Mouse Lymphoma Cells Destined to Undergo Apoptosis in Response to Thapsigargin Treatment Fail to Generate a Calcium-mediated grp78/grp94 Stress Response

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Thomas S. McCormick‡‡, Karen S. McColl‡‡, and Clark W. Distelhorst‡‡
From the Department of Medicine, Case Western Reserve University School of Medicine and the Ireland Cancer Center, University Hospitals of Cleveland, Cleveland, Ohio 44106

Grp78/grp94 induction is critical for maintaining the viability of epithelial cells and fibroblasts following treatment with thapsigargin (TG), an inhibitor of Ca\(^{2+}\) uptake into the endoplasmic reticulum. In contrast to these cell types, WEHI7.2 mouse lymphoma cells undergo apoptosis when treated with TG, prompting us to examine the grp78/grp94 stress response in WEHI7.2 cells. TG treatment failed to induce grp78/grp94 transcription in WEHI7.2 cells, measured by Northern hybridization and nuclear run-on assays, even if the cells were protected from apoptosis by overexpressing bcl-2. However, grp78/grp94 transcription was induced by the glycosylation inhibitor tunicamycin, suggesting that there are at least two grp78/grp94 signaling pathways, one in response to TG-induced endoplasmic reticulum Ca\(^{2+}\) pool depletion, which is inoperable in WEHI7.2 cells, and one in response to glycosylation inhibition, which is operable in WEHI7.2 cells. Studies of additional lymphoid lines, as well as several nonlymphoid lines, suggested a correlation between grp78/grp94 induction and resistance to apoptosis following TG treatment. In conclusion, the vulnerability of TG-treated WEHI7.2 cells to apoptosis may be due to failure to signal a grp78/grp94 stress response.

The endoplasmic reticulum (ER)\(^{1}\) is the major intracellular reservoir of Ca\(^{2+}\) in nonmuscle cells (1). The ER Ca\(^{2+}\) pool is essential for a number of vital cellular functions, which include protein processing within the ER (2, 3), maintaining high translation rates of newly synthesized messages (4), preserving the structural integrity of the ER (5, 6), and regulating cell proliferation and cell cycle progression (7). Under physiological conditions, the ER Ca\(^{2+}\)-ATPase that pumps Ca\(^{2+}\) into the ER lumen from the cytoplasm (8). The ER Ca\(^{2+}\) pool can be depleted by treating cells with the Ca\(^{2+}\)-ionophore A23187 or the selective ER Ca\(^{2+}\)-ATPase inhibitor thapsigargin (TG) (9).

ER function is mediated, in part, by intraluminal Ca\(^{2+}\)-binding proteins, which include the glucose-regulated proteins GRP78 and GRP94 (5, 10, 11). GRP78 and GRP94 are found constitutively within the ER, and transcription of the genes for these proteins is elevated in response to malfolded proteins, inhibition of glycosylation, and ER Ca\(^{2+}\) pool depletion (12–14). GRP78 is a highly conserved 78-kDa protein that shares 60% amino acid homology with the 70-kDa heat shock protein HSP70. GRP78 (also known as BiP) associates transiently with nascent proteins as they traverse the ER and aids in their folding and transport (15–20). The binding of immature proteins by GRP78 requires ATP, and GRP78 has both ATP binding and ATPase activities (21). GRP94 is a 94-kDa glycoprotein that shares 50% amino acid homology with HSP90 (11, 22). GRP94 acts in concert with GRP78 to fold nascent proteins and also exhibits ATPase activity (22–24).

In epithelial and fibroblasts, grp78 and grp94 are coordinately regulated through common Ca\(^{2+}\)-responsive promoter elements that respond to ER Ca\(^{2+}\) pool depletion (10, 25). Thus, ER Ca\(^{2+}\) pool depletion, induced by either A23187 or TG, signals an increase in grp78/grp94 transcription, producing a 5–20-fold elevation of grp78/grp94 mRNA levels (25). In these cells, the loss of ER Ca\(^{2+}\) induced by TG or A23187 does not result in a loss of viability, unless the grp78/grp94 stress response is repressed by antisense, promoter competition, or ribozyme techniques (26–28). Moreover, grp78/grp94 induction restores protein synthesis under conditions where intracellular Ca\(^{2+}\) is depleted (29). This indicates that grp78/grp94 gene induction is a protective response mechanism by which cells accommodate to potentially lethal stress caused by the disruption of intracellular Ca\(^{2+}\) homeostasis.

In contrast to epithelial cells and fibroblasts, we have found that WEHI7.2 mouse lymphoma cells undergo apoptosis in response to TG-induced ER Ca\(^{2+}\) loss, unless protected by overexpression of the anti-apoptotic onconege bcl-2 (30). Given this observation, we chose to examine the grp78/grp94 stress response in WEHI7.2 mouse lymphoma cells. We report for the first time that TG-induced Ca\(^{2+}\) loss from the ER of WEHI7.2 cells does not induce grp78/grp94 transcription, even if cells are protected from undergoing apoptosis by bcl-2. Interestingly, treatment with tunicamycin (TN), an inhibitor of N-linked glycosylation, does induce grp78/grp94 transcription, suggesting that ER Ca\(^{2+}\) pool depletion and accumulation of underglycosylated proteins signal an increase in grp78/grp94 transcription through independent pathways, the former pathway being inoperative in WEHI7.2 cells. Moreover, in three breast cancer cell lines and two additional lymphoma lines, the induction of grp78 correlated with resistance to TG-induced apoptosis. These findings suggest that inherent differences in the susceptibility of cells to apoptosis induction by TG can be determined, at least in part, by the cell's capacity to mount a grp78/grp94 stress response.
EXPERIMENTAL PROCEDURES

Materials—TG was purchased from LC Laboratories, serum from Hyclone Laboratories, and TN from Calbiochem. L-Glutamine, antibiotics, and nonessential amino acids were from Life Technologies, Inc. All other chemicals, unless noted otherwise, were obtained from Sigma.

Cell Culture and Treatment Conditions—The WEHI.7.2 mouse lymphoma cell line, which does not express detectable levels of Bcl-2, was stably transfected with a cDNA encoding full-length human grp78, the protein designated by D. Bole, University of Michigan (15) or blotting as described previously (30), using a human monoclonal anti-GFP antibody (provided by D. Bole, University of Michigan) (15) or with GRP78 (provided by M. Green, St. Louis University) (32). The WEHI.7.2 derivatives were maintained in RPMI 1640 medium (Cancer Center Tissue Culture Core Facility) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.4 μg/ml nonessential amino acids at 37°C in a 7% CO2 atmosphere.

Total RNA was isolated from cells using TRIzol (Life Technologies, Inc.). 10 or 20 μg of total RNA was denatured by heating at 60°C in 6 M guanidinium thiocyanate solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sodium dodecyl sulfate, 20°C for 1 h) and was precipitated with 0.1 vol of 2 M sodium acetate (pH 5.2) and 1 vol of isopropanol. The RNA was washed by centrifugation for 10 min at 4°C and resuspended in 10 μl of sterile water.

Cell viability was determined at 37°C in a 7% CO2 atmosphere. MCF-7 human breast cancer cells (from S. Gerson, Case Western Reserve University) were cultured in RPMI 1640 medium (from S. Gerson, Case Western Reserve University) were cultured in RPMI 1640 medium (Cancer Center Tissue Culture Core Facility) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.4 μg/ml nonessential amino acids at 37°C in a 7% CO2 atmosphere.

A 1 mg/ml stock of TG was made in dimethyl sulfoxide and stored in aliquots at −20°C. A working stock was prepared by diluting TG in fresh culture medium to a final concentration of 0.1 μg/ml. TG was then added to the cell cultures to achieve the desired final concentrations as noted below. Untreated cultures received the same volumes of dimethyl sulfoxide without TG. A 5 mg/ml stock of TN was prepared in dimethyl sulfoxide and stored at room temperature. TN was added to the cell cultures to a final concentration of 0.75 μM. Untreated cultures received the same volumes of dimethyl sulfoxide without TN. Cell viability was assessed by counting cells on a hemocytometer after suspension in trypan blue dye.

Western Blotting—Levels of Bcl-2 protein were measured by Western blotting as described previously (30), using a human monoclonal anti-Bcl-2 antibody (Pharmingen). GRP78 and GRP94 levels were measured by Western blotting as described previously (31), using a monoclonal antibody to GRP78 (provided by D. Bole, University of Michigan) (15) or with PLP antibody (a-H3S) that immunocross-reacts with GRP78 (provided by M. Green, St. Louis University) (32). The WEHI.7.2 derivatives were maintained in RPMI 1640 medium (from S. Gerson, Case Western Reserve University) were cultured in RPMI 1640 medium (Cancer Center Tissue Culture Core Facility) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, and 0.4 μg/ml nonessential amino acids at 37°C in a 7% CO2 atmosphere.

Slot Blot—Deficient grp78 grp94 Stress Response

RESULTS

The susceptibility to cell death following TG treatment was investigated in WEHI.7.2 cells, which do not express Bcl-2, and in stable transfectants that express either a low level of Bcl-2 (W.Hb13) or a high level of Bcl-2 (W.Hb12 and W.Hb15) (Fig. 1A). Consistent with earlier findings (30), WEHI.7.2 cells rapidly lost viability following treatment with 100 μM TG, whereas a derivative expressing a low level of Bcl-2 (W.Hb13) was killed more slowly, and derivatives expressing a high level of Bcl-2 (W.Hb12 and W.Hb15) were resistant to TG-induced cell death (Fig. 1B).

To define the early stages of grp78 mRNA following TG treatment, the mRNAs were quantitated by Northern blot analysis (2). The constitutively expressed marker CHO-B was used to control for minor loading differences as described under "Experimental Procedures." As shown by the Northern blot in Fig. 2A, the grp78 mRNA level did not appear to increase following treatment of WEHI.7.2 cells with 100 μM TG. In multiple experiments, the ratio of post-treatment to pretreatment levels was quantitated...
at each time point by densitometry with normalization to the CHO-B standard. The maximum ratio was 1.7 ± 0.2, which did not represent a reproducible elevation above pretreatment levels (p ≥ 0.05). The failure of TG treatment to induce an elevation of the grp78 mRNA level was confirmed at several other concentrations of TG (10, 50, and 300 nM) (data not shown).

To determine whether or not the failure of TG treatment to increase grp78 mRNA levels in WEHI7.2 cells was secondary to early changes accompanying cell death, we examined the grp78 stress response in W.Hb12 cells, which are protected from apoptosis by bcl-2. As shown by the Northern blot in Fig. 2B, the grp78 mRNA level did not appear to increase following treatment of W.Hb12 cells with 100 nM TG. In multiple experiments, the maximum post-treatment to pretreatment grp78 mRNA ratio was 2.1 ± 0.4, which did not represent a significant elevation above pretreatment levels (p ≥ 0.05). Northern blot analysis of two other Bcl-2-expressing clones, W.Hb13 and W.Hb15, confirmed that grp78 mRNA levels did not increase following treatment with 100 nM TG (Fig. 3, A and B). Note that in Fig. 3, grp78 mRNA levels actually decreased relative to CHO-B levels at 16 and 24 h after TG addition. This observation was variable among experiments, including those with WEHI7.2 and W.Hb12 cells. Note that we have previously shown, in WEHI7.2 cells and derivatives expressing Bcl-2, that TG treatment inhibits the ER Ca²⁺-ATPase, producing cytosolic Ca²⁺ elevation and ER Ca²⁺ pool depletion (30, 36). Hence, the failure to significantly elevate grp78/grp94 transcription following TG treatment is not due to a failure of TG to disrupt Ca²⁺ homeostasis.

Levels of GRP78 and GRP94 proteins, assessed by Western blotting, were the same in untreated WEHI7.2 and W.Hb12 cells, indicating that bcl-2 does not affect basal levels of GRP78/GRP94 expression at the protein level (Fig. 4, A and B). Furthermore, levels of GRP78 protein did not increase following TG treatment in either WEHI7.2 or W.Hb12 cells (Fig. 4C).

Both WEHI7.2 and W.Hb12 cells up-regulated grp78 mRNA levels by 6–7-fold when treated with 0.75 μM TN (Fig. 5). Thus, although ER Ca²⁺ pool depletion failed to induce an up-regulation of grp78 mRNA, accumulation of unglycosylated proteins in the ER induced a strong up-regulation of grp78 mRNA levels. These findings suggest that there is more than one signal transduction pathway for grp78 induction (see “Discussion”).

To assess if grp94 is regulated in the same manner as grp78 in WEHI7.2 and W.Hb12 cells, we examined the steady-state level of grp94 mRNA after treatment with 100 nM TG (Fig. 6). A modest elevation of grp94 mRNA levels appeared to occur at 5 h after TG addition in both WEHI7.2 and W.Hb12 cells. In multiple experiments, however, the maximum ratio of post-treatment to pretreatment grp94 mRNA levels in WEHI7.2 cells was only 2.0 ± 0.5, which did not represent a reproducible elevation above base-line levels (p ≥ 0.05). In W.Hb12 cells, the maximum ratio was only 1.5 ± 0.2, which also did not represent a significant elevation above base-line levels (p ≥ 0.05).

The preceding findings suggest that TG treatment does not signal an increase in grp78/grp94 transcription in the WEHI7.2
lymphoma cell line or its derivatives that express Bcl-2. To confirm that this is the case, we measured the effect of TG treatment on the transcription rate of \textit{grp78} and \textit{grp94} genes by nuclear run-off assays using isolated nuclei from WEHI7.2 and W.Hb12 cells. An increase in newly expressed \textit{grp78} and \textit{grp94} message after TG treatment was not detected in WEHI7.2 cells (Fig. 7A) or W.Hb12 cells (Fig. 7B). TN treatment, however, did induce a significant increase in \textit{grp78} and \textit{grp94} transcription, which was detected by 5 and 7 h, respectively. This indicates that \textit{grp78}/\textit{grp94} transcription is not induced by TG in WEHI7.2 cells or derivatives that express Bcl-2, but is induced by TN.

Because earlier studies of \textit{grp78} regulation have emphasized epithelial cells and fibroblasts (see the Introduction), as a positive control, we examined the effect of TG treatment on \textit{grp94} mRNA levels in three epithelial breast cancer lines, Mm5MT, MDA-MB-468, and MCF-7. Mm5MT cells were highly sensitive to TG-induced cell death (Fig. 8A), but did not express \textit{grp78} mRNA (Fig. 8B). MDA-MB-468 and MCF-7 cells were also much less sensitive to TG-induced cell death (Fig. 8D), but displayed marked induction of \textit{grp78} mRNA levels in response to TG treatment (Fig. 8B and C).

To determine if the defect in TG-mediated \textit{grp78} signaling is observed in other lymphoid cells, we measured the effect of TG treatment on \textit{grp78} mRNA levels in two additional Bcl-2-negative mouse lymphoma lines, W7.MG1 and S49.1. \textit{grp78} transcription is induced by TN treatment in both of these lines (31). The level of \textit{grp78} mRNA failed to increase following TG treatment in W7.MG1 cells, which rapidly lost viability following TG treatment.
treatment, whereas the level of grp78 mRNA did increase 3–4-fold following TG treatment in S49.1 cells, which were relatively resistant to TG-induced cell death (Fig. 9). These data are consistent with the concept that a deficiency of grp78 induction increases susceptibility to TG-induced cell death.

DISCUSSION

We have discovered that the transcription of grp78 and grp94 is not significantly increased in WEHI7.2 cells in response to treatment with the ER Ca\(^{2+}\)-ATPase inhibitor TG, even when apoptosis is inhibited by overexpressing grp78. Examination of two additional lymphoma lines revealed an absence of grp78 induction in WEHI7.2 cells and 3–4-fold induction of grp78 in S49.1 cells following TG treatment. By comparison, TG treatment induced a marked elevation of grp78 mRNA levels in all three nonlymphoid lines tested (Mm5MT, MDA-MB-468, and MCF-7), consistent with studies indicating that TG treatment substantially induces grp78/grp94 transcription in epithelial cells and fibroblasts (13).

We have previously shown, in WEHI7.2 cells and derivatives expressing Bel-2, that TG treatment inhibits the ER Ca\(^{2+}\)-ATPase, producing cytosolic Ca\(^{2+}\) elevation and ER Ca\(^{2+}\) pool depletion (30, 36). Hence, the failure to significantly elevate grp78/grp94 transcription following TG treatment is not due to a failure of TG to disrupt Ca\(^{2+}\) homeostasis. Moreover, in the present study, we show that TN treatment induces a substantial grp78/grp94 transcriptional response. This observation is important for two reasons. First, it provides evidence that the grp78/grp94 stress response is not only maximally induced in WEHI7.2 cells. Second, it suggests that the grp78/grp94 stress response induced by Ca\(^{2+}\) mobilization may be regulated differently than that induced by TN. Ca\(^{2+}\) mobilization and inhibition of glycosylation have been shown to induce grp78/grp94 transcription through common promoter elements (12).

Therefore, the deficiency in the TG-induced grp78/grp94 transcriptional response observed in WEHI7.2 cells is unlikely to reside at the promoter level. One possible explanation for our findings is that two independent ER-to-nucleus grp78/grp94 signaling pathways may exist: one Ca\(^{2+}\)-mediated and the other mediated by glycosylation inhibition. Both pathways are operative in fibroblasts and epithelial cells, which induce grp78/grp94 in response to both TG and TN, but only the glycosylation inhibition signaling pathway appears to be operative in WEHI7.2 cells.

Little is known about the ER-to-nucleus signaling pathway that activates grp78/grp94 transcription. ER-to-nuclear signaling may be Ca\(^{2+}\)/calmodulin-regulated (37) or may be mediated through tyrosine kinases and/or serine/threonine kinases (38, 39). Recently, it has been shown that Ire1p (Ern1), a yeast transmembrane serine/threonine kinase required for the induction of Kar2, the yeast homologue of grp78, may play a role in the ER-to-nuclear signaling pathway mediating Kar2/grp78 up-regulation in response to misfolded proteins (40, 41). Overexpression of Ire1p in fibroblasts produced a modest increase in the ability of transfectants to up-regulate grp78 in response to TG treatment (39). The WEHI7.2 cell line described in this report may be a useful model for the delineation of ER-to-nuclear signaling pathways. For example, it will be interesting to determine whether or not expression of Ire1p/Ern1 restores Ca\(^{2+}\)-mediated grp78/grp94 transcriptional induction in these cells, thus further elucidating the role of Ire1p/Ern1 proteins in the pathway of grp78/grp94 induction.

Understanding ER-to-nucleus signaling pathways should provide insight into mechanisms that regulate apoptosis induction during ER Ca\(^{2+}\) pool depletion. Indeed, our findings suggest that cells deficient in grp78 stress response signaling are more susceptible to TG-induced apoptosis than cells that mount a grp78 stress response. These findings are consistent with those of earlier work by Lee and co-workers (26–28) in fibroblasts and epithelial cells, indicating that up-regulation of grp78 and coordinately regulated grp94, in response to ER Ca\(^{2+}\) pool depletion, prevents cell death. Hence, when the
grp78/grp94 response was inhibited, fibroblasts died in response to treatment with agents that mobilize Ca\(^{2+}\) from the ER, including TG and the Ca\(^{2+}\) ionophore A23187. Using a grp78 antisense plasmid, they demonstrated that the inability to up-regulate grp78 resulted in increased cell death following A23187 treatment (26). Similarly, when grp78 induction was inhibited by amplification of the grp78 core promoter region, an increased sensitivity to A23187 was observed (27). Furthermore, when induction of grp78/grp94 was inhibited by ribozyme cleavage of newly transcribed grp94 mRNA, increased sensitivity to A23187 and TG was observed (28). Interestingly, abrogation of the grp78/grp94 stress response did not enhance the cytotoxicity of TN, suggesting that the increase in Ca\(^{2+}\) leakiness of lymphoid cells. Second, there may be at least two signal transduction pathways that mediate the grp78/grp94 stress response, one in response to ER Ca\(^{2+}\) mobilization and the other in response to protein glycosylation inhibition. Third, regulation of the grp78/grp94 stress response may be a major factor in deciding whether a cell lives or dies in response to disruption of intracellular Ca\(^{2+}\) homeostasis. Indeed, the absence of a Ca\(^{2+}\) -mediated grp78/grp94 stress response may be the basis for the marked susceptibility of WEHI7.2 cells to TG-induced apoptosis.

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