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Caspase 12 in Calnexin-Deficient Cells†

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ABSTRACT: We investigated a role for calnexin, caspase 12, and Bap31 in endoplasmic reticulum stress-induced apoptosis in calnexin-deficient mouse embryonic fibroblasts and a calnexin-deficient human T cell line (NKR). We showed that calnexin-deficient mouse embryonic fibroblasts are relatively resistant to endoplasmic reticulum stress-induced apoptosis. Western blot analysis demonstrated that both wild-type and calnexin-deficient cells contained a caspase 12 protein. Caspase 12 expression was slightly inhibited in calnexin-deficient cells, and the protein carried out specific cleavage in the presence of thapsigargin. Immunoprecipitation experiments revealed that in the endoplasmic reticulum, caspase 12 forms complexes with Bap31 and calnexin. Treatment of wild-type cells with thapsigargin induced apoptosis and cleavage of Bap31. However, in the absence of calnexin, there was no significant cleavage of Bap31. There was also a negligible processing of caspase 8 in these cells. This work indicates that calnexin may play a role in modulating the sensitivity of a cell to apoptosis induced by endoplasmic reticulum stress, in conjunction with caspase 12 and Bap31.

The endoplasmic reticulum (ER)[1] plays a critical role in many cellular functions and has recently been implicated as playing an important role in apoptotic pathways (*1*, *2*). However, the pathways that lead from ER stress to DNA fragmentation and apoptosis are not well understood. It is well-known that caspases, a family of cysteine proteases, play a vital role during apoptosis (*3*). The activation of caspases triggers a cascade of proteolytic cleavage of specific substrates, leading to activation of nuclease activity, alterations in DNA repair processes, and modifications in membrane dynamics. One caspase specific to the ER, caspase 12, is ubiquitously expressed and localized to the ER membrane and, like other caspases, is synthesized as an inactive proenzyme consisting of a regulatory prodomain and two catalytic subunits (*4*, *5*). Caspase 12 is activated by conditions that elicit ER stress, such as disturbances in protein folding or disruption of intracellular Ca^{2+} stores (*3*, *4*). For example, pharmacological inhibition of the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) with thapsigargin, a highly specific SERCA inhibitor which disturbs cellular Ca^{2+} homeostasis (*3*), induces caspase 12-dependent apoptosis (*6*).

Recent studies using a human calnexin-deficient cell line, NKR, indicate that calnexin, an ER lectin-like chaperone, may play a role in modulating cell sensitivity to apoptosis induced by ER stress (*7*). Calnexin, involved in quality control of glycoproteins in the ER, may have the ability to respond to a buildup of misfolded protein, resulting in ER stress. The protein forms complexes with Bap31 (*7*), a 28 kDa integral membrane protein. Bap31 contains a cytoplasmic domain that associates with caspase 8, Bcl-XL, and Bcl-2 (*8*). Zuppini et al. (*7*) showed that calnexin may be important in later apoptotic events via its influence on Bap31 function. Exacerbated ER stress ultimately leads to apoptosis via ER-localized caspase 12, specifically activated under these conditions (*6*). Recent evidence has demonstrated that the majority of the human population does not have the complete gene product of caspase 12 (*14*, *15*). The relationship between calnexin and caspase 12 has not yet been investigated. In this study, we generated calnexin-deficient mouse embryonic fibroblast cell lines to investigate a relationship between caspase 12 and calnexin in ER-induced apoptotic events. We demonstrate in calnexin-deficient mouse embryonic fibroblasts that caspase 12 is involved in ER stress-induced apoptosis. Most importantly, we show that caspase 12 forms complexes with Bap31 and calnexin. This indicates that a three-way calnexin−Bap31−caspase 12 complex is important for apoptotic pathways initiated via the ER membrane.

EXPERIMENTAL PROCEDURES

Materials. Anti-Bcl-2 and anti-Grp94 antibodies were from Stressgene Inc. Anti-caspase 12 antibodies were kindly provided by D. Y. Juang (Department of Cell Biology, Harvard Medical School, Boston, MA). Anti-caspase 3 and anti-caspase 8 antibodies were from D. Nicholson (Merck

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[1] Abbreviations: ER, endoplasmic reticulum; MEF, mouse embryonic fibroblasts.

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Frost). Anti-actin and anti-GAPDH antibodies were obtained from Sigma. The anti-Bap31 antibody was described previously (*8*). Anti-calnexin, anti-calreticulin, anti-PDI, and anti-ERp57 antibodies were described previously (*9*−*11*). All other chemicals were obtained from Sigma unless otherwise specified and were of the highest available grade.

Cell Lines, Western Blot Analysis, and Immunoprecipitation. Human CEM (control) and NKR (calnexin-deficient) T lymphoblastoid leukemic cell lines were described previously (*7*). Mouse embryonic fibroblasts (MEF) were isolated from 12-day-old wild-type and calnexin-deficient embryos, immortalized and maintained as described previously (*12*). ER stress was induced by a 16 h treatment with 1 μM thapsigargin (*7*). CEM and NKR cells were also incubated for 16 h at 37 °C with 20 μM caspase 3 inhibitor (z-DEVD-fmk) or 20 μM caspase 8 inhibitor (z-IETD-fmk) in the presence or absence of 1 μM thapsigargin. Western blot analysis was carried out as described previously (*7*) and visualized using the Odyssey system (Licor Bioscience Inc.). The primary antibody was diluted in Odyssey Blocking Buffer diluted 1:1 in PBS [137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4 (pH 7.3)] in the presence of 0.1% Tween 20. The following antibodies were used: rat anti-caspase 12, goat anti-calreticulin, rabbit anti-calnexin, rabbit anti-ERp57, rabbit anti-Bap31, rabbit anti-caspase 8, rabbit anti-caspase 3, rabbit anti-BiP, rabbit anti-Grp94, rabbit anti-PDI, and rabbit anti-Bcl-2. The secondary antibodies were anti-rabbit, anti-goat, and anti-rat antibodies conjugated to Alexa-680. Quantitative analysis of protein bands was carried out as described previously (*7*), or by using Image J (http://rsb.info.nih.gov/ij/, National Institutes of Health, Bethesda, MD). Protein loading was normalized using Coomassie stain or anti-actin, anti-tubulin, and anti-GAPDH antibodies. Statistical analysis was carried out using one-way ANOVA testing. *p* values were a maximum of 0.05 and a minimum of 0.0001 for different antibodies as indicated.

For immunoprecipitation, CEM cells were lysed in a buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and a cocktail of protease inhibitors. The cell extract was centrifuged and diluted in $^2/_3$ volume of a buffer containing 50 mM Hepes (pH 7.6), 200 mM NaCl, and 2% Chaps and precleared by incubation with a 10% protein A−Sepharose bead suspension. Beads were separated by centrifugation, and 3 μL of anti-Bap31 antibody was added. After overnight incubation at 4 °C, 100 μL of a 10% protein A−Sepharose bead suspension was added followed by incubation for an additional 4 h at 4 °C. Beads were pelleted by centrifugation, washed three times with an excess of buffer containing 50 mM Hepes (pH 7.6), 200 mM NaCl, and 2% Chaps, and spun down at 4 °C. Laemmli buffer was added to the pellet, and the sample was separated via SDS−PAGE (*7*).

Immunofluorescence and Analysis of Cytoplasmic Ca^{2+} Concentrations. For immunofluorescence, MEF cells were fixed with 4% formaldehyde for 15 min at room temperature and permeabilized in PBS containing 0.1% saponin (*7*). All antibodies were diluted in PBS containing 0.1% saponin and 2% milk powder. Fixed cells were stained with rabbit anti-calnexin antibodies at a 1:800 dilution and rat anti-caspase 12 antibodies at a 1:2 dilution. Secondary antibodies were goat anti-rabbit Alexa-488 F_{ab} used at a 1:250 dilution and goat anti-rat Alexa-594 IgG used at a 1:250 dilution. Images were collected using a Zeiss confocal laser scanning microscope (LSM 510, software version LSM 3.2). The colocalization coefficient was calculated using Imaris and was between 0.5 and 0.6 for all images.

For Ca^{2+} measurements, CEM and NKR cells (2×10^7 cells/mL) were loaded with the fluorescent Ca^{2+} dye Fura-2/AM (2 μM), taking precautions to avoid dye sequestration (*13*). Fura-2 fluorescence was measured at a λ_{ex} of 340 nm using a C43 fluorometer (Photon Technology International). Cells were stimulated with 100 nM thapsigargin and 100 μM carbachol (*7*).

FACS Analysis. Binding of annexin V-FITC to MEF was carried out using the cell death detection kit as recommended by the manufacturer (BD Biosciences Inc.). MEF cells were trypsinized and collected by low-speed centrifugation (1200 rpm for 3 min). Cells were washed with PBS and suspended at a density of 1×10^6 cells/mL, followed by an annexin V labeling assay. FACS analysis was performed using a FACScan Instrument (BD Biosciences Inc.). Analysis of labeled cells was conducted using CellQuest (BD Biosciences Inc.). Statistical analysis was carried out using one-way ANOVA testing. Results were determined to be statistically significant at a *p* value of 0.02.

RESULTS

Western blot analysis using anti-rat caspase 12 antibodies (*7*) revealed that both control (CEM) and calnexin-deficient human leukemic T cells (NKR) contain a caspase 12-like protein. It was recently determined in the majority of humans that caspase 12 contains a TGA stop codon at amino acid position 125 that results in the synthesis of a truncated protein product, while in approximately 20% of the African-American populace, this polymorphism is not present and the nucleotide sequence encodes a full-length caspase 12 proenzyme (*15*, *16*). For this reason, to further identify a relationship among calnexin, Bap31, and caspase 12, we generated wild-type and calnexin-deficient mouse embryonic fibroblast cell lines (MEF).

Calnexin and ER Stress Responses in Mouse Embryonic Fibroblasts (MEF). Wild-type (8WT) and calnexin-deficient (9KO) MEF cells were used to investigate the relationship between calnexin and ER-associated caspase 12. Western blot analysis of the expression of ER resident proteins in calnexin-deficient MEF demonstrated no significant differences in the expression of calreticulin, Grp94, and PDI (Figure 1B−D). As expected, 9KO cells did not express calnexin (Figure 1A). However, 9KO cells contained approximately 40% less BiP (Figure 1E). Figure 1F shows that there was also a significant reduction in the amount of ERp57 protein in calnexin-deficient cells. This was not observed in the human CEM and NKR cells (*7*), and it might be a cell specific phenomenon. Next, we investigated whether the calnexin deficiency affected thapsigargin-induced ER stress and apoptosis. The incubation of calnexin-deficient cells with thapsigargin also resulted in a slightly decreased level of expression of Grp94 and ERp57 (Figure 1D,E).

Bcl-2, Caspase, and Bap31 Activation in Calnexin-Deficient Mouse Embryonic Fibroblasts (MEF). Levels of expression of Bcl-2 protein (Figure 2A), an anti-apoptotic

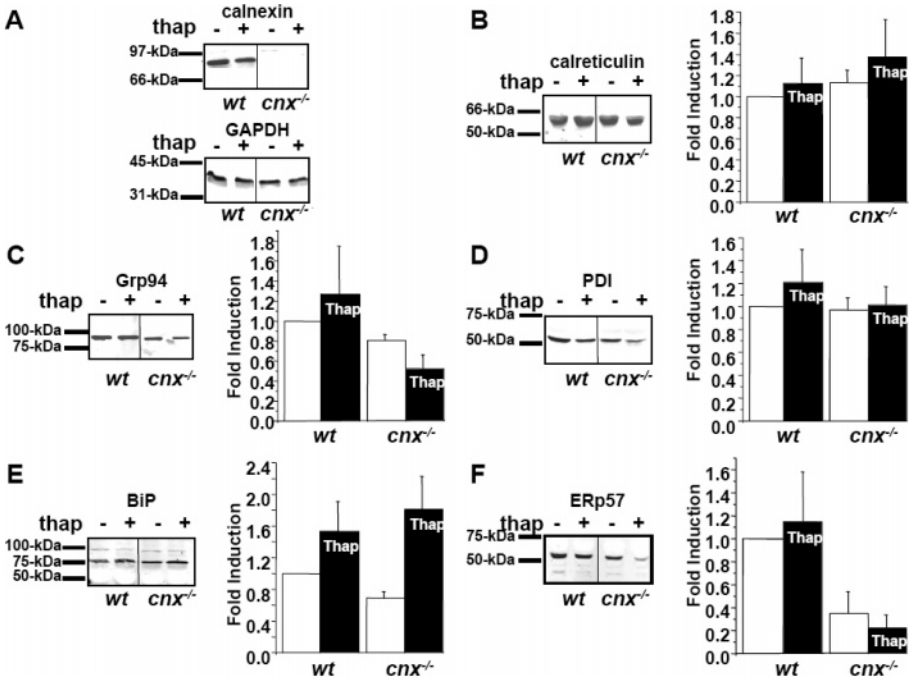

FIGURE 1: Expression of ER proteins in calnexin-deficient cells. Protein extracts were separated by SDS−PAGE, transferred onto a nitrocellulose membrane, and probed with anti-calnexin (90 kDa) (1:500 dilution), anti-GAPDH (38 kDa) (1:2500 dilution) (A), anti-calreticulin (60 kDa) (1:500 dilution) (B), anti-Grp94 (94 kDa) (1:500 dilution) (C), anti-PDI (55 kDa) (1:1000 dilution) (D), anti-BiP (78 kDa) (1:1000 dilution) (E), and anti-ERp57 (57 kDa) (1:500 dilution) (F) antibodies. Histograms represent the quantitative analysis of immunoreactive protein bands carried out as described in Experimental Procedures. Data are means ± the standard error of three or more independent experiments: 8WT, wild-type mouse embryonic cell line; 9KO, calnexin-deficient mouse embryonic cell line; white bars, untreated cells; and black bars labeled Thap, cells treated with 1 μM thapsigargin for 16 h at 37 °C. Results are statistically significant with a p value of 0.01 for BiP (E) and a p value of 0.05 for ERp57 (F) (one-way ANOVA testing).

Bcl family member reported to be present in the ER (*17*), and caspase 3 protein (Figure 2B) were reduced in the absence of calnexin. However, the absence of calnexin did not have any significant effect on thapsigargin-induced cleavage of caspase 3 (Figure 2B). Western blot analysis using anti-rat caspase 12 antibodies demonstrated that both 8WT and 9KO cells contained a caspase 12 protein (Figure 2C). The level of caspase 12 expression is slightly reduced in calnexin-deficient MEF (Figure 2C) with the protein undergoing specific cleavage in the presence of thapsigargin, indicating that a deficiency in calnexin did not interfere with thapsigargin-dependent activation of caspase 12.

Bap31 is an integral membrane protein of the ER that is also involved in apoptosis pathways (*8*, *18*). Activation of apoptosis results in the cleavage of Bap31 by caspase 8, generating a 20 kDa p20 proteolytic fragment (*18*). Treatment of wild-type (8WT) cells with thapsigargin induced apoptosis and cleavage of Bap31 to produce the p20 fragment (Figure 3A). Interestingly, in the absence of calnexin, there was no significant cleavage of Bap31 (Figure 3A), similar to that seen in human CEM and NKR cells (*7*).

Bap31 is a target of caspase 8 (*19*). Caspase 8 is synthesized as two isoforms (54 and 52 kDa) which are processed to form two heterodimers known as p18 and p10 (*20*). Next we compared the processing of caspase 8 in response to thapsigargin treatment in the control and calnexin-deficient cell lines. Surprisingly, treatment with thapsigargin induced processing of caspase 8 only in the control CEM cell line (Figure 3B). We found negligible processing of caspase 8 in the calnexin-deficient cells (Figure 3B). These observations indicate that calnexin-deficient MEF cells, similar to human calnexin-deficient T cells (*7*), have modified their apoptosis program.

Effects of Caspase Inhibitors on Bap31 Cleavage in Human Leukemic T Cells. Human Bap31 binds caspase 8 proenzyme (*21*) and calnexin (*7*). Bap31 is also cleaved upon activation of caspase cascades in HeLa cells, and this cleavage is affected by caspase inhibitors (*22*). We asked, therefore, whether caspase-dependent cleavage of Bap31 occurs in ER stress-induced apoptosis in the presence and absence of calnexin. Cells were incubated with 1 μM thapsigargin in the presence or absence of the caspase 8 inhibitor z-IETD-fmk (20 μM) and the caspase 3 inhibitor z-DEVD-fmk (20 μM). This was followed by Western blot analysis which measured the extent of Bap31 cleavage. The caspase 8 inhibitor, z-IETD-fmk, prevented considerable Bap31 cleavage in the wild-type cell line and completely prevented any cleavage of Bap31 in the calnexin-deficient cell line (Figure 3C). In conjunction, the caspase 3 inhibitor, z-DEVD-fmk, also prevented significant cleavage of Bap31 to the p20 fragment. In contrast to the caspase 8 inhibitor, the caspase 3 inhibitor, z-DEVD-fmk, did not abolish Bap31 cleavage in the calnexin-deficient cells (Figure 3C). Previous studies with HeLa cells using photodynamic therapy have shown that the caspase 3 inhibitor z-DEVD-fmk blocks cleavage of both caspase 8 and Bap31, suggesting that the processing of these two molecules occurs downstream of caspase 3 activation (*23*). We observed a similar hierarchy of activation in the control cell line during thapsigargin-induced apoptosis. However, it appears that the calnexin-deficient cells have altered regulation in the apoptosis pathways activated by thapsigargin treatment.

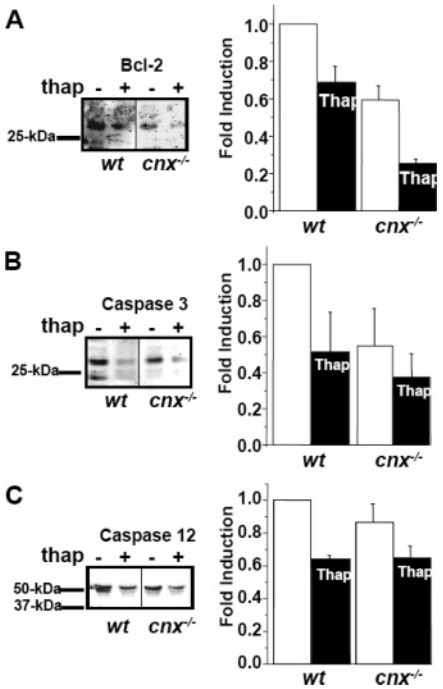

FIGURE 2: Expression of apoptotic proteins in calnexin-deficient cells. Protein extracts were separated by SDS−PAGE, transferred onto a nitrocellulose membrane, and probed with anti-Bcl-2 (30 kDa) (1:2000 dilution) (A), anti-caspase 3 (30 kDa) (1:1000 dilution) (B), and anti-caspase 12 (55 kDa) (1:20 dilution) (C) antibodies. Histograms represent the quantitative analysis of immunoreactive protein bands carried out as described in Experimental Procedures. Data are means ± the standard error of three or more independent experiments: 8WT, wild-type mouse embryonic cell line; 9KO, calnexin-deficient mouse embryonic cell line; white bars, untreated cells; and black bars labeled Thap, cells treated with 1 μM thapsigargin for 16 h at 37 °C. Results are statistically significant with a p value of 0.0001 for Bcl-2 (A), a p value of 0.1 for caspase 3 (B), and a p value of 0.007 for caspase 12 (C) (one-way ANOVA testing).

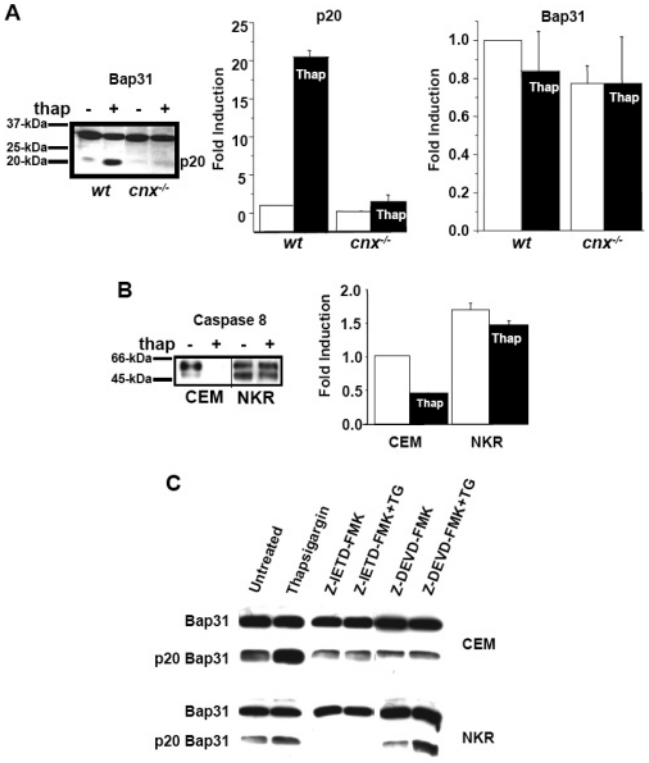

FIGURE 3: Expression of Bap31 and generation of the p20 fragment. Protein extracts were separated by SDS−PAGE, transferred onto a nitrocellulose membrane, and probed with anti-Bap31 (31 and 20 kDa) (1:2000 dilution) (A and C) or anti-caspase 8 (53 kDa) (1:2000 dilution) (B) antibodies. In panel C, cells were treated with either 20 μM caspase 8 inhibitor z-IETD-fmk or 20 μM caspase 3 inhibitor z-DEVD-fmk for 16 h at 37 °C, in the presence or absence of 1 μM thapsigargin as described in Experimental Procedures. Histograms represent the quantitative analysis of immunoreactive protein bands carried out as described in Experimental Procedures. Data are means ± the standard error of three or more independent experiments: 8WT, wild-type mouse embryonic cell line; 9KO, calnexin-deficient mouse embryonic cell line; CEM, parental human cell line; NKR, calnexin-deficient cell line; white bars, untreated cells; and black bars labeled Thap, cells treated with 1 μM thapsigargin for 16 h at 37 °C. Results are statistically significant at a p value of 0.0005 for the p20 fragment (A) (one-way ANOVA testing).

Calnexin-deficient MEF cells were significantly resistant to thapsigargin-induced apoptosis (approximately 6-fold) as measured by binding of annexin V to the cell surface (Figure 4A,C). This was in line with our earlier observations with calnexin-deficient human leukemic T cells (*7*).When the cells were treated with thapsigargin, there was also a significant number of PI positive cells in a wild-type MEF population (Figure 4B,C). Therefore, it appears that calnexin might be necessary for the cleavage of Bap31 into the p20 fragment and subsequent activation of the apoptosis pathway in both human and mouse systems.

Effects of Caspase Inhibitors on Ca^{2+} Signaling in Human Leukemic T Cells. The peak and duration of the elevation in $[Ca^{2+}]_{cyt}$ are similar in CEM and NKR cells after thapsigargin treatment, indicating that they have the same ER storage capacity (*7*). Here, we used Fura-2 to determine whether caspase inhibitors have any effect on intracellular Ca^{2+} homeostasis in the human leukemic T cell lines that were investigated. Figure 5 shows that, in cells treated for 16 h with z-IETD-fmk and z-DEVD-fmk, thapsigargin induced an immediate increase in $[Ca^{2+}]_{cyt}$ which was followed by a sustained elevation of $[Ca^{2+}]_{cyt}$. Following treatment with the caspase 8 inhibitor (z-IETD-fmk), the elevation in $[Ca^{2+}]_{cyt}$ was greater and faster in the calnexin-deficient cells (NKR) than in the control cells (CEM) (Figure 5A). In contrast, the rate of elevation in $[Ca^{2+}]_{cyt}$ in the two cell lines was indistinguishable following treatment with the caspase 3 inhibitor (z-DEVD-fmk) (Figure 5C). However, the peak of $[Ca^{2+}]_{cyt}$ was significantly higher in NKR cells treated with the caspase 3 inhibitor (Figure 5C). In another experiment, we used carbachol to elicit $InsP_3$-dependent release of Ca^{2+} from the ER in the control and calnexin-deficient cells (*7*). In the presence of z-IETD-fmk (the caspase 8 inhibitor), carbachol treatment caused a rapid release of Ca^{2+} in both cell types, with an increased $[Ca^{2+}]_{cyt}$ (Figure 5B). In the presence of z-DEVD-fmk (the caspase 3 inhibitor), carbachol again induced a rapid increase in $[Ca^{2+}]_{cyt}$ in both cell types. However, the return to basal levels of $[Ca^{2+}]_{cyt}$ (recovery time) that results from removal of Ca^{2+} from the cytoplasm was significantly longer in calnexin-deficient cells (Figure 5D), indicating an impaired function of normal Ca^{2+} removal systems, which include SERCA and/or the plasma membrane Ca^{2+}-ATPase.

Bap31 Interacts with Caspase 12 and Calnexin in Mouse Embryonic Fibroblasts. We reported previously that Bap31 and calnexin form complexes in the ER in human CEM and NKR cells (*7*). Since human cells may not express caspase 12, we set out to establish if there is a relationship between caspase 12 and Bap31 using MEF. Calnexin-deficient and

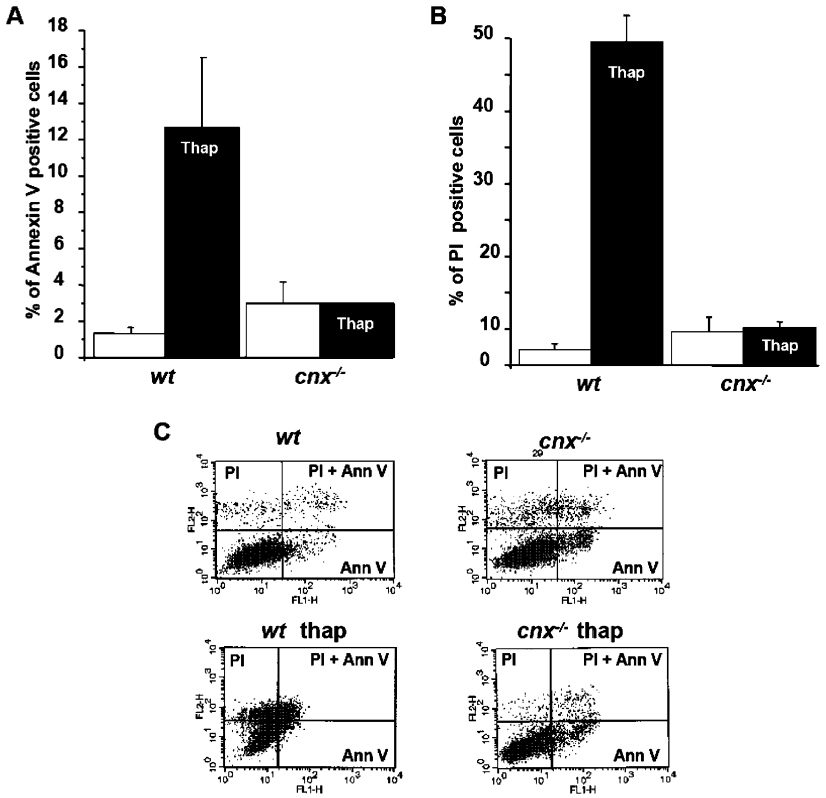

FIGURE 4: Percent of annexin V and PI positive cells, as determined by FACS analysis. Annexin V (A) and PI (B) staining of MEF was carried out as described in Experimental Procedures. Data are means ± the standard error of three or more independent experiments. Results are statistically significant at a *p* value of 0.02 (one-way ANOVA testing). A representative flow cytometry histogram of wild-type (*wt*) and calnexin-deficient ($cnx^{-/-}$) MEF is shown in panel C. Thap, thapsigargin.

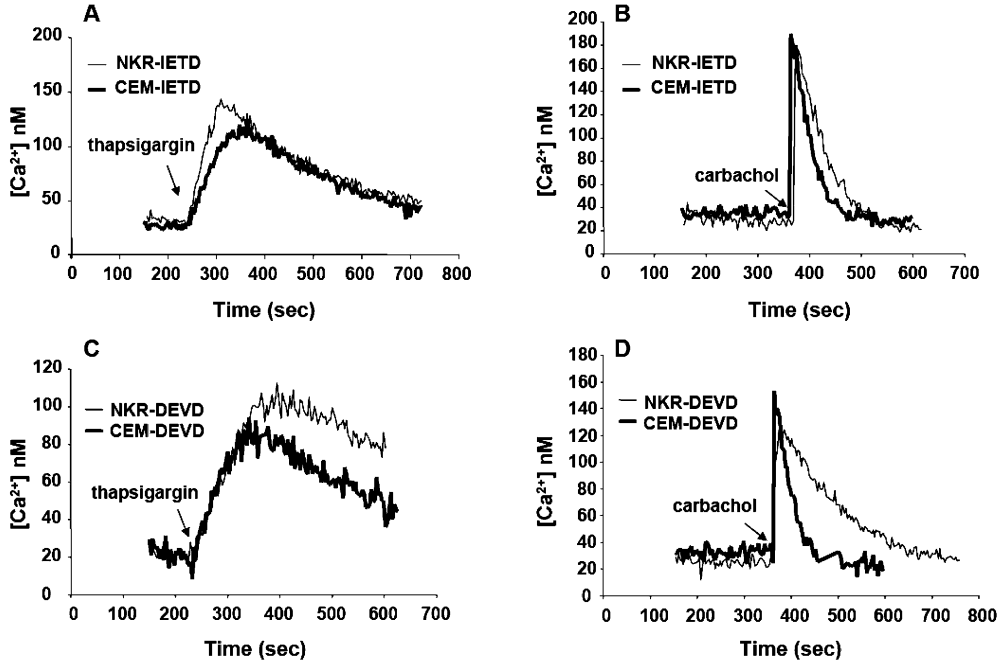

FIGURE 5: Ca^{2+} release induced by thapsigargin and carbachol in calnexin-deficient cells incubated with caspase inhibitors. Cells were incubated for 16 h with either 20 μM caspase 8 inhibitor z-IETD-fmk or 20 μM caspase 3 inhibitor z-DEVD-fmk, loaded with the fluorescent Ca^{2+} dye Fura-2/AM and stimulated with 100 nM thapsigargin (A and C) or 100 μM carbachol (B and D) as described in Experimental Procedures. Panels A and C show typical traces induced by thapsigargin in a Ca^{2+}-free medium. A significant Ca^{2+} release was obtained after addition of carbachol in both cell lines treated with the different inhibitors (B and D). Results are representative of four independent experiments.

wild-type MEF along with 3T3 fibroblasts were cultured and lysates taken for immunoprecipitation with the anti-Bap31 antibody. The anti-Bap31 antibody was able to precipitate a full-length Bap31 product (Figure 6A) as well as a product that was recognized by the anti-caspase 12 antibody (Figure 6B). Importantly, the anti-Bap31 antibody was also able to precipitate calnexin from mouse cell extracts (Figure 6C). These results indicate that Bap31 interacts with caspase 12 in MEF in the presence and absence of calnexin. Furthermore, Bap31 also forms complexes with calnexin in MEF,

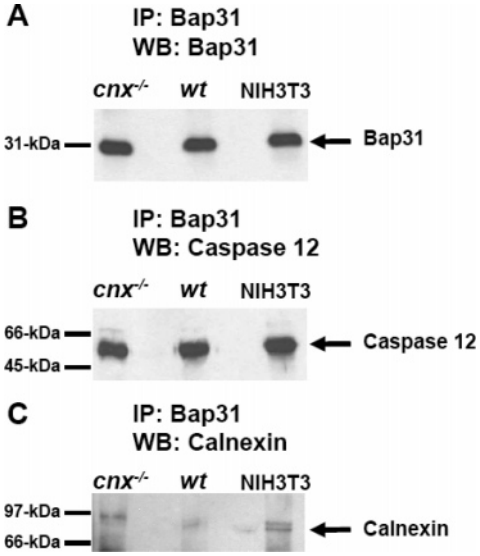

FIGURE 6: Bap31, caspase 12, and calnexin interact in mouse embryonic fibroblasts. Immunoprecipitation was carried out as described in Experimental Procedures. Protein extracts were incubated with rat anti-caspase 12 antibodies, separated by SDS−PAGE, and transferred onto a nitrocellulose membrane. Western blot analysis was carried out with rabbit anti-Bap31 (31 kDa) (1:2000 dilution) (A), rat anti-caspase 12 (55 kDa) (1:20 dilution) (B), and rabbit anti-calnexin (90 kDa) (1:500 dilution) (C) antibodies. Results are representative of three or more independent experiments.

suggesting that all three proteins may form complexes involved in signaling ER stress-induced apoptosis.

Figure 7 shows that calnexin (Figure 7A, red) and caspase 12 (Figure 7A, green), and Bap31 (Figure 7B, red) and caspase 12 (Figure 7B, green), colocalized to an ER-like network. Approximately 67.1 ± 1.6% of caspase 12 positive staining colocalized with calnexin, and approximately 81.3 ± 3.3% of caspase 12 positive staining colocalized with Bap31. We conclude that these proteins colocalize in the ER, supporting our observations that they may form complexes in the ER.

DISCUSSION

In this study, we investigated a role for calnexin, caspase 12, and Bap31 in ER stress-induced apoptosis. Human cells do not express caspase 12 (*15*, *16*); consequently, to study the relationship between caspase 12 and calnexin or Bap31, we turned to mouse embryonic fibroblast cells which express caspase 12. Using thapsigargin, we showed that calnexin-deficient mouse embryonic fibroblasts are relatively resistant to ER stress-induced apoptosis with modified apoptosis-induced cleavage of Bap31 and caspase 12 activity. Other inducers of ER stress were also tested, including tunicamycin, an inhibitor of N-linked glycosylation, Brefeldin A, an inhibitor of the ER−Golgi transport, and staurosporine, a protein kinase C inhibitor. Results from the ER stress-inducing agents (tunicamycin and Brefeldin A) were similar to those for thapsigargin treatment (results not shown). Staurosporine does not induce ER stress, and therefore, it did not induce Bap31 cleavage (results not shown). We also demonstrated that the caspase 12-like protein observed in the human CEM and NKR cells and in human A549 lung carcinoma cells (*14*) behaves in a manner similar to that of caspase 12 found in mouse cell lines, implying that some human cell lines may contain caspase 12.

In a previous study, we showed that changes in the expression of calreticulin, an ER resident homologue of calnexin, affect the sensitivity of a cell to thapsigargin-induced apoptosis (*24*). Earlier investigations of human T

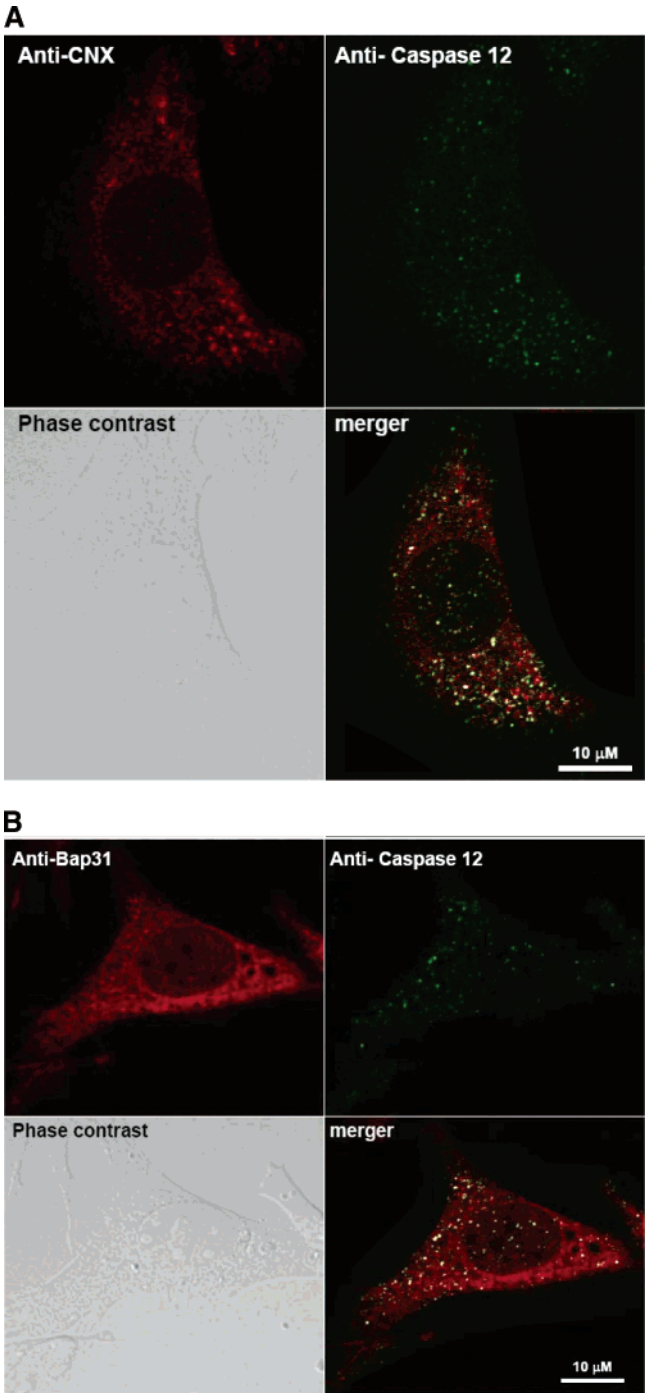

FIGURE 7: Immunolocalization of calnexin and caspase 12 and of Bap31 and caspase 12. The indirect immunofluorescence of wild-type (8WT) cells was determined as described in Experimental Procedures. In panel A, fixed and permeabilized cells were incubated with rabbit anti-calnexin (red) and rat anti-caspase 12 (green) antibodies, with the merged image indicating 67.1 ± 1.6% colocalization of caspase 12 positive staining with calnexin positive staining. In panel B, fixed and permeabilized cells were incubated with rabbit anti-Bap31 (red) and rat anti-caspase 12 (green) antibodies, with the merged image indicating 81.32 ± 3.3% colocalization of caspase 12 positive staining with Bap31 positive staining. The merged image indicates colocalization as designated in white. Data are means ± the standard error of three or more independent experiments. The colocalization coefficient value was 0.5565 for caspase 12 and calnexin (A) and 0.5495 for caspase 12 and Bap31 (B).

cell lines deficient in calnexin also indicated that calnexin might play a role in apoptotic pathways (*7*). Here we demonstrate by monitoring the processing of caspase 12 that this protein is involved in apoptosis induced by specific ER stress in both human T cells and mouse cells. Most importantly, we established that caspase 12 forms a complex with Bap31, an ER-associated protein which is involved in apoptosis. Bap31 is an integral membrane protein of the ER, which has a "death effector" cytoplasmic domain that is cleaved by caspase 8 or related caspases (*8*). The resultant p20 fragment, when expressed ectopically, induces apoptosis either by amplifying a caspase 8-initiated protease cascade (*25*, *26*) or by contributing to other pathways. The ER membrane contains a complex of Bcl-2/Bcl-XL, procaspase 8, and Bap31 (*8*, *21*), which might be involved in an apoptotic signal transduction pathway, similar in manner to the plasma membrane apoptotic receptors Fas and TNFR-1 (*27*). Also, proximity-induced activation (*28*) may represent how caspase 12 is activated, and the activated caspase 12 may be responsible for the cleavage of Bap31 under conditions of ER stress. Identification of the caspase responsible for Bap31 cleavage will take further study. In previous immunoprecipitation experiments, we have shown an interaction between Bap31 and calnexin (*7*). Here, we show that caspase 12 also interacts with calnexin and Bap31. A significant portion of caspase 12 is associated with the ER: 67.1 ± 1.6% of caspase 12 colocalized with calnexin (Figure 7A) and 81.3 ± 3.3% of caspase 12 colocalized with Bap31 (Figure 7B) in mouse fibroblasts. This subpopulation likely represents caspase 12−Bap31−calnexin complexes restricted to the ER membrane. Together, these data suggest that the Bap31 complex (*21*) might also include calnexin and caspase 12, which assist in the functioning of the signaling complex that triggers apoptosis in response to ER stress, or that caspase 12, Bap31, and calnexin may form a novel complex specifically activated under conditions of ER stress, which will take further study.

A deficiency in calnexin along with inhibition of caspases appears to lead to a disruption in the recovery from high cytoplasmic Ca^{2+} levels, implying a role for calnexin in either the signaling to these plasma membrane transporters or the chaperoning of these membrane proteins, directly affecting their function. Calnexin has been specifically linked to a number of plasma membrane proteins, such as the insulin receptor (*29*), the low-density lipoprotein receptor (*30*), the cystic fibrosis transmembrane regulator (*31*), and the voltage-gated Kv1 transporter (*32*).

Our work indicates that in the absence of calnexin, there is a disruption in the transduction of the apoptosis signal. Interestingly, even though thapsigargin-induced programmed cell death is significantly reduced in calnexin-deficient cells (*7*), the activation of caspase 12 and caspase 3 was not altered. A previous study, looking at apoptosis induced by photodynamic therapy, suggested that processing of caspase 8 and Bap31 might occur downstream of caspase 3 activation (*23*). However, we found that caspase 8 is not activated in calnexin-deficient human leukemic T cells (NKR). Despite these changes, thapsigargin treatment induces mitochondrial apoptotic pathways normally (with the consequent release of cytochrome *c* from the mitochondria) in both parental and calnexin-deficient cell lines (*7*). Our findings support the idea that calnexin plays a role in sensing and modulating the sensitivity of a cell to apoptosis induced by ER stress in conjunction with caspase 12 and Bap31. The interaction among Bap31, calnexin, and caspase 12 must be important in the apoptotic cascade stimulated by ER stress in two ways: (1) in perception of ER stress with the consequent activation of caspase 12 and (2) in the cleavage of Bap31 followed by generation of the apoptotic p20 fragment and stimulation of the mitochondrial pathway. The human leukemia T cells as well as the mouse embryonic fibroblasts are able to perceive the signal that results from thapsigargin treatment and initiate the programmed cell death cascade, in particular activating the mitochondrial pathway. However, calnexin deficiency apparently affects this apoptotic cascade by blocking the cleavage of Bap31. Calnexin may be a link sensing misfolded proteins and ER stress which allows activation of caspase 8 or caspase 12 and the consequent cleavage of Bap31 to produce the apoptosis inducer fragment p20. Our results are consistent with the view that calnexin, Bap31, and caspase 12 may form functional complexes which play an important role in ER stress-induced apoptosis.

NOTE ADDED AFTER ASAP PUBLICATION

In the version published October 17, 2006, Figure 1 was partially eclipsed by Figure 2. The correct version was published October 23, 2006.

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