# Calnexin Deficiency and Endoplasmic Reticulum Stress-Induced Apoptosis†

Anna Zuppini,‡ Jody Groenendyk,‡ Lori A. Cormack,‡ Gordon Shore,§ Michal Opas,|| R. Chris Bleackley,⊥ and Marek Michalak*,‡,⊥

*CIHR Research Group in Molecular Biology of Membrane Proteins and Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, Department of Biochemistry, McGill University, Montreal, Quebec, Canada, and Department of Anatomy and Cell Biology, University of Toronto, Toronto, Ontario, Canada*

*Received November 21, 2001; Revised Manuscript Received December 18, 2001*

ABSTRACT: In this study, we used calnexin-deficient cells to investigate the role of this protein in ER stress-induced apoptosis. We found that calnexin-deficient cells are relatively resistant to ER stress-induced apoptosis. However, caspase 3 and 8 cleavage and cytochrome *c* release were unchanged in these cells, indicating that ER to mitochondria "communication" during apoptotic stimulation is not affected in the absence of calnexin. The Bcl-2:Bax ratio was also not significantly changed in calnexin-deficient cells regardless of whether the ER stress was induced with thapsigargin or not. $Ca^{2+}$ homeostasis and ER morphology were unaffected by the lack of calnexin, but ER stress-induced Bap31 cleavage was significantly inhibited. Immunoprecipitation experiments revealed that Bap31 forms complexes with calnexin, which may play a role in apoptosis. The results suggest that calnexin may not play a role in the initiation of the ER stress but that the protein has an effect on later apoptotic events via its influence on Bap31 function.

The endoplasmic reticulum (ER)[1] plays a critical role in a variety of processes, including the maintenance of intracellular $Ca^{2+}$ homeostasis, and the synthesis, post-translational modification, and folding of membrane-associated, secreted, and integral membrane proteins. Recent evidence also indicates that the ER may play a critical role in the regulation of apoptosis (*1*−*9*). Among its many proteins, the ER membrane contains a number that are involved in apoptosis, including the Bcl-2 family (*2*), Bap31 (*3*), and caspase 12 (*6*). Thapsigargin, an inhibitor of SERCA (*10*), perturbs intracellular free $Ca^{2+}$ concentrations and activates apoptotic cell death in several cell types (*1*, *11*−*13*). Also, changes in the lumen of the ER play a role in modulating cell sensitivity to apoptosis (*5*). Calreticulin, which resides in the lumen of the ER, shares functional and structural similarities with calnexin, an integral membrane protein in the ER (*14*, *15*). Calreticulin and calnexin are unique ER chaperones in that they can bind monoglucosylated, high-mannose carbohydrate and/or the polypeptide portion of newly synthesized (glyco)proteins (*14*, *16*, *17*). While it appears that calreticulin affects cell sensitivity to apoptosis, the role of calnexin in these processes is unclear.

Here we have used calnexin-deficient cells (*18*) to examine the role of calnexin in apoptosis resulting from ER stress. In these studies, we have shown that the calnexin deficiency affects the sensitivity of the NKR cells to apoptosis but does not have an effect on $Ca^{2+}$ homeostasis in the ER. We show that Bap31 forms complexes with calnexin, and these may be affected by Bap31 function. The results suggest that calnexin may not play a role in the initiation of the ER stress but that the protein has an effect on later apoptotic events via its influence on Bap31 function.

## EXPERIMENTAL PROCEDURES

*Materials.* Anti-Bcl-2 and anti-Bax antibodies were from UBI. Anti-caspase 3 and anti-caspase 8 were from D. Nicholson (Merck Frost, Canada). Anti-cytochrome *c* and anti-PARP [poly(ADP-ribose)polymerase] were from Pharmingen. Anti-BiP and anti-SERCA2 were kindly provided by L. M. Hendershot (Department of Medical Biochemistry and Genetics, Texas A&M University, College Station, TX) and K. P. Campbell (Howard Hughes Medical Institute, University of Iowa, Iowa City, IA), respectively.

*Cell Culture, SDS−PAGE, Western Blot Analysis, and RT-PCR.* The human CEM and NKR T-lymphoblastoid leukemia cell lines (a gift from T. Elliott) were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and a 1% penicillin/streptomycin mixture. Cells, at a density of $2 \times 10^6$ cells/mL, were harvested by centrifugation (5 min at 1000 rpm), washed two times with

† Supported by grants from the CIHR (to M.M., M.O., R.C.B., and G.S.) and from the HSFA (to M.M.) and HSFO (to M.O.). R.C.B. is a CIHR Distinguished Scientist, AHFMR Medical Scientist, and HHMI International Scholar. M.M. is a CIHR Senior Investigator and AHFMR Medical Scientist.

* To whom correspondence should be addressed: Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. Telephone: (780) 492-2256. Fax: (780) 492-0886. E-mail: marek.michalak@ualberta.ca.

‡ CIHR Research Group in Molecular Biology of Membrane Proteins, University of Alberta.

§ McGill University.

|| University of Toronto.

⊥ Department of Biochemistry, University of Alberta.

[1] Abbreviations: ER, endoplasmic reticulum; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SERCA, sarco-(endo)plasmic reticulum $Ca^{2+}$-ATPase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ERp57, endoplasmic reticulum protein with a molecular weight of 57 000; SD, standard deviation; SE, standard error.

10.1021/bi015967+ CCC: $22.00 
Published on Web 01/26/2002

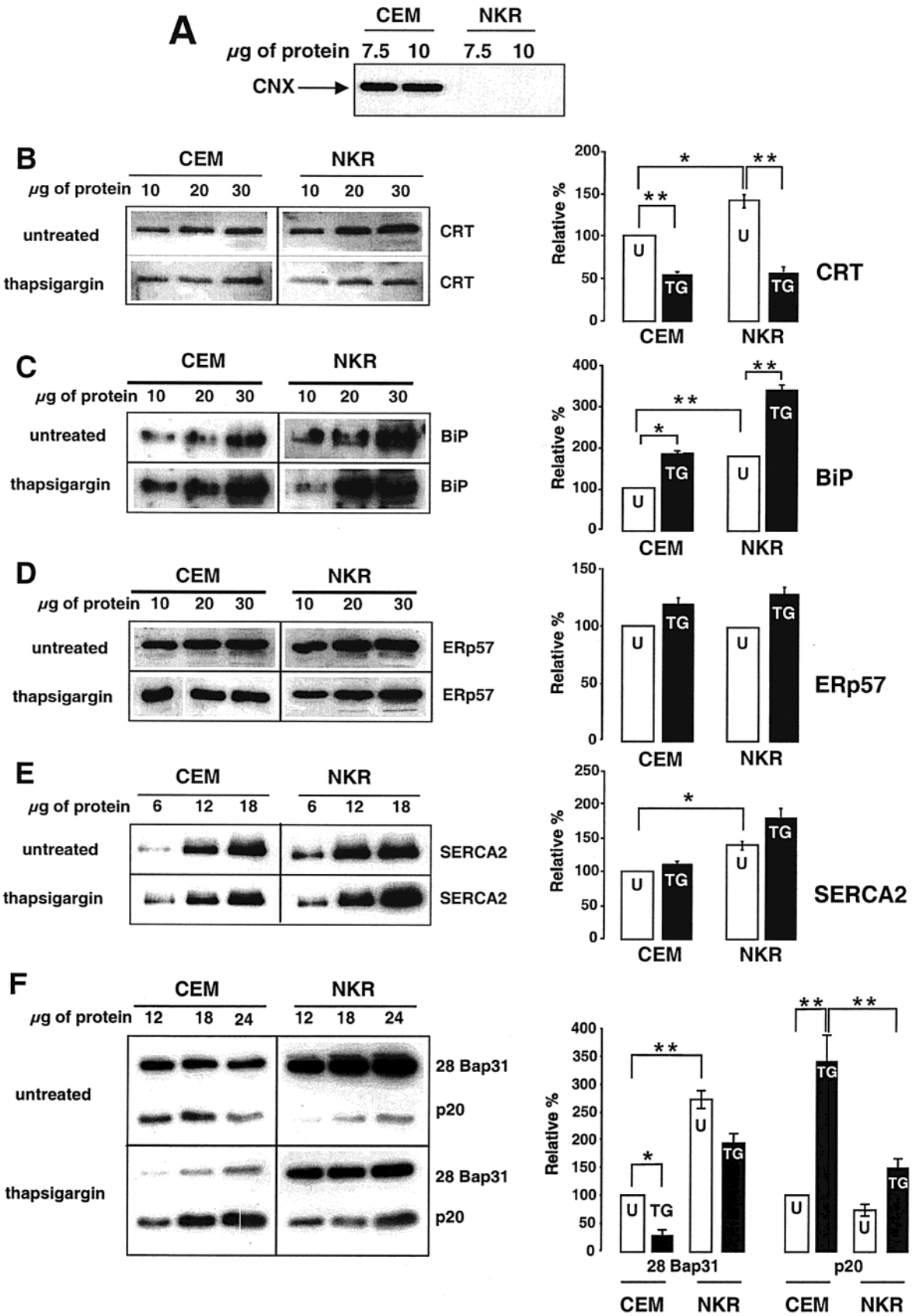



FIGURE 1: Expression of ER-associated proteins in CEM and NKR cells. CEM and NKR cells were harvested and lysed with RIPA buffer. Protein extracts were separated via SDS−PAGE, transferred onto nitrocellulose membranes, and probed with anti-calnexin (A), anti-calreticulin (B), anti-BiP (C), anti-ERp57 (D), anti-SERCA2 (E), or anti-Bap31 antibodies (F). Quantitative analysis was carried out by densitometric scanning of immunoreactive protein bands as described in Experimental Procedures. In panel F, quantitative analysis of Bap31 (28 Bap31) and its proteolytic fragment, p20, was carried out independently: (white bars) untreated cells (U) and (black bars) thapsigargin-treated cells (TG). Cells were treated with 1 $\mu$M thapsigargin for 16 h at 37 °C. The 100% value corresponds to untreated CEM cells. Data are means ± SD of three independent experiments. CEM, parental cell line; NKR, calnexin-deficient cell line; CNX, calnexin; CRT, calreticulin. Statistically significant at $p < 0.001$ (two asterisks) and $p < 0.005$ (one asterisk).

PBS, and solubilized with a solution containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM benzamidine, and 0.5 mM PMSF. Protein extracts were separated by SDS−PAGE (7.5, 10, and 12.5% acrylamide) (*19*), transferred to nitrocellulose membranes, and probed with specific antibodies, and immunoreactive protein bands were detected using horseradish peroxidase-conjugated secondary antibodies followed by an ECL reaction (Amersham) and exposure to X-ray film (*19*). Quantitative immunoblotting was carried out and analyzed as previously described (*5*, *20*, *21*). Briefly, immunoreactive protein bands were scanned, and the slope of the correlation between the amount of protein loaded on the SDS−PAGE gel and the optical density of the protein band was determined. The slopes were compared to determined changes in the level of protein in different cell lines or after thapsigargin treatment.

RNA was isolated as described previously (*19*). RT-PCR was carried out using the Gibco RP-PCR kit as recommended by the manufacturer. The following oligodeoxynucleotides were then used for PCR-driven amplification of calreticulin cDNA fragments: 5′-GATAAAGGGTTGCAGACAAGC-3′ and 5′-CCCAGACTTGACCTGCC-3′. Levels of calreticulin mRNA were normalized by comparison with levels of actin mRNA. Actin mRNA was amplified using the following primers: 5′-GACGAGGCCCAGAGCAAGAG-3′ and 5′-CCAGACAGCACTGTGTTGGC-3′. PCR products were separated on 1% agarose gels and visualized by staining with ethidium bromide.

*Apoptosis and Caspase Assay.* For detection and quantification of apoptosis, we used the *In situ* cell death detection kit, fluorescein (Roche Diagnostics). To induce apoptosis, cells ($2 \times 10^7$ cells/mL) were treated with 1 $\mu$M thapsigargin for 16 h at 37 °C. Cells were then washed in PBS containing 1% BSA and subjected to a TUNEL assay, as recommended by the manufacturer. DNA fragmentation was detected by flow cytometry. In each experiment, a negative control received the label solution without the terminal transferase instead of the TUNEL reaction mix. Prior to the TUNEL reaction, DNA strand breaks in positive controls were induced by treating cells with 1 $\mu$g of DNase I/mL, for 10 min at room temperature, in a buffer containing 50 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 1 mg/mL BSA.

Caspase 3 activity was measured in microtiter plates using the Caspase 3 Activity Assay (Roche Diagnostics) as described by the manufacturer. Cytosolic extracts were prepared as described previously (*22*), and cleavage of acetyl-DEVD-7-amino-4-(trifluoromethyl)coumarin (Ac-DEVD-AFC) by the cytosolic proteins (140 $\mu$g) was monitored fluorometrically, at a $\lambda_{max}$ of 485 nm, using a C43 fluorometer (PTI). Cells were also treated with 20 $\mu$M Z-DEVD-FMK (caspase 3 inhibitor II) and 20 $\mu$M Z-IETD-FMK (caspase 8 inhibitor II) for 16 h at 37 °C in the presence or absence of 1 $\mu$M thapsigargin. Cytochrome *c* release was assessed using specific anti-cytochrome *c* antibodies (*22*).

*ER and Cytoplasmic $Ca^{2+}$ Concentration.* The ER $Ca^{2+}$ capacity was estimated using a $^{45}Ca^{2+}$ equilibration method, as described previously (*21*). For measurement of the cytoplasmic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$), cells ($2 \times 10^7$/mL) were loaded with the fluorescent $Ca^{2+}$ indicator fura-2/AM (2 $\mu$M), taking precautions to avoid dye sequestration (*21*). Fura-2 fluorescence was measured at a $\lambda_{ex}$ of 340 nm using the C43 fluorometer (PTI). Cells were also stimulated with one of the following: 100 nM thapsigargin, 100 $\mu$M ATP, 100 $\mu$M carbachol, 100 nM bombesin, 2 $\mu$M ionomycin, or 2 $\mu$M ionomycin with 100 nM thapsigargin.

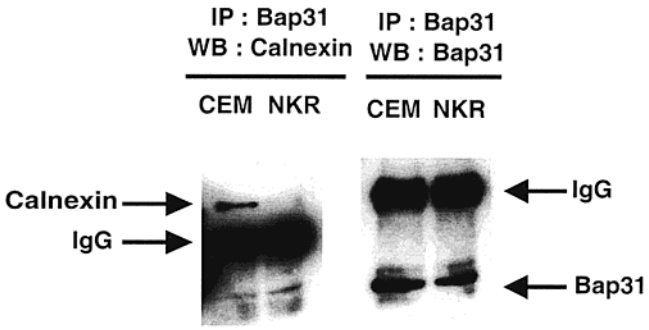


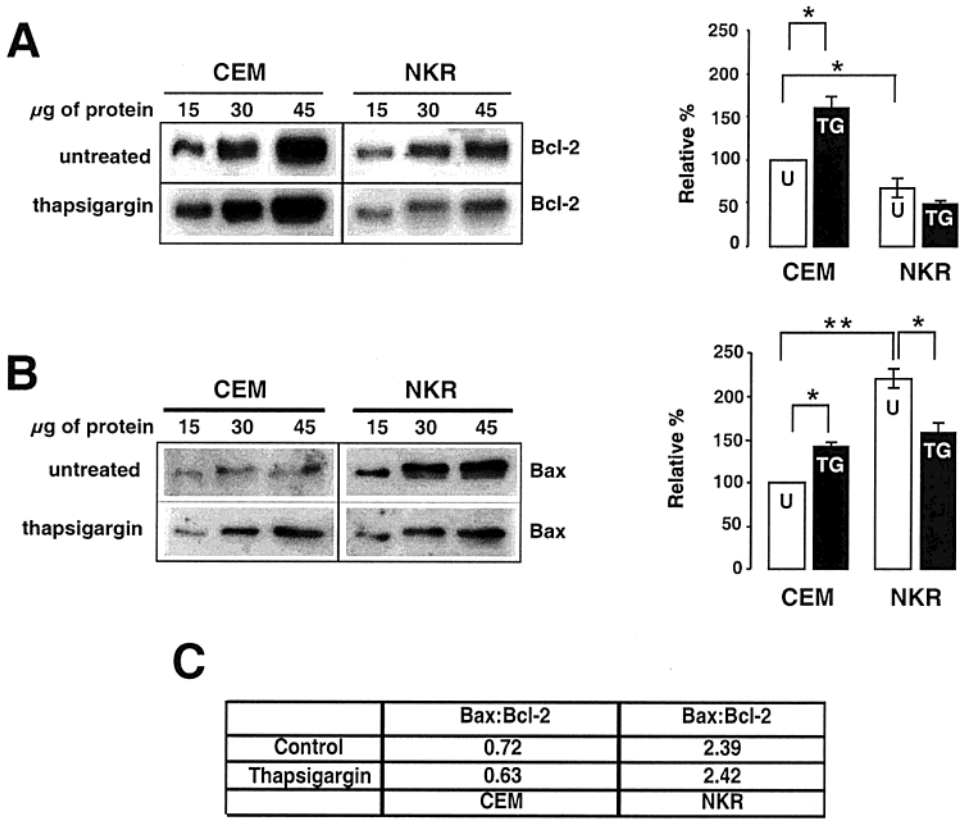

FIGURE 2: Bap31 and calnexin complex. Cellular extracts were incubated with anti-Bap31 antibodies, followed by SDS−PGAE, transfer to nitrocellulose membrane, and Western blot analysis with either anti-calnexin (left) or anti-Bap31 (right) antibodies as described in Experimental Procedures.

*Immunoprecipitation.* Cells were lysed for 30 min on ice in a solution containing 50 mM Hepes (pH 7.6), 200 mM NaCl, 2% Chaps, 0.5 mM benzamidine, and 0.5 mM PMSF (2% Chaps/HBS). The protein extract was centrifuged, then diluted in $^2/_3$ volume of 2% Chaps/HBS, and precleared by rotating for 30 min at 4 °C with $^1/_{15}$ volume of a 10% protein A−Sepharose CL-4B bead suspension in a solution containing 50 mM Hepes (pH 7.6) and 200 mM NaCl. Beads were then separated by centrifugation at 4 °C for 20 s (14 000 rpm), and 3 $\mu$L of anti-BaP31 antibody was added to the supernatant. After overnight incubation at 4 °C, 100 $\mu$L of a 10% protein A−Sepharose beads suspension was added to the samples and incubation extended for an additional 4 h. Beads were spun down at 4 °C for 20 s (14 000 rpm) and washed three times with 1% Chaps/HBS and once with HBS. A Laemmli sample buffer was added, and the samples were subjected to SDS−PAGE.

*Immunolocalization and Electron Microscopy.* For confocal immunofluorescence analysis, cells were attached to poly(L-lysine)-coated coverslips followed by incubation with specific antibodies as described previously (*5*). For electron microscopy, cells, harvested at confluency, were fixed for 1.5 h at room temperature in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.9). Samples were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1.5 h and dehydrated in ethanol and propylene oxide. Cells were then embedded in Araldite epoxy resin, and sections were analyzed with an electron microscope.

## RESULTS

*Calnexin-Deficient Cells and Thapsigargin-Induced ER Stress.* The calnexin-deficient cell line (NKR) that we used in these experiments was previously derived from an NK-sensitive parental cell line, CEM (*18*, *23*). We used NKR (calnexin-deficient) cells and their CEM parental cell line to investigate ER function when calnexin was absent. Figure 1A shows that the NKR cells did not express calnexin, as expected. However, these cells expressed significantly higher levels of calreticulin compared with the parental, CEM cell line (Figure 1B), and semiquantitative RT-PCR analysis




C

| | Bax:Bcl-2 | Bax:Bcl-2 |
|---|---|---|
| Control | 0.72 | 2.39 |
| Thapsigargin | 0.63 | 2.42 |
| | CEM | NKR |

FIGURE 3: Bax and Bcl-2 in calnexin-deficient cells. Cells were lysed and proteins separated by SDS−PAGE followed by Western blot analysis as described in Experimental Procedures. Protein blots were probed with anti-Bax (A) or anti-Bcl-2 (B) antibodies. Quantitative analysis was carried out by densitometric scanning of immunoreactive protein bands, and the results are shown on the right: (white bars) untreated cells (U) and (black bars) thapsigargin-treated cells (TG). The 100% value corresponds to untreated CEM cells. Data are means ± SD of three independent experiments. Panel C shows relative Bax:Bcl-2 ratio in CEM and NKR cells. CEM, parental cell line; NKR, calnexin-deficient cells. Statistically significant at $p < 0.001$ (two asterisks) and $p < 0.005$ (one asterisk).

revealed that the calnexin-deficient cells (NKR) contained approximately 3-fold more calreticulin mRNA than the CEM cells (not shown). The NKR cells also expressed more BiP and SERCA2 (Figure 1C,E). No significant differences in the expression of ERp57 were observed (Figure 1D).

Since an increased level of expression of BiP is associated with ER stress (*24*), we investigated whether the calnexin deficiency affected thapsigargin-induced ER stress. Thapsigargin inhibits SERCA pumps in the ER, leading to the depletion of intracellular $Ca^{2+}$ stores, ER stress, and, consequently, apoptosis (*25*). Figure 1C shows that, in both cell lines, thapsigargin increased the level of expression of BiP [(1.9 ± 0.1)-fold (mean ± SE); $n = 3$]. The incubation of cells with thapsigargin also resulted in a slightly increased level of expression of ERp57 and SERCA2 [(1.2 ± 0.1)-fold (mean ± SE); $n = 3$] (Figure 1D,E). Previous studies have shown that ER stress resulting from thapsigargin treatment leads to upregulation of the calreticulin gene and high levels of expression of this protein (*15*). However, thapsigargin treatment of both the CEM and calnexin-deficient (NKR) cells resulted in significantly reduced levels of calreticulin (Figure 1B). Consistent with this observation, in both cell lines, Northern blot analysis and semiquantitative RT-PCR indicated that levels of calreticulin mRNA were also reduced after thapsigargin treatment (data not shown). This was a surprising result because it is well-documented that thapsigargin treatment induces high-level expression of the calreticulin protein and mRNA in several cell lines (*26*−*28*). At present, there is no explanation for why thapsigargin treatment results in decreased levels of calreticulin in these cells, but it may be a cell-specific phenomenon.

*Bap31 in Calnexin-Deficient Cells.* Bap31 is an integral membrane protein of the ER that is involved in apoptotic pathways (*3*, *4*). Activation of apoptosis results in the cleavage of Bap31 by caspase 8, generating a 20 kDa, p20, proteolytic fragment (*4*). Using Western blot analysis, we found that the calnexin-deficient NKR cells expressed almost 3-fold more Bap31 than the parental CEM cell line (Figure 1F). Figure 1F also shows that, in the CEM cells, thapsigargin-induced apoptosis resulted in efficient cleavage of Bap31 as shown by the appearance of the p20 fragment. In contrast, when we treated the calnexin-deficient NKR cells with thapsigargin, Bap31 was significantly resistant to proteolysis (Figure 1F).

*Interactions between Bap31 and Calnexin.* As shown above, we found that the expression and function of ER-associated Bap31 are altered in calnexin-deficient cells, indicating that calnexin may play a role in apoptosis. To investigate whether calnexin and Bap31 may form protein complexes, we carried out immunoprecipitation experiments. We incubated cellular proteins with anti-Bap31 antibodies and followed this with Western blot analysis using anti-calnexin antibodies. Figure 2 shows that anti-Bap31 antibodies immunoprecipitated a protein complex, which contained immunoreactive calnexin in CEM cells, indicating that calnexin and Bap31 form a complex in the ER. As expected, no calnexin was found in Bap31 immunoprecipitates derived from the NKR calnexin-deficient cells (Figure 2). Western

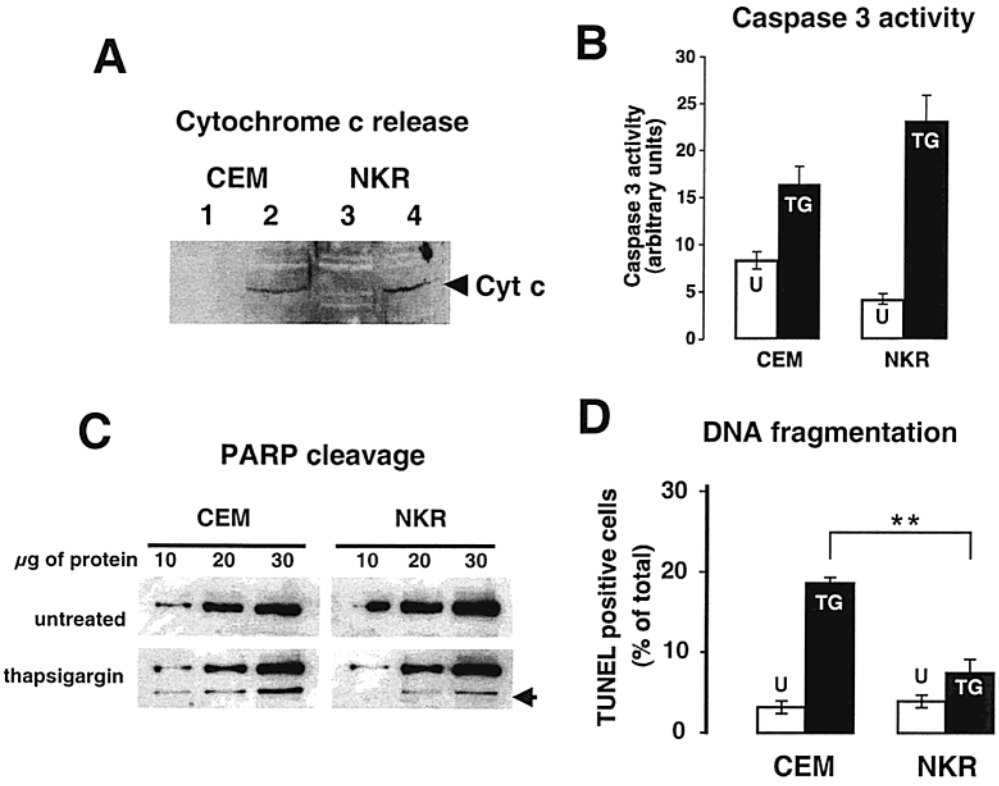



FIGURE 4: ER stress-induced apoptosis in calnexin-deficient cells. Cytochrome *c* release (A), caspase 3 activity (B), PARP cleavage (C), and DNA fragmentation (D) were assessed in CEM and calnexin-deficient (NKR) cells as described in Experimental Procedures. (A) Cytochrome *c* accumulation in the cytosol was assessed by Western blot analysis: lanes 1 and 3, cytosolic extracts from untreated cells; lanes 2 and 4, cytosolic extracts from cells incubated with 1 $\mu$M thapsigargin. (B) The caspase 3 activity in control and thapsigargin-treated cells was measured using the fluorimetric substrate Ac-DEVD-AFC. Results are means ± SD of three independent experiments. (C) The PARP cleavage was identified by Western blot analysis (the arrow) in control (untreated) and thapsigargin-treated cells. (D) DNA fragmentation was monitored by a TUNEL assay in control, untreated (−), and thapsigargin-treated (+) cell populations. Results are means ± SD of three independent experiments. Statistically significant at $p < 0.001$ (two asterisks) and $p < 0.005$ (one asterisk).

blot analysis revealed that the immunoprecipitated calnexin−Bap31 complex did not contain any calreticulin, ERp57, or Bcl-2 (data not shown).

*Bax and Bcl-2 in Calnexin-Deficient Cells.* It has been reported that the Bcl family of proteins is present in the ER (*2*). Bcl-2 is an antiapoptotic protein, whereas Bax is a proapoptotic protein; importantly, in leukemic cell lines, the Bax:Bcl-2 ratio is critical in determining whether cells will resist drug-induced apoptosis (*29*). We found a significantly greater level of expression of Bcl-2 in the CEM cells than in the calnexin-deficient (NKR) cells (Figure 3A). In contrast, the level of expression of Bax was greater in the calnexin-deficient cells (Figure 3B). Following treatment with thapsigargin, and the induction of ER stress, the expression of Bcl-2 and Bax was upregulated in CEM cells, by (1.5 ± 0.1)-fold (mean ± SE; $n = 3$) and (1.4 ± 0.1)-fold (mean ± SE; $n = 3$), respectively (Figure 3A,B). In contrast, the expression of Bcl-2 and Bax was downregulated in the calnexin-deficient cells (Figure 3A,B). Although Bcl-2 and Bax are expressed at different levels in the CEM and calnexin-deficient cells, in both cell types the relative ratio of Bax to Bcl-2 was unaffected by treatment with thapsigargin (Figure 3C).

*Apoptosis Induced by ER Stress in Calnexin-Deficient Cells.* To examine the events that link ER stress to apoptosis, we monitored cytochrome *c* release, caspase activity, PARP cleavage, and DNA fragmentation in calnexin-deficient cells after treatment with thapsigargin. The release of cytochrome *c* from mitochondria is considered a key event in apoptosis (*30*). We assessed the release of cytochrome *c* by cell fractionation and Western blot analysis. Figure 4A shows thapsigargin-induced release of cytochrome *c* in wild-type (CEM) and calnexin-deficient (NKR) cells. Neither the CEM nor the NKR cells contained detectable cytoplasmic cytochrome *c* (Figure 4, lanes 1 and 3). After treatment with thapsigargin, the release and accumulation of cytochrome *c* were similar in CEM and NKR cells (Figure 4, lanes 2 and 4).

In both cell lines, treatment with thapsigargin activated caspase 3 as estimated on the basis of the rate of cleavage of the fluorometric substrate DEVD-AFC (Figure 4B). In the CEM cells, the activity was increased (2.0 ± 0.2)-fold (mean ± SE; $n = 3$), and in the calnexin-deficient (NKR) cells, it was increased (4.0 ± 0.6)-fold (mean ± SE; $n = 3$) (Figure 4B). PARP cleavage, however, was similar in both CEM and calnexin-deficient cells (Figure 4C). Last, we analyzed DNA fragmentation using a TUNEL assay. Thapsigargin treatment of CEM cells resulted in a (7.4 ± 0.2)-fold (mean ± SE; $n = 3$) increase in TUNEL positive cells, whereas treatment of calnexin-deficient NKR cells resulted in a (1.8 ± 0.3)-fold (mean ± SE; $n = 3$) increase (Figure 4D). These results indicate that the calnexin-deficient cells (NKR) were more resistant than the parental cell line (CEM) to apoptosis induced by ER stress.

*Endoplasmic Reticulum in Calnexin-Deficient Cells.* Calnexin is an integral membrane protein of the ER, which has

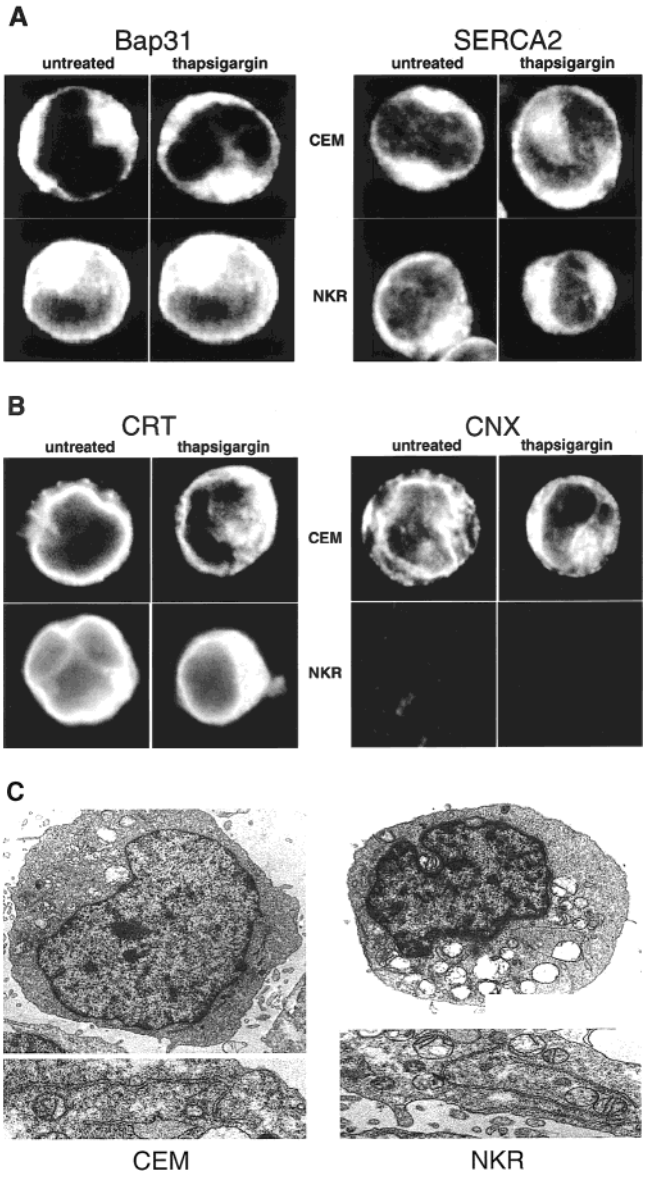



FIGURE 5: Immunofluorescence and electron microscope analysis of calnexin-deficient cells. Immunolocalization of Bap31 and SERCA2 (A) and calreticulin and calnexin (B) in CEM (wild-type) and NKR (calnexin-deficient) cells was carried out as described in Experimental Procedures. Cells were also treated with 1 $\mu$M thapsigargin followed by immunostaining with specific antibodies. In all cell lines, Bap31, SERCA2, calreticulin (CRT), and calnexin (CNX) were localized to the ER-like network. Calnexin-deficient NKR cells do not contain calnexin, and therefore, they did not stain with anti-calnexin antibodies (B). ER, electron micrographs of wild-type (CEM) and calnexin-deficient (NKR) cells.

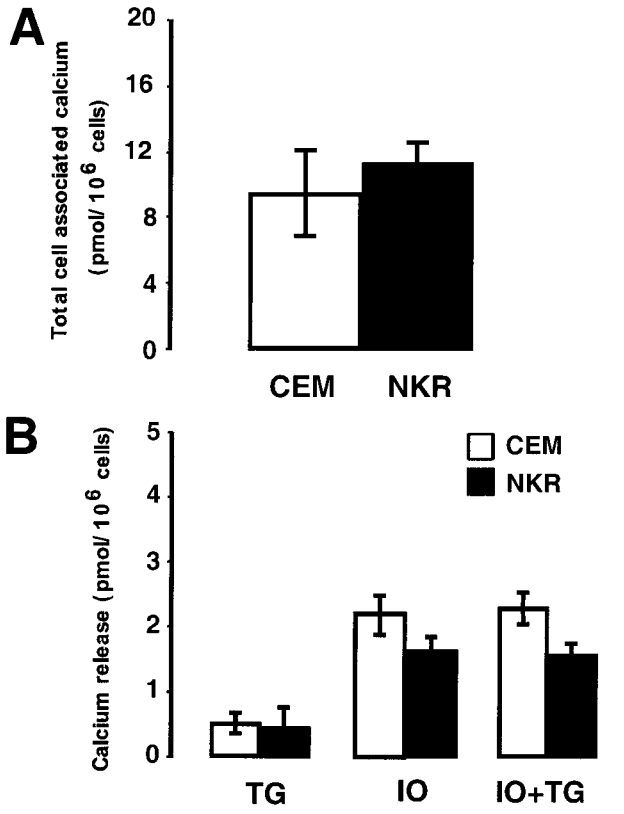



FIGURE 6: Total ER $Ca^{2+}$ content of calnexin-deficient cells. A total cellular $Ca^{2+}$ content was determined using equilibrium incubation with $^{45}Ca^{2+}$ followed by addition of thapsigargin (estimates the $Ca^{2+}$ pool in thapsigargin-sensitive $Ca^{2+}$ stores) or ionomycin (estimates the $Ca^{2+}$ pool in thapsigargin-insensitive $Ca^{2+}$ stores) as described in Experimental Procedures. The $Ca^{2+}$ content was measured in wild-type (CEM) and calnexin-deficient (NKR) cells. TG, thapsigargin; IO, ionomycin. Results are means ± SD of three independent experiments.

been implicated in affecting SERCA function (*31*). Calnexin, a homologue of calreticulin, has significant effects on intracellular $Ca^{2+}$ homeostasis (*15*). Therefore, we tested whether the relative resistance of the calnexin-deficient cells to apoptosis, shown above, might be explained by structural changes to the ER and/or modification of $Ca^{2+}$ homeostasis. First, we compared wild-type (CEM) and calnexin-deficient (NKR) cells using immunofluorescence and electron microscopy. The morphological appearance of the two cell lines was indistinguishable (Figure 5). Further, both CEM and NKR cells expressed Bap31, SERCA2, and calreticulin, and these proteins were localized similarly to an ER-like network and in the nuclear envelope. As expected, there was no expression of calnexin in the NKR cells (Figure 5B). Morphologically, in electron micrographs, the ER appeared intact in both cell lines (Figure 5C). We also observed typical nuclear and mitochondrial morphology in both cell lines. Importantly, thapsigargin treatment of CEM and calnexin-deficient (NKR) cells had no effect on the intracellular localization of Bap31, SERCA, and calreticulin (Figure 5). Our results show that calnexin deficiency and ER stress (thapsigargin treatment) did not affect the localization of ER proteins (integral membrane proteins Bap31 and SERCA and peripheral membrane protein calreticulin). Calnexin deficiency and ER stress also did not affect the morphology of the ER.

*$Ca^{2+}$ Homeostasis in Calnexin-Deficient Cells.* Changes in $Ca^{2+}$ concentration in the cytoplasm and in the lumen of the ER affect apoptosis. Calnexin, an integral ER membrane protein, might affect $Ca^{2+}$ uptake and release from the ER in several ways, including direct regulation of the SERCA $Ca^{2+}$ pump (*31*). These actions could either directly affect apoptotic processes or lead to changes in the expression of proteins involved in apoptosis (*5*, *8*, *9*). In the following experiments, we investigated whether calnexin deficiency affects the ER $Ca^{2+}$ capacity or cytoplasmic $Ca^{2+}$ concentrations in NKR cells. The CEM cells contained 9.5 ± 2.6 pmol of $Ca^{2+}/10^6$ cells (mean ± SE; $n = 3$), and the calnexin-deficient NKR cells contained 11.3 ± 1.2 pmol of $Ca^{2+}/10^6$ cells (mean ± SE; $n = 3$) (Figure 6). Thus, the absence of

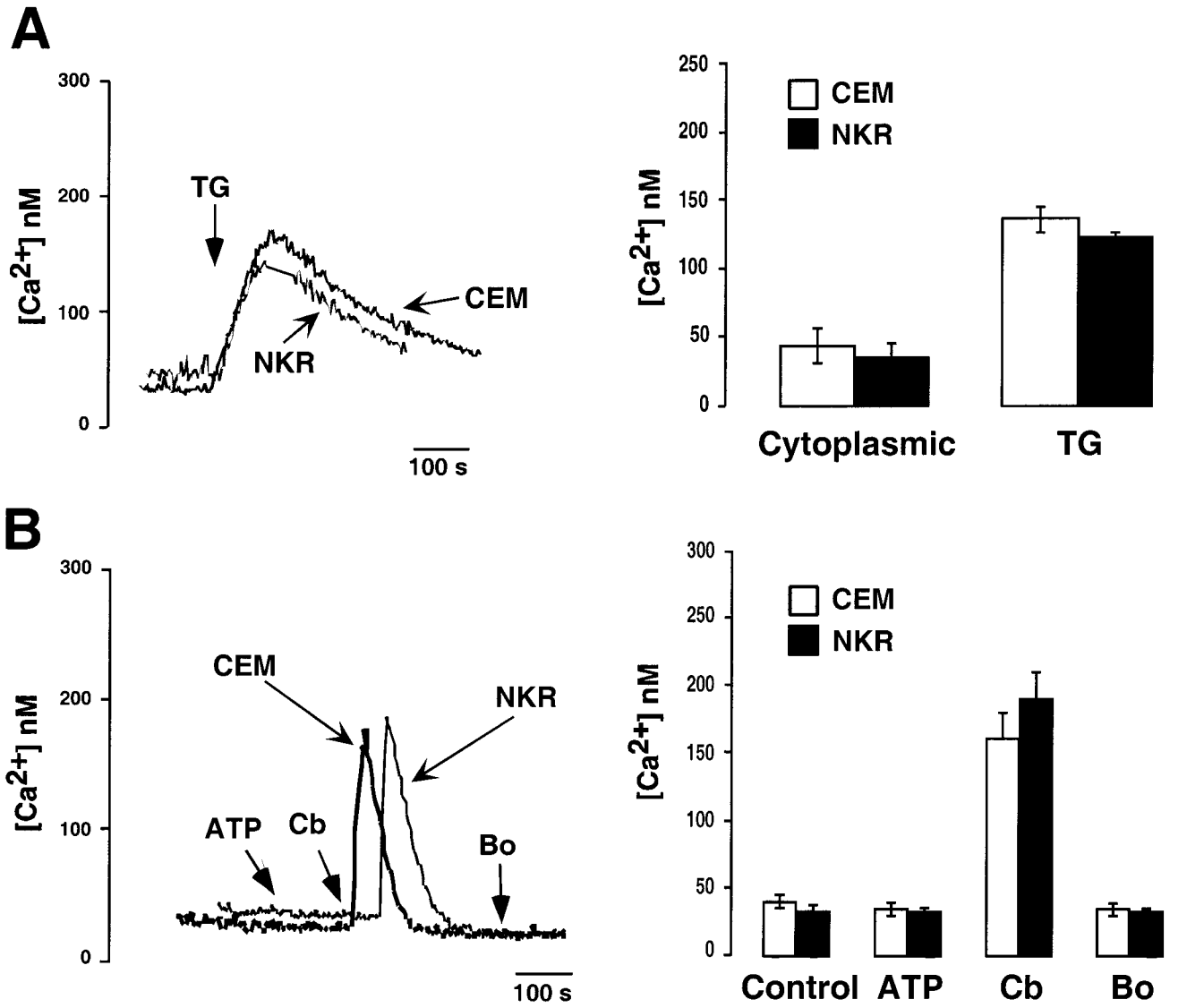



FIGURE 7: Agonist-induced $Ca^{2+}$ release in calnexin-deficient cells. Cells were loaded with the fluorescent $Ca^{2+}$ indicator fura-2 and stimulated with thapsigargin (A) or 100 $\mu$M ATP and 100 $\mu$M carbachol (Cb) or 100 nM bombesin (Bo) (B). CEM, wild-type cells; NKR, calnexin-deficient cells. Panel A shows typical traces showing thapsigargin (TG) stimulation of cells in a $Ca^{2+}$-free medium. (B) There was no $Ca^{2+}$ released by ATP or bombesin but a significant $Ca^{2+}$ release by addition of carbochol. There was not a significant difference in carbachol-induced $Ca^{2+}$ release in the cell lines that were investigated. Data are means ± SE ($n = 3$).

calnexin does not affect the $Ca^{2+}$ storage capacity of the ER in the NKR cells.

Next, we used a $Ca^{2+}$-sensitive fluorescent dye, fura-2, to investigate the effects of calnexin deficiency on cytoplasmic $Ca^{2+}$ concentrations ($[Ca^{2+}]_c$). Basal $[Ca^{2+}]_c$ values in control (CEM) and calnexin-deficient (NKR) cells were similar [~34.9 ± 3.7 nM (mean ± SD); $n = 3$]. When cells were treated with thapsigargin, the peak and duration of the $[Ca^{2+}]_c$ elevations were comparable (Figure 7A). Next, we compared agonist-induced $Ca^{2+}$ release in the CEM and calnexin-deficient NKR cells. In preliminary experiments, we tested the effect of 100 $\mu$M carbachol, 100 $\mu$M ATP, and 50 nM bombesin on $Ca^{2+}$ release. Of these agonists, only carbachol resulted in $Ca^{2+}$ release from both CEM and NKR cells (Figure 7B), so we used it in our subsequent experiments. Carbachol, a muscarinic agonist, induces $Ca^{2+}$ release from the ER via an $InsP_3$-dependent pathway (*32*). We found that carbachol induced a rapid and transient increase in the $[Ca^{2+}]_c$ in both CEM and NKR cell lines (Figure 7B). Similarly, increases in $[Ca^{2+}]_c$ were observed when both cell types were stimulated with ionomycin [204 ± 21 nM (mean ± SE); $n = 3$] or ionomycin and thapsigargin [212.3 ± 35 nM (mean ± SE); $n = 3$]. In all cases, the peak and the duration of the elevations in $[Ca^{2+}]_c$ were comparable in CEM and calnexin-deficient NKR cells. In summary, our results indicate that calnexin deficiency affected neither the storage capacity of the ER nor thapsigargin- and $InsP_3$-dependent $Ca^{2+}$ release from the ER.

## DISCUSSION

In this study, we investigated the potential role of calnexin in thapsigargin-induced (ER stress-induced) apoptosis. To do this, we used a calnexin-deficient, leukemic T-cell line (*18*, *23*). In DNA fragmentation TUNEL assays, we found that calnexin-deficient cells are relatively resistant to apoptosis induced by ER stress. We also found that, in calnexin-deficient cells, the cleavage of Bap31 that results from ER stress was significantly inhibited. While the extent of Bap31 cleavage was reduced, the cleavage of caspase 3 and 8 was unchanged by thapsigargin treatment. Therefore, the reduc-

tion in the level of Bap31 cleavage may be responsible for the observed resistance of the calnexin-deficient cells to apoptosis induced by ER stress.

In a previous study, we showed that changes in the expression of calreticulin, an ER resident homologue of calnexin, affect cell sensitivity to thapsigargin-induced apoptosis (*5*). For example, we found that calreticulin-deficient cells are significantly resistant to apoptosis. This resistance is accompanied by a decrease in the level of release of cytochrome *c* from mitochondria and by low levels of caspase 3 activity (*5*). This indicates that the lumen of the ER is involved in the release of cytochrome *c* from mitochondria and in the increased caspase activity that occurs during apoptosis. This suggests "communication" between the ER and mitochondria, which likely involves $Ca^{2+}$ and which plays an important role in determining cell sensitivity to apoptosis. At present, the most likely explanation for the effects of calreticulin deficiency is that calreticulin functions as an ER $Ca^{2+}$ storage protein (*5*, *33*). Calreticulin-deficient cells are more resistant to apoptosis, and at the same time, the level of $Ca^{2+}$ contained in their ER is significantly reduced (*33*). In keeping with this, it has been shown that cells which overexpress Bcl-2 are also relatively resistant to apoptosis and have significantly reduced luminal ER $Ca^{2+}$ levels (*8*, *9*). However, in the leukemic T-cell lines used in this study, we found that ER-dependent $Ca^{2+}$ homeostasis was not affected in calnexin-deficient cells, indicating that calnexin does not play any role in $Ca^{2+}$ storage in the ER. Despite this, the calnexin-deficient cells were relatively resistant to apoptosis induced by ER stress. This indicates that the role of calnexin deficiency in apoptosis is not mediated via $Ca^{2+}$, allowing the suggestion that luminal and integral membrane proteins of the ER may act with different mechanisms during apoptosis.

The role of the ER integral membrane proteins in apoptosis has only recently been considered (*1*−*3*, *6*, *34*). In particular, roles for Bap31, caspase 12, and the Bcl-2 family of proteins have been suggested (*2*, *3*, *6*). One central finding of this study is that Bap31 and calnexin form a protein complex (*35*). It is unlikely that the interaction between calnexin and Bap31 reflects the molecular chaperone function of calnexin because we have not detected any ERp57 in the immunoprecipitate. We also did not detect any interactions between Bap31 and calreticulin and Bcl-2. Bap31 is an integral membrane protein of the ER, which contains a "death effector" cytosolic domain that is cleaved by caspase 8 or related caspases (*3*). The cleavage product, p20, induces apoptosis either by amplifying a protease cascade initiated by caspase 8 (*36*) or by contributing to other pathways. Ng et al. (*3*) identified an ER membrane complex, including Bcl-2/Bcl-$X_L$, procaspas 8, and Bap31, that could be involved in apoptosis signal transduction. They suggested this complex would work like the plasma membrane apoptotic receptors Fas and TNFR-1 (*37*). In this study, we found that Bap31 associates with calnexin. Ng and Shore (*4*) hypothesized a Bap31 complex, including calnexin, which functions as a signaling complex to trigger apoptosis during ER stress. Our finding that the cleavage of Bap31 is significantly inhibited in calnexin-deficient cells, with a resultant low level of p20, is in keeping with this hypothesis. The cytoplasmic "tail" of calnexin may play a role in caspase 8-dependent cleavage of Bap31 (*35*). Alternatively, the absence of calnexin may prevent the formation of the Bap31, Bcl-2/Bcl-$X_L$, and procaspase 8 complex. In conclusion, we show here that calnexin may not be essential during initiation of the ER stress but that the protein plays an important role in later events involving ER stress-induced Bap31 cleavage and DNA fragmentation. These findings further support a notion that apoptosis may depend on both the presence of external apoptosis-activating signals and an internal factor represented by the ER and other intracellular organelles.

## ACKNOWLEDGMENT

We thank Monika Dabrowska and Ewa Dziak for superb technical assistance.

## REFERENCES

1. Lam, M., Dubyk, G., Chen, L., Nunez, G., Miesfeld, R. L., and Distelhorst, C. W. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6569−6573.
2. Zhu, W., Cowie, A., Wasfy, G. W., Penn, L. Z., Leber, B., and Andrews, D. W. (1996) *EMBO J. 15*, 4130−4141.
3. Ng, F. W., Nguyen, M., Kwan, T., Branton, P. E., Nicholson, D. W., Cromlish, J. A., and Shore, G. C. (1997) *J. Cell Biol. 139*, 327−338.
4. Ng, F. W. H., and Shore, G. C. (1998) *J. Biol. Chem. 273*, 3140−3143.
5. Nakamura, K., Bossy-Wetzel, E., Burns, K., Fadel, M., Lozyk, M., Goping, I. S., Opas, M., Bleackley, R. C., Green, D. R., and Michalak, M. (2000) *J. Cell Biol. 150*, 731−740.
6. Nakagawa, T., Zhu, H., Morishima, N., Li, E., Xu, J., Yankner, B. A., and Yuan, J. (2000) *Nature 403*, 98−103.
7. Nakagawa, T., and Yuan, J. (2000) *J. Cell Biol. 150*, 887−894.
8. Pinton, P., Ferrari, D., Magalhaes, P., Schulze-Osthoff, K., Di Virgilio, F., Pozzan, T., and Rizzuto, R. (2000) *J. Cell Biol. 148*, 857−862.
9. Foyouzi-Youseffi, R., Arnaudeau, S., Borner, C., Kelley, W. L., Tschopp, J., Lew, D. P., Demaurex, N., and Krause, K.-H. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 5723−5728.
10. Thastrup, O., Cullen, P. J., Drobak, B. K., Hanley, M. R., and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2466−2470.
11. Jackisch, C., Hahm, H. A., Tombal, B., McCloskey, D., Butash, K., Davidson, N. E., and Denmeade, S. R. (2000) *Clin. Cancer Res. 6*, 2844−2850.
12. Saleh, H., Schlatter, E., Lang, D., Pauels, H. G., and Heidenreich, S. (2000) *Kidney Int. 58*, 1876−1884.
13. Peiro, C., Vallejo, S., Cercas, E., Llergo, J. L., Lafuente, N., Matesanz, N., Rodriguez-Manas, L., and Sanchez-Ferrer, C. F. (2000) *J. Cardiovasc. Pharmacol. 36*, 676−680.
14. Williams, D. B. (1995) *Biochem. Cell Biol. 73*, 123−132.
15. Michalak, M., Corbett, E. F., Mesaeli, N., Nakamura, K., and Opas, M. (1999) *Biochem. J. 344*, 281−292.
16. Saito, Y., Ihara, Y., Leach, M. R., Cohen-Doyle, M. F., and Williams, D. B. (1999) *EMBO J. 18*, 6718−6729.
17. Ihara, Y., Cohen-Doyle, M. F., Saito, Y., and Williams, D. B. (1999) *Mol. Cell 4*, 331−341.
18. Scott, J. E., and Dawson, J. R. (1995) *J. Immunol. 155*, 143−148.
19. Milner, R. E., Baksh, S., Shemanko, C., Carpenter, M. R., Smillie, L., Vance, J. E., Opas, M., and Michalak, M. (1991) *J. Biol. Chem. 266*, 7155−7165.
20. Van Delden, C., Favre, C., Spat, A., Cerny, E., Krause, K.-H., and Lew, D. P. (1992) *Biochem. J. 281*, 651−656.
21. Mery, L., Mesaeli, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K.-H. (1996) *J. Biol. Chem. 271*, 9332−9339.
22. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. (1998) *EMBO J. 17*, 37−49.
23. Malyguine, A. M., Scott, J. E., and Dawson, J. R. (1998) *Immunol. Lett. 61*, 67−71.
24. Kozutsumi, Y., Segal, M., Normington, K., Gething, M. J., and Sambrook, J. (1988) *Nature 332*, 462−464.

25. Lam, M., Dubyak, G., and Distelhorst, C. W. (1993) *Mol. Endocrinol. 7*, 686−693.
26. Nguyen, T. O., Capra, J. D., and Sontheimer, R. D. (1996) *Mol. Immunol. 33*, 379−386.
27. Llewellyn, D. H., Kendall, J. M., Sheikh, F. N., and Campbell, A. K. (1996) *Biochem. J. 318*, 555−560.
28. Waser, M., Mesaeli, N., Spencer, C., and Michalak, M. (1997) *J. Cell Biol. 138*, 547−557.
29. Salomons, G. S., Brady, H. J., Verwijs-Janssen, M., Van Den Berg, J. D., Hart, A. A., Van Den Berg, H., Behrendt, H., Hahlen, K., and Smets, L. A. (1997) *Int. J. Cancer 71*, 959−965.
30. Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I., and Green, D. R. (2000) *Nat. Cell Biol. 2*, 156−162.
31. Roderick, H. L., Lechleiter, J. D., and Camacho, P. (2000) *J. Cell Biol. 149*, 1235−1248.
32. Felder, C. C., Poulter, M. O., and Wess, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 509−513.
33. Nakamura, K., Zuppini, A., Arnaudeau, S., Lynch, J., Ahsan, I., Krause, R., Papp, S., De Smedt, H., Parys, J. B., Müller-Esterl, W., Lew, D. P., Krause, K.-H., Demaurex, N., Opas, M., and Michalak, M. (2001) *J. Cell Biol. 154*, 961−972.
34. Chami, M., Gozuacik, D., Lagorce, D., Brini, M., Falson, P., Peaucellier, G., Pinton, P., Lecoeur, H., Gougeon, M. L., le Maire, M., Rizzuto, R., Brechot, C., and Paterlini-Brechot, P. (2001) *J. Cell Biol. 153*, 1301−1314.
35. Chevet, E., Nantel, A., Thomas, D., and Bergeron, J. J. M. (2000) *Mol. Biol. Cell 11*, 417a.
36. Muzio, M., Salvesen, G. S., and Dixit, V. M. (1997) *J. Biol. Chem. 272*, 2952−2956.
37. Nagata, S. (1997) *Cell 88*, 355−365.

BI015967+