culture period, cells were centrifuged, and the pellets were resuspended in 5% formalin containing 4 μg/ml Hoechst 33258. Cells were observed under fluorescence microscopy and scored for apoptotic morphology. Cells exhibiting nuclear condensation/fragmentation were defined as apoptotic. A miniature microscopy and scored for apoptotic morphology. Cells exhibiting nuclear condensation/fragmentation were defined as apoptotic.

**Clonogenic Assay**—To measure clonogenicity, HL-60 or resistant clone cells (5 × 10<sup>4</sup>) were incubated in 100 μl of RPMI 1640 medium supplemented with 5% FBS for 24 h in the presence or absence of different concentration of drugs. Unless indicated, all drugs were purchased from Sigma. For treatment with econazole, cells were incubated in serum-free media for 2 h, resuspended in RPMI with 5% FBS, and cultured for an additional 22 h. 2000 treated cells were plated in 0.3% agar containing 20% FBS in RPMI 1640. Colonies were counted after 10 days incubation in humidity at 5% CO<sub>2</sub>, 37 °C.

**Clonogenicity Assays**—Colonies were counted following 10 days of incubation. A, econazole; B, thapsigargin; C, tunicamycin; and D, DTT. Open circles, HL-60; closed circles, E2R2; open squares, E2R4; closed squares, E2R6.

**Protein Synthesis**—Cells (2 × 10<sup>5</sup>/sample) were collected, washed with PBS, and then resuspended in RPMI supplemented with fatty acid-free bovine serum albumin (0.05%; Sigma). Cells were treated with Ec (0, 15 μM) or Tg (0, 100 nM) for 15 min. After centrifugation (2500 rpm; 5 min), cells were pulse-labeled with <sup>3</sup>H]leucine (50 Ci/ml) for 10 min (37 °C; 5% CO<sub>2</sub>) in leucine-free RPMI. After two washes in RPMI, pellets were lysed with Triton X-100 (0.5% in PBS) followed by trichloroacetic acid (10% w/v) during the experiments, and then harvested in trichloroacetic acid (5% w/v), and the protein pellets were resuspended in microscintillant (Packard Instrument Co.) and measured using a microplate scintillation counter (Packard Instrument Co.).

**Microarray Analysis**—The microarrays used in the present study are from the human 19K2 microarrays (Clinical Genomics Centre, CGC, Toronto, Canada) that contain 18,980 human cDNAs and 220 positive and negative control features (Invitrogen). A complete gene list and protocols for the production of the microarrays can be found at University Health Network CGC web site (www.microarrays.ca). HL-60 and E2R2 cells were grown under log phase conditions at equivalent cell densities. The cells were collected, and total RNAs from HL-60 or E2R2 were extracted; cDNAs were labeled, respectively, with Cy3 and Cy5 fluorescent dye through reverse transcription, purified, and hybridized.
Eto/H9262 cells were pelleted and fixed with 5% formalin and stained with 4 μg/ml Hoechst 33258. Morphology was viewed through fluorescent microscope. Ctl, control; Ec, econazole (12 μM); Tg, thapsigargin (100 μM); Tu, tunicamycin (0.5 μg/ml); DTT, dithiothreitol (1.5 mM). B, comparison of the percentage of the apoptotic cells in two cell lines following treatment of ER stress-inducing agents or other drugs. Ctl, control; Ec, econazole (12 μM); Tg, thapsigargin (100 μM); Tu, tunicamycin (0.5 μg/ml); DTT, dithiothreitol (1.5 mM); Eto, etoposide (5 μM); FasL, Fas ligand (100 μg/ml). Data were representative of three separate experiments.

**Northern Blot Analysis**—Expression of four ribosomal protein genes was further evaluated by Northern blot. Briefly, RNA samples extracted from equal cell numbers from both wild type and E2R2 cells were fractionated on an 1.5% agarose gel containing formaldehyde. After transfer, the nylon membranes were UV cross-linked. Prehybridization and hybridization were performed at 50 °C in DigEasy hybridization solution (Roche Applied Science). The probes for the ribosomal protein genes S12, L27, and L12 were obtained from ATCC, and the probe for the Large P2 gene was cloned reverse transcriptase-PCR. The probe for the Large P2 gene was cloned reverse transcriptase-PCR. The probes were prepared by random priming labeling (Invitrogen) with [α-32P]dCTP. The membranes were also probed for the glyceraldehyde-3-phosphate dehydrogenase gene as a control for total RNA loading. For quantitation, densitometry analysis of the blots was performed and analyzed by NIH Image 1.62 software.

**Ribosomal Protein Isolation and Quantitation**—1 × 10⁸ HL-60 or resistant clone cells cultured under log phase conditions were collected, washed with cold PBS, and fractionated according to the method described by Madjar et al. Cells were pelleted at 2000 × g and washed with PBS, and then treated with a hypotonic solution containing 5 mM MgCl₂, 200 mM sucrose, and 5 mM Tris-Cl (pH 8.0) and disrupted by sonication for 30 s. The ribosome pellets were resuspended in 300 μl of sample buffer and disrupted by incubation in 60 mM EDTA on ice for 30 min. Ribosomal RNAs were extracted with TRIzol and analyzed by electrophoresis on a 0.8% agarose gel stained with EtBr. Total ribosomal proteins were separately extracted from equal cell numbers from both wild type and E2R2 cells using a hypotonic solution containing 5 mM MgCl₂, 200 mM sucrose, and 5 mM Tris-Cl (pH 8.0) and disrupted by sonication for 30 s. The ribosome pellets were resuspended in 300 μl of sample buffer and disrupted by incubation in 60 mM EDTA on ice for 30 min. Ribosomal RNAs were extracted with TRIzol and analyzed by electrophoresis on a 0.8% agarose gel stained with EtBr. Total ribosomal proteins were separately analyzed by electrophoresis on a 12% SDS-PAGE gel. The gel was then stained with Coomassie Brilliant Blue to visualize the protein bands.

**Western Blot**—Assays for the expression of Bip (GRP78), an ER stress marker, were performed by Western blot. Cells were treated with different ER stress-inducing agents as described for the clonogenicity assays. After 12 h of treatment, the cells were collected and lysed in chilled RIPA buffer (Nonidet P-40 (1% v/v), sodium deoxycholate (0.05% w/v), SDS (0.1% w/v)), containing phenylmethylsulfonyl fluoride (10 μg/ml) and protease inhibitor mixture I (Sigma), by sonication (30 s) followed by an incubation period (30 min; 4 °C). The proteins in the supernatants (18,000 × g; 20 min; 4 °C) were resolved by 12% SDS-PAGE gel as described above.

**Phosphorylation of eIF2α**—To examine phosphorylation of eIF2α, both wild type and E2R2 cells were treated with Ec (15 μM) or Tg (1 μM) for 1 h. Cells were lysed in RIPA buffer containing phosphatase inhibitor mixture and protease inhibitor mixture I (Sigma), and total protein samples were resolved on 12% SDS-PAGE gel as described above. Phosphorylation of eIF2α was detected by Western blot using an antibody recognizing phosphorylated eIF2α (serine 51, Cell Signaling). The same samples were also probed for total eIF2α as a control.

**FIG. 2. Morphological changes following the treatment of ER stress-inducing drugs.** A, HL-60 or E2R2 cells (5 × 10⁶ cells/ml) were treated with different ER stress-inducing agents for 24 h; cells were pelleted and fixed with 5% formalin and stained with 4 μg/ml Hoechst 33258. Morphology was viewed through fluorescent microscope. Ctl, control; Ec, econazole (12 μM); Tg, thapsigargin (100 μM); Tu, tunicamycin (0.5 μg/ml); DTT, dithiothreitol (1.5 mM). B, comparison of the percentage of the apoptotic cells in two cell lines following treatment of ER stress-inducing agents or other drugs. Ctl, control; Ec, econazole (12 μM); Tg, thapsigargin (100 μM); Tu, tunicamycin (0.5 μg/ml); DTT, dithiothreitol (1.5 mM); Eto, etoposide (5 μM); FasL, Fas ligand (100 μg/ml). Data were representative of three separate experiments.
FIG. 3. Store-operated calcium influx induced by thapsigargin in HL-60 and mutant cells. A, cells were loaded with indo-1AM in Tyrode's buffer (with Ca²⁺) and treated with 3 µM of Tg. B, cells were pretreated with Ni²⁺ (5 mM) and then with Tg (3 µM) to mobilize the Tg-sensitive intracellular calcium store (ER store). C, cells were loaded with indo-1AM in the same buffer as above, washed 3 times with calcium-free Tyrode's buffer, and resuspended in calcium-free buffer. EGTA (0.5 mM) was added to the cell suspension before the experiments. Tg was used to empty the ER store and to activate the SOC influx with the addition of CaCl₂ (2 mM). Inset on the upper right corner is the amplification of Tg-induced ER calcium release in C. D and E, calcium influx and Tg-sensitive calcium stores in different resistant clones were measured using the methods described in A and B. F, linear regression analysis of calcium influx (represented by SOC) and relative resistance (represented by log fold resistance) to Ec. The correlation coefficient is 0.925, p = 0.038.
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TABLE I

|                | Peak [Ca^{2+}]/(Tg) | Δ[Ca^{2+}]/(Tg) | AUC | AUC (Ni^{2+} + Tg) | SOC |
|----------------|---------------------|-----------------|-----|--------------------|-----|
| HL60           | 513 ± 94            | 378 ± 54        | 141 ± 6 | 68 ± 13          | 73 ± 9 |
| E2R2           | 766 ± 223           | 636 ± 17        | 288 ± 18 | 62 ± 9           | 226 ± 11 |

* p < 0.05.

† p < 0.001.

‡ p < 0.001. SOC values were derived by subtracting area under the curve values in the presence of Ni^{2+} from area under the curve values in its absence.

** Fig. 4. E2R2 cells are resistant to econazole-induced ER Ca^{2+} depletion.** A, both cell lines were exposed to econazole (45 μM, without preincubation) followed by Tg (3 μM) to demonstrate the emptying of the Tg-sensitive calcium stores in response to high concentration of econazole. B, HL-60 or E2R2 cells were incubated with 15 μM econazole for 2 h at 37 °C. Cells were then treated with Tg (3 μM) in the presence of Ni^{2+} (5 mM).

**Statistical Analysis—** Colony data were analyzed by two-way analysis of variance, with differences between individual means determined by Bonferroni’s post-tests. SOC calcium influx was analyzed by area under the curve. Other data were analyzed by Student’s t test. Data were expressed as means ± S.E.

**RESULTS**

**Selection of Econazole-resistant HL-60 Cells**—In order to study the mechanism of action of the ER stress-inducing agent Ec, we developed Ec-resistant variants of the human promyelocytic leukemia cell line HL-60 by treating the cells with stepwise increasing concentrations of the drug followed by recovery in the absence of drug over a period of 6 months. We then subcloned these cells by limiting dilution assay. Six clones of Ec-resistant cells designated E2R1 to E2R6 were generated, and 3 of the clones exhibiting the highest levels of resistance were analyzed. Fig. 1A showed different degrees of resistance to the drug Ec. At the highest concentration used, the fold increase of resistance for mutant clones ranged from 20 to more than 300 compared with wild type HL-60 cells. Among these mutant clones, E2R2 cells displayed the highest relative resistance to Ec by clonogenicity assay, and the variant E2R4 was the least resistant (Fig. 1A). By challenging the mutant cells with Ec once a month, we have maintained the resistant phenotype for more than 2 years.

**Resistant Clones Are Cross-resistant to ER Stress-inducing Agents**—Previous work from our laboratory indicated that Ec induces the depletion of Ca^{2+} from the ER resulting in profound inhibition of protein synthesis and cell death. We therefore investigated the sensitivity of resistant clone cells to other ER stress-inducing agents. By using a colony forming assay, we found that all the mutant clones tested were 100–200 times more resistant to Tg, tunicamycin, and DTT (Fig. 1, B–D). In contrast, no significant difference in sensitivity to DNA-damaging drugs such as doxorubicin and etoposide was observed in these cell lines (data not shown). Because E2R2 cells displayed the highest relative resistance to all tested stress-inducing agents and were found to be the most stable, they were chosen for further analysis.

Morphological analysis of HL-60 cells treated for 24 h with the agents mentioned above indicated typical characteristics of apoptosis such as nuclear condensation and fragmentation. In agreement with this observation, we have detected cleavage of caspase 3 and caspase 12 in cells treated with Ec, Tg, and tunicamycin. In contrast, E2R2 cells displayed only limited levels of apoptosis in response to these agents (Fig. 2A), so for drug concentrations that lead to more than 50% of ER stress-related apoptosis in HL-60 cells, none of the drugs were toxic to E2R2 cells (Fig. 2B).

The cross-resistance to these agents was not observed with all

*Y. Zhang and S. A. Berger, unpublished observations.
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**TABLE II**

| Accession no. | Gene description               | Expression ratio (E2R2/HL60) |
|---------------|--------------------------------|-----------------------------|
| AA046808      | Ribosomal protein S27 isofrom  | 3.03 ± 0.22                 |
| AA114861      | Ribosomal protein L27          | 3.00 ± 0.17                 |
| AA121296      | Ribosomal protein L37          | 2.83 ± 0.12                 |
| N746701       | Ribosomal protein S3a          | 2.80 ± 0.11                 |
| T62596        | Ribosomal protein S29          | 2.69 ± 0.19                 |
| AA939258      | Ribosomal protein L31          | 2.60 ± 0.04                 |
| W329609       | Ribosomal protein L37a         | 2.46 ± 0.14                 |
| N81091        | Ribosomal protein L6            | 2.28 ± 0.09                 |
| H471134       | Ribosomal protein L23a         | 2.17 ± 0.20                 |
| T48772        | Ribosomal protein L12          | 2.10 ± 0.23                 |
| W93580        | Ribosomal protein S12          | 1.71 ± 0.13                 |
| W93065        | Ribosomal protein L21          | 1.68 ± 0.20                 |
| AA156680      | Ribosomal protein S27          | 1.66 ± 0.11                 |
| N70526        | Ribosomal protein large P2     | 1.56 ± 0.05                 |
| H45241        | Ribosomal protein L11          | 1.50 ± 0.10                 |

Inducers of cell death because both cell lines exhibited similar sensitivity to etoposide and Fas ligand (Fig. 2B), agents that are not thought to target the ER. These results therefore show that E2R2 cells selected for resistance to Ec exhibit specific cross-resistance to ER stress-associated agents.

**Increase in Capacitive Calcium Influx in Resistant Clones**—Because Ec is known to stimulate ER Ca\(^{2+}\) depletion and block influx, we investigated the dynamic Ca\(^{2+}\) content of the mutant cells. Intracellular calcium concentrations ([Ca\(^{2+}\)]\(_{i}\)) were measured in indo-1AM loaded HL-60 and resistant clone cells by flow cytometry. In the unchallenged state, basal [Ca\(^{2+}\)]\(_{i}\) for HL-60 and E2R2 cells was similar and found to be between 50 and 100 nM. Tg is an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase and causes rapid release of Ca\(^{2+}\) from the ER coupled with capacitive influx (14). As shown in Fig. 3A, stimulation with thapsigargin (3 \(µ\)M) led to a biphasic increase of [Ca\(^{2+}\)]\(_{i}\), which was seen in both cell lines consisting of a rapid but small magnitude “plateau” followed by a prolonged and further elevation of [Ca\(^{2+}\)]\(_{i}\), likely representing influx through SOC Ca\(^{2+}\) channels. Both the peak and total magnitude of the Ca\(^{2+}\) signal were significantly greater in the resistant clone compared with WT cells. The peaks of the transients for two cells were 767 ± 225 nM in E2R2 cells and 514 ± 94 nM in HL-60 cells (Table I, n = 7, p < 0.05).

In order to distinguish between mobilization and influx, cells were treated with Tg in the presence of Ni\(^{2+}\), a nonspecific Ca\(^{2+}\) influx antagonist, to block all influx (Fig. 3B). In this case, the peak change (188 ± 63 nM in HL-60 versus 192 ± 36 nM in E2R2 cells, n = 5, p > 0.05) and total Ca\(^{2+}\) were similar indicating that Tg-releasable ER Ca\(^{2+}\) content was the same in both cell types but that the mutant cells exhibited greater store-operated influx. In order to confirm this difference, Ca\(^{2+}\) influx was measured directly by restoring Ca\(^{2+}\) to the culture medium following Tg treatment in Ca\(^{2+}\)-free medium. As shown in Fig. 3C, both the peak influx and area under the curve were significantly larger in E2R2 compared with WT cells. We therefore conclude that E2R2 cells exhibit similar resting cytoplasmic Ca\(^{2+}\) levels and similar ER Ca\(^{2+}\) content compared with WT. However, capacitive Ca\(^{2+}\) influx is much higher in E2R2 cells in response to Tg than WT.

By using the same calcium measurement method, we found that the other resistant clones E2R4 and E2R6 also showed significantly increased calcium influx (Fig. 3D) while retaining similar ER calcium content as WT cells (Fig. 3E). The increased calcium influx (SOC, \(\mu\)ER) observed in these resistant clones was found to correlate strongly with the relative resistance (log fold resistance, logR) to Ec (Fig. 3F; \(r = 0.93, p = 0.038\)), supporting the conclusion that increased Ca\(^{2+}\) influx correlates with ER stress resistance.

**ER Calcium Store Depletion by Prolonged Incubation with Ec**—In a previous study (11), we showed that Ec suppressed protein synthesis of murine bone marrow mast cells through sustained depletion of ER calcium stores. In order to investigate whether E2R2 cells were also resistant to ER calcium depletion, the cells were incubated with very high levels of Ec. Both E2R2 and WT cells were observed to release similar levels of Ca\(^{2+}\) from Tg-sensitive stores (Fig. 4A). The level of Ca\(^{2+}\) released into the cytoplasm is consistent with mobilization from intracellular stores in the absence of influx. However, when the two cell lines were incubated with econazole (15 \(µ\)M) in Tyrode’s buffer for 2 h at 37 °C and Ca\(^{2+}\) content in the Tg-sensitive ER store was measured, as shown in Fig. 4B, the ER Ca\(^{2+}\) store in WT cells was completely depleted, whereas the mutant cells retained significant levels of ER Ca\(^{2+}\). These results indicate that while Ec can still stimulate release from the ER and block influx in E2R2 cells, E2R2 cells are capable of refilling their ER and the WT cells are not.

**Grp78 Expression**—Previous studies from other laboratories had shown that overexpression of the ER-resident chaperone Grp78 can contribute to resistance to ER stress (21–23). Given the cross-resistance phenotype of the E2R2 cells, we therefore investigated the expression levels of Grp78. As shown in Fig. 6A, Northern blot analysis of ribosomal protein genes, Total RNAs were extracted from HL-60 or E2R2 cells and fractionated on 1.5% agarose gel containing formaldehyde. Probes for ribosomal protein genes L12, L27, S12, and Large P2 were labeled, respectively, by random priming and hybridized to membranes. A, Northern blots showing expression level of selected ribosomal protein genes; RP, ribosomal protein; H, HL-60; E, E2R2. B, quantitation of the expression by densitometry analysis of the blots. Arbitrary units (AU) were used in the graph. The values were also normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.
Ribosomal protein fraction. H, HL-60; E, E2R2 cells. Ribosomal protein bands were resolved in 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue to view ribosomal protein bands. Total ribosomes were sedimented by ultracentrifuge on 1 M sucrose cushion, resuspended in 300 µl of RIPA protein buffer, and disrupted with 60 mM EDTA. The concentration of the total ribosomal protein was calculated based on the absorbance of the samples (A$_{260}$). rRNA was extracted with Trizol. Data were representative of four separate experiments; *, p < 0.05. C, ribosomal proteins or total protein lysates from the two cell lines were resolved in 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue to view ribosomal protein bands. M, marker; H, HL-60; E, E2R2; TP, total protein; RP, ribosomal protein fraction. D, 2 µg of RNA samples purified from ribosome fraction of HL-60 or E2R2 cells were run on 0.8% agarose gel and stained with ethidium bromide.

5A, GRP78 expression was found to be overexpressed in both E2R2 and HL-60 cells 12 h following treatment with ER stress-inducing agents. However, E2R2 cells did not exhibit any higher levels of GRP78 than HL-60 cells. Therefore, GRP78 overexpression does not appear to contribute to the cross-resistance phenotype in E2R2 cells.

**GADD153 Expression**—GADD153 (also known as CHOP) is a leucine zipper transcription factor that is highly inducible in response to cellular stress including ER stress (24–26). Because induction of GADD153 expression in response to ER stress promotes death, we determined expression levels of GADD153 following exposure to different ER stress-inducing agents. Surprisingly, neither HL-60 cells nor E2R2-resistant cells were induced to express GADD153 protein (Fig. 5B), indicating that alterations in GADD153 expression are not associated with the resistance phenotype.

**Generalized Overexpression of Ribosomal Protein Genes Is Observed in Cross-resistant E2R2 Cells**—In order to further investigate differences between the mutant and WT cells, we used microarray analysis to compare the gene expression profile of E2R2 cells with that of the wild type HL-60 cells. The two cell lines were grown under identical tissue culture conditions and to similar cell densities in order to suppress differences associated with growth status. From 10 comparisons with 4 independent RNA extractions, we observed ~86 genes whose expression was reproducibly increased in E2R2 cells compared with HL-60 cells. Remarkably, we observed that at least 15 family members of the ribosomal protein genes were overexpressed in E2R2 cells at levels ranging from 1.5- to 3-fold. Overexpressed genes include ribosomal proteins of the large subunit of the ribosome (rpL6, rpL12, rpL21, rpL23a, rpL27, rpL31, rpL37, rpL41, and rpL42) and ribosomal proteins of the small subunit of the ribosome (rpS3a, rpS12, rpS27, rpS29, and rpS37) (Table II).

To further validate overexpression of ribosomal protein genes observed by microarray, the expression of selected ribosomal protein genes was analyzed by Northern blot in wild type and E2R2-resistant clones. As shown in Fig. 6A, ribosomal protein genes L12, L27, S12, and Large P2 were all found to be up-regulated in E2R2 cells compared with HL-60 cells. Consistent with the microarray results, quantitation of the expression levels of the ribosomal protein genes based on Northern blot in these two cell lines showed 1.4–2.8-fold increases in E2R2 cells (Fig. 6B).

In order to investigate whether these increases in gene expression were translated into protein, we measured ribosomal protein content in the wild type and resistant clone cell lines. Ribosomal protein-enriched fractions were prepared by centrifugation through a sucrose cushion. The presence of ribosomal protein in this fraction was confirmed by Western blot with human autoimmune antisera recognizing ribosomal P antigens (Fig. 7A). As shown in Fig. 7B, 1326 ± 94 and 979 ± 26 µg of total ribosomal proteins were extracted from ribosome fractions obtained from 1 × 10⁶ E2R2 or HL-60 cells, respectively (p < 0.05). As well, increased ribosomal protein P2 was detected by Western blot (Fig. 7A; compare lanes 3 and 4), which was one of the ribosomal protein genes identified in the microarray analysis. Increased ribosomal content was also observed by SDS-PAGE followed by Coomassie Blue staining analysis (Fig. 7C). Increased ribosomal RNA (445 ± 32 versus 324 ± 10 µg, p < 0.05) in E2R2 cells was also observed (Fig. 7D). Quantitation of ribosomes in E2R4 and E2R6 clones also showed increased ribosomal protein levels in these cells (Fig. 7B). We therefore conclude that the increased expression of ribosomal protein genes translates into increased ribosomal protein and RNA content in resistant cells.

**E2R2 Cells Display Increased Resistance to Protein Synthesis Inhibition**—The observation of increased ribosomal content coupled with our previous demonstration that Ec inhibits protein synthesis prompted us to investigate protein synthesis in E2R2 and WT cells. As shown in Fig. 8A, protein synthesis levels in unchallenged cells were similar in the mutant and WT cells. As observed previously in mast cells, exposure of HL-60 cells to Ec or Tg inhibited protein synthesis. We found that Tg (100 nM) or Ec (15 µM) causes 32 (p = 0.19) and 30% (p = 0.01) inhibition, respectively. In contrast, E2R2 cells retained protein synthesis at unchallenged levels.
Inhibition of protein synthesis in response to ER stress is regulated primarily through phosphorylation of EIF2α at Ser-51 (27). We therefore investigated the phosphorylation status of EIF2α in response to Ec and Tg. As shown in Fig. 8A, and as previously observed in murine mast cells (11), HL-60 cells challenged with these reagents exhibited increased phosphorylation of EIF2α. In contrast, challenged E2R2 cells displayed significantly reduced EIF2α levels compared with WT, in accordance with their ability to sustain protein synthesis in the presence of Ec and Tg.

The ability to retain protein synthesis in Ec or Tg challenged cells suggests that these cells may be generally resistant to protein synthesis inhibition. As shown in Fig. 8C, E2R2 cells exhibit profound resistance to cycloheximide, the protein synthesis inhibitor. E2R2 cells were at least 130 times more resistant then HL-60 cells to cycloheximide at the highest concentration (40 μg/ml, p < 0.001) tested. We conclude that E2R2 cells also exhibit cross-resistance to the protein synthesis inhibitor cycloheximide.

Partial Reversal of Resistance by Saporin—To address the role of increased ribosomal protein content in resistance, we determined the effect of saporin, a type I ribosomal-inactivating protein isolated from the plant Saponaria officinalis, on protein synthesis and clonogenicity. Saporin inactivates ribosomes by catalyzing the hydrolysis of a specific N-glycosidic bond of large rRNA, thus suppressing protein synthesis (28, 29). Pretreatment of HL-60 cells with saporin (3 μg/ml) suppressed protein synthesis by 50% (Fig. 9A), whereas resistant clones were not affected. When the resistant cells were exposed to saporin together with Ec or Tg, however, protein synthesis was inhibited by 25–30%. Furthermore, cell viability as evaluated by clonogenicity assay showed that Ec and Tg resistance was partially reversed in all three clones to varying degrees (Fig. 9B), consistent with the interpretation that suppression of ribosome function can partially reverse resistance.

**DISCUSSION**

In this study, we have generated and characterized a group of resistant clones from HL-60 cells by selection with increasing concentrations of Ec. These mutant cells exhibit resistance to Ec as well as cross-resistance to a variety of other ER stress-inducing drugs but not other toxic agents such as etoposide, doxorubicin, or Fas ligand. Phenotypic analysis revealed that the mutant cells have normal levels of cytoplasmic and ER Ca²⁺ levels but display increased capacity of Ca²⁺ influx, resistance to ER depletion by Ec, increased ribosomal content, maintain protein synthesis levels, and suppress EIF2α phosphorylation following exposure to Ec or Tg. The increased calcium influx in the mutant clones is closely correlated with the relative resistance to Ec, and partial reversal of resistance is induced with the ribosome inactivating protein saporin. Taken together, these results define a novel phenotype of ER stress-associated multidrug resistance.

Although the mechanism coupling ER calcium depletion to SOC activation remains elusive, it is clear that SOC calcium influx, apart from extending the temporally limited Ca²⁺ signal induced by agonist, also plays an important role in replenishing the ER store (5, 30). We observed that a 2-h incubation with Ec (15 μM) depleted the Tg-sensitive ER store in HL-60 cells but not in E2R2 cells (Fig. 4A). This concentration of Ec has no immediate effect on [Ca²⁺], but higher concentrations trigger similar emptying of the ER store in both cell lines (Fig. 4B). Coupled with the observation that E2R2 cells have similar levels of Ca²⁺ in their ER (Fig. 3B) and increased SOC influx (Fig. 3A), we conclude that the increased SOC activity associated with E2R2 cells is likely responsible for the continuous...
replenishment of calcium into the ER store, compensating for the ER calcium store depletion caused by Ec.

Although resistance to ER depletion by Ec may be linked to increased capacitive influx, our data do not yet indicate whether this increased capacitive influx also contributes to the cross-resistance to other ER stress agents. E2R2 cells remain sensitive to the Ca\(^{2+}\)/H\(\text{11001}\)-mobilizing effects of Tg, despite their profound resistance to its toxic effects. An analysis of the role of Ca\(^{2+}\) influx in toxicity associated with these agents is in progress.

Recent studies directed toward identifying channel proteins responsible for SOC influx have focused on the transient receptor potential (TRP) gene superfamily. Three categories of TRP genes, i.e. TRPC, TRPV, and TRPM, have been cloned. Some of the TRPC family members (TRP1 to TRP7) and Cat1, Cat2 from the TRPV family, have been shown to function as SOC

![Diagram of Partial reversal of ER stress resistance by saporin.](http://www.jbc.org/Downloaded from)

Fig. 9. Partial reversal of ER stress resistance by saporin. A, HL-60 or resistant cells (2 \(\times 10^6\)/sample) were collected, washed with PBS, and then resuspended in RPMI and incubated with 3 \(\mu\)g/ml saporin for 1 h and then treated with Ec (0, 15 \(\mu\)M) or Tg (0, 100 nM) for 15 min. After centrifugation (2500 rpm; 5 min), cells were pulse-labeled with \(^{3}\text{H}\)-leucine (50 \(\mu\)Ci/ml) for 10 min (37 °C; 5% CO\(_2\)) in leucine-free RPMI. Protein synthesis rates were determined according to the method described in Fig. 7. Data were representative of three separate experiments. Ctl, control; Ec, econazole; Tg, thapsigargin; *, \(p < 0.05\); **, \(p < 0.01\). B, resistance clones were first incubated with saporin (3 \(\mu\)g/ml) for 1 h followed by treatment of different concentrations of Ec or Tg for 24 h. Cells were collected and plated for the growth of colonies as indicated under "Materials and Methods." Colonies were counted after 10 days of incubation. Interactions were analyzed by analysis of variance. *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\).
Ca\(^{2+}\) and Ribosomal Changes in ER Stress Resistance

channels in different cells (31, 32). Surprisingly, by using reverse transcriptase-PCR, we were unable to detect expression of any of the TRP genes mentioned above in either HL-60 or E2R2 cells (data not shown). It is possible that a previously unidentified channel protein is associated with the increased influx observed in E2R2 cells. Further investigation is underway to identify the channel or channels expressed in resistant cells. Increased expression of GRP78 (Bip) is commonly observed in response to ER stress, and GRP78 overexpression has been shown to confer resistance to apoptosis triggered by ER stress or other toxic events (21–23, 33). Although we observed increased levels of GRP78 in response to ER stress treatments, a similar response was seen in both HL-60 and E2R2 cells, both by Western blot and by microarray analysis. These observations suggest that GRP78 overexpression is not associated with the increased resistance in E2R2 cells. Recently, a calcium cell survival pathway was defined among some pathogenic fungi that promotes resistance to azole antifungal drugs and also confers a survival advantage in the context of ER stress induced by other drugs such as tunicamycin (34). However, this pathway was described as being sensitive to FK506, and we have observed that E2R2 resistance is unaffected by FK506.\(^2\) suggesting that the resistance mechanism in E2R2 cells is unrelated to the calcium cell survival pathway.

Our findings that multiple ribosomal genes were upregulated in E2R2 cells prompted us to examine ribosomal content and protein synthesis. We observed that ribosomal content in E2R2 cells was increased by −30%. Increased ribosomal content was also observed in resistant E2R4 and E2R6 cells. Although protein synthesis rates were the same in unchallenged E2R2 cells compared with HL-60 cells, the increased level of ribosomes may have contributed to the sustained protein synthesis observed in the presence of Ec and Tg. Ribosomal overexpression may also have contributed to the observed resistance to cycloheximide.

Our observation that the ribosome-inactivating protein saporin partially reverses resistance in all three resistant clones is consistent with a role for ribosomal protein overexpression in resistance to ER stress. We believe that a threshold level of ribosome function may be needed to maintain normal levels of protein synthesis and cell viability. Consequently, sublethal concentrations of saporin do not inhibit protein synthesis in resistant clones containing increased ribosome content, whereas the combination of saporin with Ec or Tg reduces protein synthesis below the threshold required for cell viability. The fact that resistant clones also exhibit other phenotypic changes such as increased SOC influx may explain why the resistance could not be completely reversed.

In agreement with our observation that E2R2 cells maintained protein synthesis in the presence of Ec and Tg, we also observed much reduced EIF2\(\alpha\) phosphorylation levels in these cells during challenge with these reagents. Because EIF2\(\alpha\) is the main regulatory point for controlling protein synthesis levels in response to ER stress (27), it is possible that alterations in EIF2\(\alpha\) phosphorylation may contribute to resistance. Alternatively, decreased EIF2\(\alpha\) phosphorylation may simply reflect decreased stress due to the ability of E2R2 cells to maintain Ca\(^{2+}\) in the ER. These possibilities are currently under investigation.

Ribosomal protein genes are members of the TOP family of mRNAs characterized by terminal oligopyrimidines (35). Although translational regulation of TOP mRNAs has been well studied (36), less is known of the transcriptional control of these genes. Further analysis may reveal common mechanisms of transcriptional control for this family. Nevertheless, because there is as yet no direct evidence linking increased Ca\(^{2+}\) influx with ribosomal protein overexpression, we suspect that these two changes in E2R2 cells represent independent events contributing to overall resistance.

In conclusion, our development and characterization of mutant leukemia cell lines resistant to Ec have identified Ca\(^{2+}\) influx and ribosomal content as two major phenotypic changes correlated with resistance. Furthermore, our demonstration of cross-resistance of these cells to other ER stress-associated agents provides a novel phenotype of multidrug resistance associated specifically with ER stress. Further characterization of the relative importance of these other changes will provide insight into how normal and transformed cells handle ER stress.

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