Changes in Endoplasmic Reticulum Luminal Environment Affect Cell Sensitivity to Apoptosis

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Abstract. To test the role of ER luminal environment in apoptosis, we generated HeLa cell lines inducible with respect to calreticulin and calnexin and investigated their sensitivity to drug-dependent apoptosis. Overexpression of calreticulin, an ER luminal protein, resulted in an increased sensitivity of the cells to both thapsigargin- and staurosporine-induced apoptosis. This correlated with an increased release of cytochrome c from the mitochondria. Overexpression of calnexin, an integral ER membrane protein, had no significant effect on drug-induced apoptosis. In contrast, calreticulin-deficient cells were significantly resistant to apoptosis and this resistance correlated with a decreased release of cytochrome c from mitochondria and low levels of caspase 3 activity. This work indicates that changes in the lumen of the ER amplify the release of cytochrome c from mitochondria, and increase caspase activity, during drug-induced apoptosis. There may be communication between the ER and mitochondria, which may involve Ca²⁺ and play an important role in conferring cell sensitivity to apoptosis. Apoptosis may depend on both the presence of external apoptosis-activating signals and, as shown in this study, on an internal factor represented by the ER.

Key words: apoptosis • calreticulin • endoplasmic reticulum • calcium-binding protein

Introduction

The ER plays a critical role in a variety of processes including the maintenance of intracellular Ca²⁺ homeostasis, synthesis, posttranslational modification and folding of membrane and cytosolic proteins. The ER is involved in the regulated expression and cell cycle progression (Pozzan et al., 1994). Evidence now indicates that changes in intracellular Ca²⁺ homeostasis also play a role in modulation of apoptosis (Oppenheimer, 1991; Barres et al., 1992; Preston and Berlin, 1992; Baffy et al., 1993; Lam et al., 1994; Distelhorst et al., 1996; Marin et al., 1996; McConkey et al., 1996; Reynolds and Eastman, 1996; Szalai et al., 1999), a naturally occurring form of cell death which is important for proper development and homeostasis in many tissues (Jacobson et al., 1997; Nagata, 1997).

Calreticulin is a major Ca²⁺-binding chaperone found in the lumen of the ER (Michalak, 1996; Krause and Michalak, 1997; Meldolesi and Pozzan, 1998; Michalak et al., 1999). It is also involved in the regulation of intracellular Ca²⁺ homeostasis, steroid-sensitive gene expression and cell adhesion (Krause and Michalak, 1997; Michalak et al., 1999). Calreticulin shares amino acid sequence identity with calnexin, an integral ER membrane chaperone (Bergeron et al., 1994; Hammond and Helenius, 1995). Calnexin and calreticulin are both chaperones believed to play a critical role in quality control processes during protein synthesis and folding (Bergeron et al., 1994; Hammond and Helenius, 1995).
Calnexin may also play a role in control of Ca\textsuperscript{2+} homeostasis (John et al., 1998) recently.  John et al. (1998) reported that calreticulin and calnexin may interact with SERCA 2b resulting in a lower capacity for Ca\textsuperscript{2+} transport by the Ca\textsuperscript{2+}-ATPase.  In this study, we have investigated the possible role of the ER in modulation of apoptosis. We altered the expression of calreticulin and calnexin, ER luminal and integral membrane proteins, and then initiated apoptosis. We show that increased expression of the ER luminal protein calreticulin increased cell sensitivity to apoptosis, whereas overexpression of calnexin, an integral ER membrane protein, had no significant effect on the drug-induced apoptosis. Furthermore, we show that calreticulin-deficient cells, from calreticulin knockout mice, were significantly resistant to staurosporine-induced apoptosis. This resistance to apoptosis was accompanied by reduced cytochrome c release from mitochondria and lower caspase 3 enzyme activity. Our findings suggest that the ER, via specific components of its luminal environment, may play an important role in the modulation of cell sensitivity to apoptosis.

**Materials and Methods**

**Materials**

\[^{3}H\]thymidine and \[^{4}C\]Ca\textsuperscript{2+} were from A mersham Pharmacia B iotech. H.e.L a Tet-On cells, pTRE and pTK-Hyg plasmids, fetal bovine serum for the Tet system, doxycycline, hygromycin B, and FITC-labeled A nnexin-V were purchased from CLR NTECH Laboratories, Inc. D.ME, fetal bovine serum, trypsin-EDTA, \(-\)glutamine, penicillin, streptomycin, G418 (Geneticin) and RPMI 1640 were purchased from GIBCO BRL. Staurosporine, bradykinin and thapsigargin were from Sigma. Fura-2 AM was from Molecular Probes. In Situ Cell Death Detection for the TUNEL assay (TdT-mediated dUTP nick end labeling) was from Boehringer. Restriction endonuclease and DNA-modifying enzymes were obtained from Boehringer, GIBCO BRL, and Bio/Can Scientific. Goat anti-calreticulin antibodies were prepared as described previously (Milner et al., 1991). A rabbit anti-calnexin antibodies were purchased from StressGen. FITC-conjugated secondary antibodies were from Bio/Can Scientific. A nticytochrome c antibody (7H8.2C12) was from PharMingen. A c-DEVAD- AFC was from BioMol. All chemicals were of the highest grade available.

**Plasmids**

cDNA encoding full-length rabbit calreticulin and canine calnexin was subcloned into the pTRE plasmid to generate pTRE-CRT and pTRE-CNX expression vectors, respectively. These were used for generation of the Tet-On-inducible cell lines. The nucleotide sequence of all cDNAs was confirmed by the DNA Sequencing Laboratory in the Department of Biochemistry, using an Applied Biosystems DNA/RNA synthesizer. To initiate glucocorticoid-dependent apoptosis, cells were incubated for 12 h in RPMI containing 3% charcoal-treatment fetal calf serum, followed by incubation for 24 h in RPMI containing 25-500 nM dexamethasone (Dex) or pRML alone.

Hela and Tet-On cells were maintained in DME supplemented with 10% fetal bovine serum, 100 U penicillin/ml, 100 \mu g DME streptomycin/ml, and 2 mM \(-\)glutamine. DME was also supplemented with 10% tetracycline-negative fetal bovine serum for Tet system. 2 \mu g Dox/ml was added into the culture medium to induce expression of calreticulin or calnexin in Tet-On cell lines.

Mouse embryonic fibroblasts (MEF) were isolated from calreticulin-deficient crt\textsuperscript{\textasciitilde} and wild-type embryos. crt\textsuperscript{\textasciitilde} and wild-type embryos were dissociated, trypsinized for 30 min, and cultured in 6-well tissue-culture plates. To generate immortal MEF cell lines, cells were transfected with a pSV-7 encoded sequence of the SV-40 large T antigen (Conzen and Cole, 1995). A vector 2 wk in culture individual colonies of immortalized MEF were picked and used for experiments. Cells were maintained in DME containing 20% fetal calf serum. To induce apoptosis, cells were incubated with 2 \mu M, 100 nM, or 10 nM staurosporine for 90 min. This treatment was followed with an A nnexin-V binding assay, which was carried out as recommended by the manufacturer and always including propidium staining assay.

**Immunoblotting and Immunocytochemistry**

Cells were washed three times with ice-cold PBS and then lysed as described by (Mery et al., 1996). Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose membrane (Towbin et al., 1979). Goat anti-calreticulin and rabbit anti-calreticulin (affinity-purified CRT283) were prepared as described earlier (Miler et al., 1991; Michalak et al., 1996). Rabbit anti-R P57 antibodies, a generous gift of D. Thomas (Genetics Group, Biotechnology Research Institute, National Research Council of Canada, Montreal, Canada), were used at 1:1,000 (Zapun et al., 1998). Immunoblotting was carried out as described by (Nakamura et al., 1995). For analysis of cytochrome c release from mitochondria, preparation of extracts and immunoblotting was carried out as described by (Bosby-Wetzell et al., 1998; Bosby-Wetzell and Green, 1999). Indirect immunofluorescence of K N1 and K N2 cells was carried out as described by (Opas et al., 1991). In brief, K N1 and K N2 cells were plated on coverslips and cultured in the presence or absence of 2 \mu g Dox/ml for 24 h. Cells were washed with PBS and fixed with 4% paraformaldehyde. Coverslips were mounted in V i nol 2055 and examined with a Bio-Rad Laboratories confocal fluorescence microscope (model MRC-600) equipped with a krypton/argon laser.

**Apoptosis Assay**

For the A nnexin-V binding assay, cells were plated on 6-cm dishes at 5 \times 10^5/cm\textsuperscript{2} and incubated for 24 h in the presence of 2 \mu g DEX: ml for 24 h. Cells were washed with PBS and fixed with 4% paraformaldehyde. Coverslips were mounted in V inol 2055 and examined with a Bio-Rad Laboratories confocal fluorescence microscope (model MRC-600) equipped with a krypton/argon laser.

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duce apoptosis. The cells were washed with PBS, harvested and incubated with the TUNEL reaction mixture, as recommended by the manufacturer. This was followed by flow cytometry or fluorescence microscopy. Caspase 3 activity was measured by the DEVD-AFC cleavage assay (Bossy-Wetzel and Green, 1999). 50 μg protein extract was diluted in a buffer containing 200 mM A c-DEVD-AFC, 20 mM Pipes, pH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, and 10 mM DTT (caspase buffer). The kinetics of substrate cleavage were measured at 37°C under constant agitation using a spectrophotometer at λex = 400 nm and λem, at 505 nm. A poptosis was also quantified by staining cells with eosin and hematoxylin (Bossy-Wetzel et al., 1998). A apoptotic cells were counted using a light microscope. A poptosis was detected by cell shrinkage, chromatin condensation and nuclear fragmentation.

**Ca²⁺ Measurements**

In KNI cells, the total Ca²⁺ in ERCa²⁺ stores was estimated using ⁴⁰Ca²⁺ (10 μCi/ml) as described earlier (Mery et al., 1996). For measurement of cytoplasmic Ca²⁺ concentration ([Ca²⁺]cyt), cells (1.5 x 10⁶/ml) were loaded with the fluorescent Ca²⁺ indicator fura-2/AM (2 μM) taking precautions to avoid dye sequestration as described by (Mery et al., 1996). Fura-2 fluorescence was measured at λex = 340 nm. To measure changes in cytoplasmic Ca²⁺ concentration, cells were stimulated with 1 μM thapsigargin or with 200 nM bradykinin (Mery et al., 1996; Waser et al., 1997).

**Miscellaneous**

Protein assays were carried out as described by (Bradford, 1976). All recombinant DNA techniques were conducted according to standard protocols. Transgenic mice expressing the GFP reporter gene under control of the calreticulin promoter were generated as described (Mesaeli et al., 1999). Mouse embryos were dissected, fixed and prepared for fluorescence microscopy (Mesaeli et al., 1999). Images were reconstructed using the Adobe Photoshop program.

**Results**

A poptosis is an active and genetically controlled process of eliminating unwanted cells during development, tissue turnover and metamorphosis (Jacobson et al., 1997). While studying transactivation of the calreticulin promoter and expression of the protein during mouse embryogenesis we noticed that the calreticulin promoter was highly active in several tissues including cells destined for apoptosis during mouse development. We monitored transactivation of the calreticulin promoter by detection of the fluorescent signal obtained from calreticulin promoter-driven expression of the green fluorescent protein (GFP) reporter gene in transgenic mice (Mesaeli et al., 1999). Fig. 1, A and B, shows section of a developing eye (A) and a limb bud (B) of a 14.5-d-old transgenic mouse embryo. The highest fluorescent signal, indicative of high expression of GFP, was found in the central retina (Fig. 1 A, R), the lens vesicle (Fig. 1 A, LV) and in the interdigital cells (Fig. 1 B, IC). It is well established that central retina and lens vesicle cells undergo apoptosis during normal development (Ganan et al., 1996; Zou and Niswander, 1996). A poptosis is also responsible for eliminating the cells between developing digits (Ganan et al., 1996; Zou and Niswander, 1996). Furthermore, in preliminary biochemical experiments with A11 cells (a cloned cell line derived from T cells), we found that when we downregulated calreticulin, using anti-sense oligodeoxynucleotides (Fig. 2 A), there was a protective effect against Dex-induced apoptosis as assessed by determining the degree of DNA fragmentation (Fig. 2 B). Since calreticulin is involved in Ca²⁺ homeostasis these findings are in line with earlier studies which showed that Ca²⁺ release from ER may affect Dex-induced apoptosis in W7MG1 mouse lymphoma cells (Lam et al., 1993) or T lymphocytes (Jayaraman and Marks, 1997). Taken together, these findings suggested to us that changes in expression of calreticulin, an ER luminal protein, may play a role in cell sensitivity to apoptosis induced by external stimuli.
Expression of Calreticulin and Calnexin in Tet-On–inducible HeLa Cells

To investigate the role of calreticulin and calnexin on cell sensitivity to apoptosis, we generated Tet-On HeLa cell lines that are inducible with respect to calreticulin (designated KN1) and calnexin (designated KNX2). In Tet-On cells, gene expression is turned on when doxycycline (Dox) is added to the culture medium. Incubation of the KN1 and KNX2 cells with Dox resulted in 2.3 ± 0.2-fold (mean ± SD; n = 4) and 2.2 ± 0.2-fold (mean ± SD; n = 4) induction in the expression of calreticulin (Fig. 3 A) and calnexin (Fig. 3 B), respectively. A's internal control we tested for expression of ER p57, an E R luminal chaperone, in KN1 and KNX2 cells. Fig. 3, A and B, shows that Dox had no effect on expression of ER p57. Expression of other E R proteins including BiP, E Rp72, protein disulfide isomerase, Grp94, SERCA2, and InsP3 receptor was also not affected by Dox (not shown). The doubling time of these cell lines was ~20 h and was not affected by the addition of Dox. After induction of protein synthesis with Dox in KN1 and KNX2 cells calreticulin and calnexin were both localized to the E R (Fig. 3 C). There was no immunoreaction with anti-calreticulin or anti-calnexin anti-bodies in the cytoplasm, in the nucleus, or on the cell surface. This indicates that the Dox-dependent induction of calreticulin and calnexin resulted in an increased accumulation of these proteins in the E R and not in other intracellular compartments.

Induction of Apoptosis in KN1 and KNX2 Cells

Staurosporine (Raff et al., 1993; Bertrand et al., 1994; Jacobson et al., 1994) and thapsigargin (Lam et al., 1994) both induce apoptosis. To investigate the possible involvement of calreticulin and E R in apoptosis, we treated KN1 and KNX2 cells with Dox to induce overexpression of calreticulin and calnexin, respectively. We then incubated the cells with either thapsigargin or staurosporine, and measured apoptosis by A nexin-V binding or TUNEL assay. By itself, Dox did not induce apoptosis in HeLa cells, mock transfected control cells, or KN1 and KNX2 cells (Figs. 4 and 5, HeLa-On). When thapsigargin was used to induce apoptosis, we found that cells overexpressing calreticulin were more sensitive. A s shown in Fig. 4, after treatment with thapsigargin, A nixin-V binding was greater in cells that were overexpressing calreticulin. Fig. 5 A shows that after treatment with staurosporine, A nixin-V binding was also increased in Dox-treated KN1 cells as compared with untreated KN1 cells. T his shows that the cells overexpressing calreticulin were more sensitive to staurosporine. Similar results were obtained with 2 μM and 10 nM staurosporine (data not shown). In contrast, in KNX2 cells overexpressing calnexin, a small and statistically insignificant reduction in sensitivity to both thapsigargin (Fig. 4) and staurosporine (Fig. 5 A) was observed. These results indicate that the overexpression of calnexin did not affect apoptosis.

The sensitivity of KN1 and KNX2 cells to staurosporine was also evaluated by TUNEL analysis (Fig. 5 B). In keeping with the A nixin-V binding results described above, we found that Dox-dependent overexpression of calreticulin increased the sensitivity of KN1 cells to apoptosis (Fig. 5 B). A fer incubation with staurosporine, ~30% of cells overexpressing calreticulin (Dox-treated KN1) were TUNEL positive, compared with only 18% of control cells. In contrast, Dox-dependent overexpression of calnexin did not affect the sensitivity of KNX2 cells to apoptosis. A fter treatment with staurosporine, the proportion of cells that were TUNEL positive was the same in Dox-treated and control cells.

We also carried out a time course analysis of staurosporine-induced apoptosis in KN1 cells overexpressing calreticulin (Fig. 5, C and D). Increased sensitivity of KN1 cells overexpressing calreticulin to staurosporine-induced apoptosis was observed after 0–4 h of incubation with the drug (Fig. 5, C and D). Time course for both A nixin-V binding (Fig. 5 C) and the appearance of TUNEL-positive cells (Fig. 5 D) were comparable indicating that overexpression of calreticulin likely affected the rate of apoptosis rather than the sequence of events associated with apoptosis.

Our results show that KN1 cells are more sensitive to apoptosis when they are overexpressing calreticulin. To determine whether or not apoptosis was occurring via similar pathways in control and test cells, we measured cyto-
Overexpression of calreticulin was induced with Dox, and then apoptosis was induced by treatment with staurosporine or etoposide. We prepared cell extracts, which were devoid of mitochondria, and then we assessed cytochrome c levels by immunoblotting. In KN1 cells treated with staurosporine or etoposide there was a detectable release of cytochrome c from the mitochondria (Fig. 5E). In untreated KN1 cells there was no release of cytochrome c from the mitochondria (Fig. 5E). In KN1 cells first incubated with Dox...
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**Figure 4.** Thapsigargin-dependent induction of apoptosis in cells overexpressing calreticulin and calnexin. KN1 and KNX2 cells were incubated with 2 μg Dox/ml (filled bars) for 24 h to induce expression of calreticulin and calnexin, respectively. Cells were treated with thapsigargin followed by Annexin-V binding assay. HeLa-O, mock transfected Tet-On HeLa cells. (Open bars) Cells not incubated with Dox; (filled bars) Dox-treated cells. 100% value corresponds to 10,000 cells. Data are means ± SD of three independent experiments. **P < 0.001.

(overexpressing calreticulin) and then treated with staurosporine or etoposide, the release of cytochrome c from the mitochondria was consistently higher (Fig. 5 E, + Dox). To confirm the differences we had found in Annexin-V binding, TUNEL assay and cytochrome c release from mitochondria, we measured rates of DEVD cleavage (caspase activity) in cytosolic extracts from KN1 cells overexpressing calreticulin. Calreticulin expression was induced with Dox followed by a 60-min incubation with staurosporine to induce apoptosis. As expected the DEVD cleavage (caspase activity) was 1.75-fold higher in calreticulin overexpressing (Dox- and staurosporine-treated) KN1 cells than in the KN1 cells incubated with staurosporine alone.

**Ca²⁺ Homeostasis in KN1 Cells**

It is well documented that calreticulin plays a role in the regulation of intracellular Ca²⁺ homeostasis (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998; Masaalil et al., 1999). Therefore, we investigated the effect on Ca²⁺ homeostasis of overexpressing calreticulin in KN1 cells. First, we used equilibrium loading techniques to determine whether the Ca²⁺ content of intracellular Ca²⁺ stores was modified in KN1 cells that were overexpressing calreticulin (Mery et al., 1996). Control KN1 cells contained 70 ± 10 pmol of Ca²⁺/10⁶ cells (mean ± SD; n = 3) whereas the KN1 cells treated with Dox (overexpressing calreticulin) contained 148 ± 12 pmol of Ca²⁺/10⁶ cells (mean ± SD; n = 3). Similar increases in the Ca²⁺ content of intracellular Ca²⁺ stores have been reported for cells transiently (Bastianutto et al., 1995) and stably (Mery et al., 1996) transfected with a calreticulin expression vector.

Second, we used a Ca²⁺-sensitive fluorescent dye, fura-2, to investigate the effects of overexpressing calreticulin on [Ca²⁺]c. In KN1 cells, basal cytoplasmic Ca²⁺ concentration ([Ca²⁺]c) in control and Dox-treated KN1 cells was similar (∼130 ± 10 nM; mean ± SD; n = 3). To measure [Ca²⁺]c, we used thapsigargin, an inhibitor of SERCA (Thastrup et al., 1990). When cells were treated with thapsigargin, the peak and duration of the [Ca²⁺]c elevations were comparable in control and Dox-treated KN1 cells (not shown). In contrast to this, we found that control and Dox-treated KN1 cells differed in their response to bradykinin. Bradykinin causes the release of Ca²⁺ from internal stores via activation of endogenous, agonist-dependent pathways. In both control and Dox-treated KN1 cells, bradykinin caused a rapid and transient increase in [Ca²⁺]c. However, the amplitude of this [Ca²⁺]c elevation was ∼1.5-fold greater in cells overexpressing calreticulin (620 ± 25 nM; mean ± SD; n = 3) than in control cells (350 ± 20 nM; mean ± SD; n = 3). It remains unclear why increased elevation of [Ca²⁺]c in KN1 cells overexpressing calreticulin was observed with bradykinin but not with thapsigargin. It is possible, that in the case of thapsigargin the slow Ca²⁺ release allows negative feedback.

**Figure 5.** Induction of apoptosis in cells overexpressing calreticulin and calnexin. KN1 and KNX2 cells were incubated with 2 μg Dox/ml (filled bars) for 24 h to induce expression of calreticulin and calnexin, respectively. Cells were treated with staurosporine (A–D) followed by Annexin-V binding assay (A and C) or TUNEL analysis (B and E). Open bars, cells not incubated with Dox; filled bars, Dox-treated cells. 100% value corresponds to 10,000 cells. Data are means ± SD of three independent experiments. In E, KN1 cells were incubated with staurosporine for the time indicated in the figure followed by Annexin-V binding assay (C) or TUNEL analysis (D). Control KN1 cells contained 10,000 cells. Data are means ± SD of three independent experiments. In E, KN1 cells were incubated with 100 nM staurosporine (STS) or 100 μM etoposide. Cytosolic extracts were prepared devoid of mitochondria, separated by SDS-PAGE and immunoblotted with anti-cytochrome c antibodies (Bossy-Wetzel and Green, 1999). The position of cytochrome c (Cyt. c) is indicated by the arrow. **P < 0.001 and *P < 0.005.
mechanism such as extrusion of Ca\(^{2+}\) via the plasma membrane Ca\(^{2+}\)-ATPase to obscure the differences in Ca\(^{2+}\) release in relation to the level of [Ca\(^{2+}\)] sub.

In summary, we show that Dox-dependent overexpression of calreticulin in KN1 cells results in increased storage of Ca\(^{2+}\) in the ER and in elevated agonist-dependent Ca\(^{2+}\) release from internal stores. In contrast, we found no significant changes in total intracellular Ca\(^{2+}\) or in Ca\(^{2+}\) release in KNX2 cells overexpressing calnexin.

**Induction of Apoptosis in Calreticulin-deficient Cells**

Recently, we have generated a calreticulin knockout embryo (Mesaeli et al., 1999). For this study we established an immortalized calreticulin-deficient, MEF cell line (for details see Materials and Methods). Since crt\(^{-/-}\) cells do not express calreticulin (Fig. 6 A) they provide an ideal tool to investigate the effects of calreticulin deficiency on apoptosis. Fig. 6 B compares staurosporine-induced apoptosis in wild-type cells (containing calreticulin) and calreticulin-deficient cells (crt\(^{-/-}\)), as measured by Annexin-V binding. Interestingly, the calreticulin-deficient cells were significantly resistant to apoptosis (Fig. 6 B). We also performed a kinetic analysis of apoptosis induced by etoposide, staurosporine and UV (UVB; Fig. 6 C). Fig. 6 C shows that calreticulin-deficient cells were consistently more resistant to apoptosis compared with the wild-type cells.

In parallel with these studies, we also tested the effects of calreticulin deficiency on Ca\(^{2+}\) homeostasis, using the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. We reported earlier (Mesaeli et al., 1999) that in wild-type and calreticulin-deficient MEF resting [Ca\(^{2+}\)] sub. was similar (80 ± 15 nM; mean ± SD; n = 3). When cells were treated with thapsigargin the peak and duration of the [Ca\(^{2+}\)] sub. elevations were comparable in control and calreticulin-deficient MEF (not shown; Mesaeli et al., 1999). In contrast, bradykinin-dependent Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores was inhibited in calreticulin-deficient MEF (Mesaeli et al., 1999).

To determine whether apoptosis was occurring by similar pathways in wild-type and calreticulin-deficient cells, we measured cytochrome c release from the mitochondria. Wild-type and calreticulin-deficient MEF were treated with UV, etoposide or staurosporine, and then cytosolic extracts, devoid of mitochondria, were prepared and analyzed by immunoblotting. As expected, in untreated cells there was no release of cytochrome c from the mitochondria (Fig. 7 A, untreated). Treatment of wild-type MEF with UV, etoposide or staurosporine resulted in cytochrome c release from the mitochondria (Fig. 7 A, +/-). However, cytochrome c release from mitochondria of calreticulin-deficient cells, after treatment with UV, etoposide or staurosporine, was reduced (Fig. 7 A, -/-). During apoptosis, cytochrome c release from mitochondria activates caspase 9, which then cleaves and activates procaspase 3 (Srinivasula et al., 1998). To confirm the differences we had found in cytochrome c release from mitochondria, we measured rates of DEVD cleavage in cytosolic extracts, using the fluorometric substrate DEVD-AFC. We treated wild-type and calreticulin-deficient MEF with UV, etoposide or staurosporine, and then we prepared cytosolic extracts from the cells. No caspase activity was detected in untreated cells (Fig. 7 B, untreated). DEVD cleavage (caspase) activity was high in wild-type MEF treated with UV or etoposide (Fig. 7 B, wt), but was reduced in the calreticulin-deficient cells (Fig. 7 B, crt\(^{-/-}\)). There was no detectable DEVDase activity in the staurosporine-treated crt\(^{-/-}\) (Fig. 7 B). However, there was significant release of cytochrome c from mitochondria of crt\(^{-/-}\)-treated with staurosporine (Fig. 7 A). This apparent dis-
crepancy is not surprising since mitochondrial cytochrome c release does not always depend on caspase activity in many apoptotic systems (Xiang et al., 1996; Yucheng et al., 2000).

Discussion

In this study, we found that overexpression of calreticulin increased cell sensitivity to both thapsigargin-induced apoptosis and staurosporine-induced apoptosis. However, the overexpression of calnexin, another Ca\(^{2+}\)-binding chaperone of the ER, had no significant effect on drug-induced apoptosis. Conversely, we found that cells lacking calreticulin were considerably resistant to drug-induced apoptosis. Conversely, we found that cells lacking calreticulin had no significant effect on drug-induced apoptosis. This is likely due to the fact that ER luminal environment in general and calreticulin-deficient cells bradykinin-dependent elevation of [Ca\(^{2+}\)] was inhibited (Mesaeli et al., 1999). We found that calnexin-deficient MEF do not have a measurable InsP\(_3\) receptor pathway. Camacho’s group has proposed that calreticulin may modulate the function of the InsP\(_3\) receptor and/or SERCA (Camacho and Lechleiter, 1995). Recently, John et al. (1998) reported that calreticulin may interact with SERCA 2b, resulting in a lower transport capacity for the Ca\(^{2+}\)-ATPase. We have shown that changes in Ca\(^{2+}\) homeostasis, particularly agonist-dependent Ca\(^{2+}\) release when stimulated with bradykinin (Mesaeli et al., 1999), indicating that calreticulin plays a role in modulating InsP\(_3\) receptor pathway. Calreticulin is a ubiquitous Ca\(^{2+}\)-binding protein, which is located in the ER and which has been implicated in such diverse functions as regulation of intracellular Ca\(^{2+}\) homeostasis, chaperone activity, gene expression and cell adhesion (Michalak et al., 1999). Given the diversity of its activities, there are many routes via which calreticulin might affect cell sensitivity to drug-induced apoptosis. In this study, we have shown that the lectin-like activity of calreticulin is probably not involved. The ER proteins calreticulin, calnexin, and calreticulin share many chaperone functions; both are lectin-like chaperones which preferentially bind monoglycosylated oligosaccharides (Berg et al., 1994; Sprio et al., 1996; Hellenius et al., 1997; Vassilakos et al., 1998) and misfolded proteins (Ihara et al., 1999; Saito et al., 1999). We found that altered expression of calnexin had no significant effect on cell sensitivity to apoptosis. It is, therefore, unlikely that calreticulin’s chaperone activity shared with calnexin is involved in the observed changes in cell sensitivity to apoptosis. However, it cannot be ruled out that calreticulin chaperone activity towards a calreticulin-specific substrate may also play a role in modulation of cell sensitivity to apoptosis.

Calreticulin is a Ca\(^{2+}\)-binding protein that modulates intracellular Ca\(^{2+}\) homeostasis (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998; Mesaeli et al., 1999) and Ca\(^{2+}\) plays an important role in apoptosis (Preston and Berlin, 1992; Baffy et al., 1993; Lam et al., 1994; Distelhorst et al., 1996; Marin et al., 1996; M cConkey et al., 1996; Reynolds and Eastman, 1996). Differential expression of calreticulin affects Ca\(^{2+}\) storage capacity of the ER, and most importantly, it modulates Ca\(^{2+}\) release from the ER (Liu et al., 1994; Bastianutto et al., 1995; Camacho and Lechleiter, 1995; Mery et al., 1996; Coppolino et al., 1997; Fasolato et al., 1998; John et al., 1998; Mesaeli et al., 1999). For example, we found that overexpression of calreticulin in the Tet-On inducible system led to increased Ca\(^{2+}\) storage in the ER, and to increased elevation of [Ca\(^{2+}\)] in response to bradykinin. Furthermore, we have previously found that calreticulin-deficient MEF do not have a measurable InsP\(_3\)-dependent Ca\(^{2+}\) release when stimulated with bradykinin (Mesaeli et al., 1999), indicating that calreticulin plays a role in modulating InsP\(_3\) receptor pathway. Camacho’s group has proposed that calreticulin may modulate the function of the InsP\(_3\) receptor and/or SERCA (Camacho and Lechleiter, 1995). Recently, John et al. (1998) reported that calreticulin may interact with SERCA 2b, resulting in a lower transport capacity for the Ca\(^{2+}\)-ATPase. We have shown that changes in Ca\(^{2+}\) homeostasis, particularly agonist-dependent Ca\(^{2+}\) release from the ER of calreticulin-deficient cells, correlate well with changes in cell sensitivity to staurosporine-induced apoptosis.

One most important implication of this current work is that changes in the lumen of the ER modify the mitochondrial cytochrome c/caspase apoptosis signaling pathway. For example, we found that, when cells are induced to undergo apoptosis, cytochrome c release from mitochondria and cytosolic caspase activity are both lower in calreticulin-deficient cells than in wild-type cells. Rizzuto et al. (1998) have shown that there are numerous close contacts between the ER and mitochondria, and that opening of the InsP\(_3\)/Ca\(^{2+}\) channel in the ER affects Ca\(^{2+}\) homeostasis in mitochondria. Close proximity between ER and mitochondria may play a role in the regulation of Ca\(^{2+}\) signaling (Rizzuto et al., 1998) and cell sensitivity to apoptosis.

There is a growing body of evidence that the ER may play an important role in the apoptotic signaling processes, which lead to downstream activation of caspases and other proteases. For example, under normal conditions, Bcl-2 and Bcl-X\(_L\) colocalize to the mitochondria and ER (Zhu et
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