The Endoplasmic Reticulum Chaperone Glycoprotein GRP94 with Ca\(^{2+}\)-binding and Antiapoptotic Properties Is a Novel Proteolytic Target of Calpain during Etoposide-induced Apoptosis*

(Received for publication, April 6, 1999, and in revised form, July 13, 1999)

Ramachandra K. Reddy, Jun Lu, and Amy S. Lee‡

From the Department of Biochemistry and Molecular Biology and the USC/Norris Comprehensive Cancer Center, University of Southern California School of Medicine, Los Angeles, California 90033

GRP94 is a 94-kDa chaperone glycoprotein with Ca\(^{2+}\)-binding properties. We report here that during apoptosis induced by the topoisomerase II inhibitor etoposide, a fraction of GRP94 associated with the endoplasmic reticulum membrane undergoes specific proteolytic cleavage, coinciding with the activation of the caspase CPP32 and initiation of DNA fragmentation. In vitro, inhibitors of caspases able to block etoposide-induced apoptosis can only partially protect GRP94 from proteolytic cleavage, whereas complete inhibition is observed with calpain inhibitor I but not with the proteasome inhibitor. In vitro, GRP94 is not a substrate for CPP32; rather, it can be completely cleaved by calpain, a Ca\(^{2+}\)-regulated protease. The cleavage of GRP94 by calpain is Ca\(^{2+}\)-dependent and generates a discrete polypeptide of 80 kDa. In contrast, calpain has no effect on other stress proteins such as GRP78 or HSP70. Further, immunohistochemical staining reveals specific co-localization of GRP94 with calpain in the perinuclear region following etoposide treatment. We further showed that reduction of GRP94 by antisense decreased cell viability in etoposide-treated Jurkat cells. Our studies provide new evidence that the cytoprotective GRP94, as in the case of the antiapoptotic protein Bcl-2, can be targets of proteolytic cleavage themselves during the apoptotic process.

Programmed cell death, or “apoptosis” is a fundamental process in multicellular organisms for normal development as well as the maintenance of homeostasis (1). Cellular stress causes severe constraints on numerous physiological functions, damages cellular macromolecules and structures, and frequently leads to cell death. To understand the in vivo regulation of apoptosis, it is important to identify proteins that can protect cells from undergoing cell death. As part of their program to escape host destruction, many viruses express proteins that protect the infected cells from cell death (2, 3). However, little is known about cellular proteins that can confer resistance to apoptosis. To date, the best characterized endogenous cellular proteins known to be able to inhibit apoptosis induced by a variety of stimuli are Bcl-2, which is an integral intracellular membrane protein, and related members of the Bcl-2/Ced9 family (4).

Cells respond to environmental or physiological stress by adaptive changes that include the induction of a set of heat shock proteins (HSPs)\(^1\) or the glucose-regulated proteins (GRPs) (5–7). Depending on the target of the stress-inducer, the HSPs and GRPs can be induced separately, simultaneously, or reciprocally (8, 9). Evidence is emerging that these stress proteins represent a novel class of apoptosis regulators that when expressed at high levels can protect the host cell against cell death (10–16). The function of the HSPs and GRPs as molecular chaperones are well documented as they participate in protein translocation, protein folding and assembly, and regulation of protein secretion (17). Further, it has been proposed that the ER-localized GRPs such as GRP94 and GRP78 with Ca\(^{2+}\)-binding properties (18, 19) protect cells against flux in Ca\(^{2+}\) homeostasis, which could result in cell death (20–24).

Despite these significant advances, the relationship between the GRPs and the apoptotic machinery is not known. While the central role of mitochondria in initiating cell death has been widely studied (25), the involvement of the ER in the apoptotic process is not well understood. Apoptosis is triggered by the activation of several intracellular cysteine proteases of the interleukin-converting enzyme family related to caspases (26). Many of the protein targets identified so far during apoptosis are involved in morphological and biochemical changes that accompany programmed cell death, or in the sensing of DNA damage and repair (27, 28). Recently, it was discovered that Bcl-2 undergoes specific proteolytic cleavage mediated by the caspases after induction of apoptosis by Fas ligation and interleukin-3 withdrawal (29). Strikingly, the cleavage of Bcl-2 not only inactivates its antiapoptotic function but converts the carboxyl-terminal Bcl-2 cleavage product into a proapoptotic molecule, accelerating Sindbis virus-induced apoptosis.

Calpains are another family of cysteine proteases with two major isoforms (\(\mu\)- and \(m\)-calpain) that are ubiquitously expressed (30, 31). It is believed that these cytoplasmic proteases are activated at the cellular membranes (32). In addition to its role in platelet aggregation, neuronal long-term potentiation, neutrophil activation, and oocyte maturation, calpain also appears to be a necessary upstream regulator for cell death pathways that require new protein synthesis but not those that are independent of new protein synthesis (33). Many putative calpain substrates are cytoskeleton-associated proteins located at or near cellular membranes, while some are nuclear proteins (31–33). Notably, the stability of p53, a major regulator of apoptosis, is regulated by calpain (34). Further, Bax with pro-

\(^1\) The abbreviations used are: HSP, heat shock protein; GRP, glucose-regulated protein; \(z\)-VAD-fmk, benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; \(z\)-DEVD-fmk, benzoylxy-carbonyl-Asp-Glu-Val-Asp-fluoromethylketone; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; ER, endoplasmic reticulum; CMV, cytomegalovirus.
inhibitors

in 2-deoxyglucose, a classical inducer of GRPs, reaffirming previous observations that GRP94 possesses antiapoptotic properties (20, 23). This prompted us to examine the status of stress proteins, in particular GRP94 and GRP78, under apoptotic conditions other than stress conditions targeted to the ER (8). In this report, we focus on etoposide-induced apoptosis in three cell models: the SN cells, the human acute T cell leukemia line, Jurkat, and the hamster fibroblast K12 cell line, where the induction of GRPs has been extensively studied (37). Fortuitously, we discovered that GRP94, but not GRP78, is proteolytically cleaved during etoposide-induced apoptosis. However, in vivo, only a fraction of GRP94 appears to be accessible to calpain cleavage. Its cleavage in vivo is completely blocked by calpain inhibitor I and is partially inhibited by z-VAD-fmk, a general caspase inhibitor. In in vitro assays, calpain but not CPP32 is capable of cleavage of GRP94, with the generation of a 80-kDa carboxyl polypeptide. The cleavage of GRP94 in vitro by calpain is Ca$^{2+}$-dependent and can be reproduced in purified microsome preparations. In contrast, neither GRP78 nor HSP70 are substrates for calpain. We further demonstrate by laser confocal microscopy that during etoposide-induced apoptosis, calpain becomes co-localized with GRP94 in the perinuclear region. The cytoprotective function of GRP94 in etoposide-treated cells is confirmed, since transfection with an antisense vector directed against GRP94 increased cell lethality. Our findings provide new evidence that the antiapoptotic stress protein GRP94 is a direct target of the apoptotic machinery. Our results are consistent with the existence of a subpopulation of GRP94 associated with the ER membrane, where it becomes a specific substrate for calpain cleavage concurrent with the progression of apoptosis induced by DNA damage mediated by etoposide.

**EXPERIMENTAL PROCEDURES**

**Cells and Culture Conditions**—The SN cell line is a human hematopoietic cell line, HL60, stably transfected with the wild type p53 gene (36). The human acute T cell leukemia line, Jurkat, and the SN cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum containing 1% penicillin/streptomycin/neomycin antibiotics. The K12 hamster fibroblast cell line was maintained in Dulbecco’s modified Eagle’s medium containing 4.5 μg/ml glucose supplemented with 10% fetal bovine serum and 1% antibiotics (37).

To induce apoptosis by etoposide (Calbiochem), the cells were exposed to 30 μM etoposide for 1–10 h. To test the effect of caspase inhibitors in vivo, the cells were treated with either 50 μM z-VAD-fmk (Calbiochem) or 2 μM z-DEVD-fmk (Calbiochem) for 3 h. For inhibition of calpain or proteasome activity during apoptosis, the cells were pretreated with 45 μM calpain inhibitor I (Roche Molecular Biochemicals) or 50 μM proteasome inhibitor N-Benzoyloxycarbonyl-Leu-Leu-Valinal (Sigma) for 4 h prior to the subsequent addition of etoposide.

**Cell Lysis and Immunoblotting**—Conditions for preparation of cell lysate and immunoblotting were described as (38). Recombinant GRP78 and antisera against GRP78, GRP94, and HSP70 were purchased from StressGen (Victoria, Canada). Antisera used were I:1000 dilution of the anti-GRP94 or anti-GRP78 monoclonal antibody, 1:10,000 dilution of anti-HSP70 polyclonal antibody, 1:5000 dilution of the anti-β-actin monoclonal antibody (Sigma), or 1:3000 dilution of the anti-CPP32 monoclonal antibody (Transduction Laboratories, Lexington, KY). The rat anti-GRP94 antibody (SPA-850) was raised against purified chicken GRP94. The mouse anti-GRP78 antibody (SPA-827) was raised against the SEKDEL peptide shared by ER luminal proteins. Under our experimental conditions, it recognizes most strongly GRP78 and a 45-kDa protein referred to as X, with minor but detectable reactivity toward GRP94. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Rabbit anti-rat antibody (Cappel-ICN, Costa Mesa, CA) at I:4000 dilution was used against anti-GRP94 antibody; goat anti-rabbit antibody (StressGen) at I:5000 dilution was used against anti-HSP70 antibody; sheep anti-HSP70 antibodies (Cappel-ICN) at I:5000 dilution were used against anti-GRP78, anti-CPP32, and anti-β-actin antibodies. Immune complexes were detected with the ECL system (Amersham Pharmacia Biotech), according to the manufacturer’s instructions. The bands were visualized by autoradiography and quantitated by densitometry using the Bio-Rad Imaging Densitometer.

**DNA Fragmentation Assays**—5 × 10$^4$ cells harvested at different times of drug treatment were incubated at 37 °C in 0.5 ml of a buffer containing 10 mM Tris- HCl, pH 8.0, 100 mM EDTA, 10 mM EGTA, 0.5% (v/v) SDS. DNA-free RNSase A was added (20 μg/ml of lysate) and incubated at 37 °C with gentle shaking, followed by the addition of proteinase K (100 μg/ml of lysate), and a further 4-h incubation at 56 °C. The DNA was extracted with phenol-chloroform and precipitated with ethanol. Five μg of genomic DNA was loaded onto a 1.9% agarose gel. DNA was visualized with ethidium bromide (0.5 μg/ml) under UV light.

**In Vitro Proteolytic Cleavage Assay**—For $^{[35]}$Smethionine-labeled GRP94, 80% confluent Jurkat cells were labeled with 100 μCi of $^{[35]}$Smethionine (NEN Life Science Products) in a 10-cm dish containing 5 ml of methionine-free medium with 10% dialyzed fetal calf serum. After 2 h of labeling, proteins were isolated, and GRP94 protein was immunoprecipitated as described (39). To test CPP32 and calpain cleavage of GRP94 in vitro, 0.1 μg of recombinant CPP32 (gift of Drs. T. Rudel and G. Bokoch; Ref. 40) resuspended in 100 mM HEPES, pH 7.5, 10% sucrose, 0.1% CHAPS, 10 mM dithiothreitol, or 0.2 unit of calpain (Sigma) resuspended in 50 mM Tris, pH 7.4, 1.5 mM β-mercaptoethanol, 5 mM CaCl$_2$, or buffer alone was added to the immunocomplex and kept at room temperature for 30 min. For the cleavage of recombinant GRP78, the same buffer was used with calpain added at 0.2 unit/μg of protein, and the reaction was performed at 30 °C. The incubation was stopped by the addition of equal volumes of 2× Laemmli buffer (1× Laemmli buffer: 50 mM Tris- HCl, pH 6.8, 2.5% (v/v) β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, and 10% glycerol) and resolved on 8% SDS-PAGE.

In other reactions, total lysates prepared from K12 cells were resuspended in radioimmune precipitation buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS). Calpain was added at 0.2–0.6 units/200 μg of protein with various amounts of CaCl$_2$, and the reaction was performed at 30 °C. The levels of GRP94, GRP78, HSP70, and X were probed in immunoblot assays described above.

**Immunofluorescence Staining**—Fibroblast K12 cells grown in chamber slides (Nalge Nunc International, Naperville, IL) were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde made in phosphate-buffered saline for 10 min. For the detection of GRP94, the cells were incubated with anti-GRP94 rat monoclonal antibody (1:100 dilution) as primary antibody and the fluorescein anti- rat IgG (1:200 dilution) (Vector Laboratories Inc., Burlingame, CA) as secondary antibody. For the detection of calpain, the cells were stained with mouse monoclonal anti-calpain antibody (MA9–940) at 1:100 dilution (Affinity Bioreagents, Inc., Golden, CO) and Texas Red anti-mouse IgG antibody (1:200 dilution) (Vector Laboratories) as primary and secondary antibody, respectively. The stained cells were examined by confocal laser scanning microscopy.

**Isolation of Microsomes and Protease Digestion**—Conditions for preparation of microsomes were as described (41). Essentially, K12 cells were trypsinized and after washing with cold phosphate-buffered saline were lysed by incubation in 10 volumes of cold hypotonic buffer (10 mM Tris-HCl, pH 7.4) and Dounce homogenization. The lysate was immediately adjusted to 0.25 M sucrose, 1 mM MgCl$_2$ and centrifuged at 1000 × g for 10 min at 4 °C to remove nuclei and cell debris. The supernatant was further centrifuged at 10,000 × g for 20 min at 4 °C. The supernatant was re centrifuged at 100,000 × g for 90 min. The pellet, representing microsomes, was rinsed briefly with cold water and resuspended in 50 mM Tris–HCl, pH 7.4, and used for proteolytic digestion.

For separation of ER membranes from luminal proteins, the microsome pellet was resuspended in 10 volumes of 100 mM sodium carbonate, pH 11.5, and incubated on ice for 30 min. The suspension was then centrifuged for 1 h at 200,000 × g at 4 °C. The pellet, which represents ER membrane, was rinsed with cold water and resuspended in SDS-PAGE sample loading buffer and analyzed by Western blot. Proteins
reduction of its pro-form indicates its activation. The 32-kDa form of CPP32 is the proenzyme form, and the
immunoblot analysis of CPP32, GRP94, or GRP78 protein levels during apoptosis. The 32-kDa form of CPP32 is the proenzyme form, and the

Calpain Cleavage of GRP94 during Etoposide-induced Apoptosis

Fig. 1. Reduction of GRP94 protein level at the onset of etoposide-induced apoptosis. Human myeloid p53− SN cells were treated with 30 μM etoposide, and the cells were harvested at hourly intervals during the treatment as indicated. The harvested cell pellet was divided into half: one part for measuring oligonucleosomal degradation and the other part for the Western blot analysis. A, kinetics of DNA fragmentation. 5 μg of genomic DNA prepared from each time point (h) as indicated at the top was applied into a 1.9% agarose gel, B, immunoblot analysis of CPP32, GRP94, or GRP78 protein levels during apoptosis. The 32-kDa form of CPP32 is the proenzyme form, and the reduction of its pro-form indicates its activation.

present in the ER lumen were recovered from the supernatant by the addition of trichloroacetic acid to a final concentration of 10%. The pellet was solubilized in the Laemmli buffer and analyzed by Western blot.

For calpain digestion reactions, approximately 100 μg of microsomes in 25 μl of 50 mM Tris-HCl, pH 7.4, was incubated with 0.2 unit of calpain in the presence of 5 mM CaCl2 at 30 °C for 30 min. For trypsin digestion reactions, the microsomes were incubated with trypsin (25 μg/ml) either in the presence or absence of 0.5% Triton X-100 for 30 min at 30 °C. The proteolytic cleavage reactions were terminated by the addition of Laemmli buffer and boiling at 100 °C. 10–20 μg of total protein from each reaction was analyzed by Western blotting.

Cytotoxicity Assays—Transfection was performed according to the protocol for Superfect provided by Qiagen (Valencia, CA). About 7.5 × 104 Jurkat cells were seeded into 5 ml of culture medium. An expression vector of CMV-β-galactosidase was used as a reporter gene. It was co-transfected with either CMV-grp94-AS, containing the antisense version of the full-length rat grp94 cDNA driven by the CMV promoter, CMV-neo-Bcl-2 (gift of Dr. C.M. Zacharchuk, National Institutes of Health; Ref. 42), or the CMV vector alone. After the addition of the transfection complexes into the cells for 24 h at 37 °C, one half of the cells were untreated and the other half were treated with 30 μM of etoposide for 10 h. The cells were harvested, washed with phosphate-buffered saline, solubilized in 250 mM Tris-HCl, pH 7.5, and, following freeze-thawing for three times, assayed for β-galactosidase activity. The percentage cell viability was calculated by the ratio between the number of β-galactosidase activity remaining in the etoposide-treated and untreated cells. For the experiments performed with the grp94 antisense vector, each dosage was repeated two to three times. The transfection efficiency was measured by the fraction of control cells stained positive for β-galactosidase expression. Protein extracts were prepared from the transfected cells for measurement of GRP94 protein level by Western blots.

RESULTS

Cleavage of GRP94 during Etoposide-induced Apoptosis—Using the human hematopoietic cell line SN, which was stably transfected with p53 as a model system to explore the relationship between the GRPs and apoptosis, we tested whether the steady state levels of the GRPs were affected during apoptosis. Exponentially growing SN cells were incubated with etoposide and harvested at 1-h intervals for 4 h. The onset of apoptosis was monitored by the appearance of DNA ladders and the activation of CPP32 caspase through cleavage of its inactive proenzyme form (Fig. 1, A and B). Concomitantly, the level of

GRP94 and GRP78 were measured by Western blot analysis (Fig. 1B). The apoptotic process was evident after 3 h of etoposide treatment, as judged from the DNA fragmentation pattern and the sharp disappearance of the proenzyme form of CPP32. For GRP94 and GRP78, within the first 2 h of drug treatment, their level was relatively constant. Strikingly, we observed that the level of GRP94, but not GRP78, started to decrease at 3 h as DNA fragmentation became evident and CPP32 was activated. Nonetheless, in contrast to CPP32, which was cleaved completely by 4 h, there appeared to be a subpopulation of GRP94 that remained resistant to this proteolytic cleavage process.

To determine whether this novel observation can be extended to other cell systems, the levels of GRP94, GRP78, and β-actin were monitored in the Jurkat human leukemia cell line, since etoposide has been shown to induce apoptosis in these cells in a relatively short time with high efficiency (43). Further, the same experiments were performed with K12 hamster fibroblast cells subjected to etoposide treatment. The protein bands were quantitated by densitometry. The kinetics and magnitude of the decrease of GRP94 protein level in the SN, Jurkat, and K12 cells during the time course of etoposide treatment are shown in Fig. 2. For all the three cell lines, the levels of GRP78 and β-actin remained relatively constant for the whole treatment period. In contrast, in all the three cell lines, a decrease in GRP94 level was detectable after 3 h. With SN and Jurkat cells, by 4 h, the level of GRP94 was reduced to about 40%. With K12 cells, which were more resistant to etoposide-induced cell death (data not shown), the GRP94 level was reduced to about 70% of the untreated cells. However, in all three cell lines, a residual amount of GRP94 remained uncleaved even after 6 h of etoposide treatment.

Inhibition of GRP94 Cleavage during Etoposide-induced Apoptosis by Calpain Inhibitor—The reduction in GRP94 protein level could be due to selective proteolytic cleavage by proteases activated during apoptosis. Since the activation of caspase CPP32 coincided with the reduction in GRP94 level and most apoptotic substrates identified have been shown to be cleaved by caspase family members (44), we first tested whether the in vivo cleavage of GRP94 is mediated by CPP32 or its related members. For this purpose, Jurkat cells were pretreated with caspase inhibitors prior to induction of apoptosis by etoposide. Two caspase inhibitors were used: one set of cells was treated with a general caspase family inhibitor, a cell-permeable synthetic tripeptide, z-VAD-fmk, which can inhibit both caspase-1- and caspase-3-like protease activities (45); the other set of cells were treated with tetrapeptide z-DEVD-fmk, which is specific for the caspase-3 (CPP32) activity (28). Our results showed that pretreatment of the cells with the general caspase inhibitor z-VAD-fmk partially prevented GRP94 cleavage during apoptosis (Fig. 3A). For cells pretreated with z-DEVD-fmk, the cleavage of GRP94 was delayed for about 3 h; however, by 6 h the reduction of GRP94 level was observed (Fig. 3B).

Since GRP94 is a Ca2+-binding protein and its phosphorylation status is also dependent on Ca2+ (39), we tested whether the in vivo cleavage of GRP94 is sensitive to calpain, the Ca2+-activated neutral protease associated with the onset of apoptosis (31, 33). For this purpose, Jurkat cells were pretreated with calpain inhibitor I (34) prior to the addition of etoposide, and the level of GRP94 was monitored by Western blot (Fig. 3C). Our results showed that treatment of cells with calpain inhibitor completely blocked the cleavage of GRP94 during etoposide-induced apoptosis. Since calpain inhibitor I might also inhibit the activity of proteasomes in addition to calpain, we tested whether pretreatment of cells with proteasome inhibitor would prevent GRP94 cleavage. Our results showed that in
contrast to calpain inhibitor I, pretreatment of cells with the proteasome inhibitor N-benzyloxycarbonyl-Leu-Leu-norvalinal (LLnL) was unable to block GRP94 cleavage (Fig. 4B). Further, the overall protein profile remained unchanged in control cells as well as in cells treated with etoposide in the presence or absence of calpain or proteasome inhibitors (Fig. 4A). Collectively, these in vitro studies reveal that the cleavage of GRP94 during etoposide-induced apoptosis appears to be selective and is not a consequence of general protein degradation.

**Ca²⁺-dependent Cleavage of GRP94 by Calpain**—To test directly whether GRP94 is a substrate for calpain, *in vitro* proteolytic assays were utilized. Metabolically [³⁵S]methionine-labeled GRP94 from Jurkat cells was immunopurified. As shown in Fig. 5A, GRP94 was efficiently cleaved by calpain, while two other co-immunoprecipitating protein bands were largely unaffected. A slight increase in a band intensity around 80 kDa was also noted. In contrast, purified recombinant CPP32, when similarly added to radiolabeled GRP94 resulted in no cleavage of GRP94 or the co-immunoprecipitating protein

---

**Fig. 2.** Kinetics of GRP94 proteolytic cleavage in SN (A), Jurkat (B), and K12 cells (C). The cells were treated with 30 μM etoposide and harvested at different time points as indicated. From each sample, 50 μg of protein lysate was applied on a 8.5% SDS-PAGE. The protein blots were probed with antisera against GRP94 (●), GRP78 (△), and β-actin (■). The relative amount of the three proteins was quantitated and plotted against the time (h) after etoposide treatment.

**Fig. 3.** Blockage of GRP94 cleavage during apoptosis by interleukin-converting enzyme protease family inhibitor and calpain inhibitor in Jurkat cells. A, the cells were either untreated (control) or pretreated with 50 μM of interleukin-converting enzyme caspase inhibitor (z-VAD-fmk) for 2 h. The cells were then changed to fresh medium containing 30 μM etoposide, and at the indicated time points protein lysates were prepared. B, cells pretreated with 120 μM CPP32 inhibitor (z-DEVD-fmk) for 3 h prior to etoposide addition. C, cells pretreated with 45 μM calpain inhibitor I for 4 h prior to the addition of etoposide. The levels of GRP94 and β-actin as a loading control were measured by Western blots.

**Fig. 4.** Effect of calpain and proteasome inhibitors on overall protein profile and GRP94 cleavage in etoposide-treated cells. Jurkat cells were either untreated or pretreated with 45 μM of calpain inhibitor I or 50 μM proteasome inhibitor N-benzyloxycarbonyl-Leu-Leu-norvalinal (LLnL) for 4 h followed by 30 μM etoposide treatment for another 4 h. A, total cellular protein profile in SDS-PAGE. 25 μg of protein extract from each sample was separated in 10% SDS-PAGE and stained by the Coomassie Blue dye. The electrophoretic mobility of the molecular size markers (M) is indicated at the left. B, 50 μg of protein lysate was separated in 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-GRP94 antibody. Relative levels of GRP94 were quantitated and compared with the untreated cells.
Calpain Cleavage of GRP94 during Etoposide-induced Apoptosis

bands (Fig. 5B). Since the activity of the recombinant CPP32 was confirmed by its ability to cleave a synthetic calorimetric CPP32 substrate Ac-DEVD-pNA (data not shown), the inability of CPP32 to cleave GRP94 suggests that GRP94 is unlikely to be a direct substrate for this caspase. Thus, while it remains to be determined whether GRP94 is an indirect target for CPP32 or other members of the caspase protease family can mediate the cleavage of GRP94 during etoposide-induced apoptosis, our results show that GRP94 is a putative substrate for calpain.

Similarly, specific cleavage of GRP94 by calpain can be observed using cell lysates prepared from K12 cells. Further, the cleavage of GRP94 by calpain is Ca\(^{2+}\)-dependent (Fig. 6A, lanes 3–6) and is not due to autolysis in the presence of calcium, since omission of calpain resulted in no cleavage (Fig. 6A, lanes 1 and 2). Under these in vitro assay conditions, GRP94 cleavage by calpain was not detectable until a calcium concentration of 3 mM was reached (Fig. 7A). Under these same assay conditions, as shown by Western blot analysis using the same cell lysates, the levels of GRP78, HSP70, and a 45-kDa protein X, which contains a KDEL epitope, were not affected by calpain, either in the presence or absence of calcium (Fig. 6A). Further, recombinant GRP78 was resistant to calpain cleavage, even at high enzyme concentrations (Fig. 6B). These results confirmed that GRP94 is a specific substrate for calpain.

Upon resolving the proteolytic products of GRP94 in SDS-PAGE, with the disappearance of full-length GRP94, a protein band of about 80 kDa was detected (Fig. 7A, lane 8). Upon longer gel electrophoresis, the 80-kDa band could be further resolved into a set of closely clustered bands (Fig. 6A, lanes 4–6). Since the same 80-kDa polypeptide was immunoreactive against an antibody directed against the carboxyl-terminal KDEL epitope shared between GRP78 and GRP94 (Fig. 7B and data not shown), calpain cleavage is likely to occur in the amino half of GRP94, generating an 80-kDa carboxyl polypeptide. Based on the primary structure of GRP94, it is possible that a transmembrane form of GRP94 may also exist that spans the ER membrane and extends into the cytoplasm (Ref. 46; Fig. 7B).

Proteolytic Cleavage of GRP94 Associated with the ER Membrane—To test whether a fraction of GRP94 is associated with the ER membrane, established biochemical techniques were used to isolate the microsomes (41). The presence of GRP94 in the ER lumen and the membrane fractions was determined by Western blot analysis. As expected, GRP94 was enriched in the microsomes as compared with the total cell lysate (Fig. 8A, lanes 1 and 2). In agreement with earlier studies (41), a fraction of GRP94 was co-purified with the ER membrane, while the majority of GRP94 was fractionated into lumen (Fig. 8A, lanes 3 and 4). Next we determined whether GRP94 associated with the ER membrane is accessible for proteolytic digestion. Calpain treatment of the intact microsomes resulted in about 40% reduction of GRP94 protein level (Fig. 8B, lanes 1 and 2). Upon longer exposure of the x-ray film, the 80-kDa proteolytic prod-
uct of GRP94 was detected after calpain treatment (Fig. 8B, lanes 3 and 4). The topology of GRP94 was further confirmed by limited trypsin digestion of the microsomes in the presence and absence of an nonionic detergent. Prior to Triton X-100 treatment, a fraction of GRP94 was accessible to trypsin cleavage with the generation of proteolytic fragments of GRP94; upon disruption of the microsome by Triton X-100, GRP94 was completely digested by trypsin (Fig. 8B, lanes 5 and 6).

**Co-localization of Calpain with GRP94 following Etoposide Treatment**—Calpain is believed to be a cytoplasmic protein that is activated at cellular membranes. To investigate the physical interaction of the two proteins, K12 cells treated with etoposide for various times were immunostained with fluorescent antibodies against calpain and GRP94 and subjected to laser confocal microscopy (Fig. 9). In control cells without etoposide treatment, both calpain and GRP94 were detected primarily in the perinuclear region but showed minimal co-localization. Upon treatment of cells with etoposide, co-localization of calpain with GRP94 became apparent. By 4 and 6 h, strong co-localization between the two proteins in the perinuclear region was detected. In contrast, there was no co-localization between calpain and GRP78 (data not shown). These results suggest that, following etoposide-induced apoptosis, calpain is activated and interacts specifically with the ER membrane-associated GRP94.

**Cytoprotective Function of GRP94 in Etoposide-treated Cells**—To determine the consequence of reduction of GRP94 protein level in cells undergoing etoposide-induced apoptosis, we utilized a transient co-transfection system (42) to introduce an antisense vector targeted against GRP94 and a β-galactosidase reporter gene into Jurkat cells. The effect of the transiently expressed gene on apoptosis was measured quantitatively in the transfected cell population by assaying the β-galactosidase activity remaining in the viable, co-transfected cells. Using Bel-2 as a positive control, we confirmed the earlier observation that its overexpression resulted in an increase in cell viability in a dosage-dependent manner (42), with complete protection achieved at the higher doses (Fig. 10A). In the case of grp94 antisense expression vector, no effect on cell viability was observed in the control cells. Upon etoposide treatment, with increasing amounts of the antisense vector, a dosage-dependent decrease in cell viability was observed (Fig. 10B).

To confirm the effect of the grp94 antisense vector on the GRP94 protein level, Western blot was performed using total cell lysate. A reduction of 20% of the overall GRP94 level was observed (Fig. 10B, inset). Since the transfection efficiency was 35%, the effective decrease in the GRP94 level in the transfected cells was estimated to be 57%. Thus, while specific reduction of GRP94 protein level by antisense under these transient transfection conditions has no negative effect on overall cell viability, upon etoposide treatment an increase in cell death was observed.

**DISCUSSION**

Our investigation into the fate of the GRPs during the apoptotic process triggered by DNA damage mediated by etoposide reveals several unexpected and interesting results. In particular, they reveal a new relationship between GRP94, an abundant ER stress-inducible glycoprotein associated with the ER, and calpain, a nonlysosomal Ca2+-activated cysteine protease. This cleavage of GRP94 upon etoposide treatment is unexpected and unique, since under ER stress conditions mammalian cells also undergo limited apoptosis, but GRP94 is not cleaved; rather, its level is enhanced within several hours of ER stress treatment (8, 20, 39). In the case of etoposide treatment, there was no immediate activation of the GRP stress response. In addition, GRP94 was specifically cleaved within 3 h of drug treatment. This cleavage of GRP94 upon etoposide treatment is unexpected and unique, since under ER stress conditions mammalian cells also undergo limited apoptosis, but GRP94 is not cleaved; rather, its level is enhanced within several hours of ER stress treatment (8, 20, 39). In the case of etoposide treatment, there was no immediate activation of the GRP stress response. In addition, GRP94 was specifically cleaved within 3 h of drug treatment.
The total amount of DNA was adjusted to 15 reporter plasmid and various amounts of the CMV-Bcl-2 as indicated.

A assay systems, GRP78 is not cleaved by calpain. Similar to cleavage of GRP78 is not due to accessibility, since even in in vitro assay systems, GRP78 is not cleaved by calpain. Similarly, HSP70, a stress-inducible chaperone with both nuclear and cytoplasm localization, is not a substrate for calpain. Since the overall protein profile is also relatively constant when GRP94 is specifically cleaved during the apoptotic process, the cleavage of the GRP94 by calpain is a specific event rather than a general consequence of programmed cell death.

What are some of the unique features of GRP94 among the stress-inducible chaperones rendering it a plausible substrate for calpain in vivo? First, GRP94 is a Ca\(^{2+}\)-binding protein harboring several high affinity and multiple low affinity Ca\(^{2+}\)-binding sites and contains several EF-hand structures that may serve as some of the Ca\(^{2+}\)-binding sites of the protein (18, 48, 49). While the calcium requirements for calpain function in vivo are not well understood, the ability of GRP94 to sequester calcium may create a microenvironment in which its concentration would be sufficiently high for calpain to be activated and exert its proteolytic effect. Second, although GRP94 resides primarily within the lumen of ER, our biochemical analysis supports previous reports that it can also exist as a transmembrane protein with a cytoplasmic carboxyl terminus (41, 46). Recently, GRP94 has been found to physically and functionally interact with the cytoplasmic Fanconi anemia group C protein (50), suggesting that some form of GRP94 is present in the cytoplasm. Thus, it is possible that as cells progress through etoposide-induced apoptosis, calpain becomes activated and comes into contact with a subpopulation of GRP94 associated with the ER membrane, where cleavage of GRP94 occurs. Since the inhibitor of cytoplasmic proteasomes cannot rescue GRP94 cleavage in vivo, the cleavage of GRP94 in etoposide-treated cells apparently does not utilize the retrograde system for degradation of ER luminal proteins (51). Third, suppression of GRP94 induction has been shown to sensitize cells in Ca\(^{2+}\)-mediated cell death (20, 23). Here, we showed that specific reduction of GRP94 protein level in Jurkat cells by antisense resulted in a decrease of cell viability in etoposide-treated cells. Thus, if activation of calpain in etoposide-treated cells is to initiate the cell death program, it is logical that it acts to neutralize or destroy the protective function of antiapoptotic proteins such as GRP94, as exemplified by the cleavage of Bcl-2 by caspase-3 (29). In case of Bcl-2, its proteolytic cleavage product is further converted into a Bax-like death effector.

Interestingly, calpains cleave their substrates in a highly specific but limited fashion, resulting in biological active proteins (31, 32, 52). It is possible that calpain can confer its regulatory effect on the apoptotic process by selectively cleaving and thereby activating specific substrates, resulting in the enhancement of cell death. Thus, it is postulated that small amounts of limited proteolysis of Bax by calpain may be sufficient to set in motion a mitochondria-based cell death program, which in turn controls the activation of caspases (35). For GRP94, cleavage by calpain generates an 80-kDa subfragment in vitro. Future studies aimed at determining the precise calpain cleavage sites and the stability and function of the putative proteolytic products of GRP94 will address whether calpain cleavage of GRP94 leads to simple destruction of GRP94 or the generation of novel biologically active molecules. Since many of the protein targets previously identified during apoptosis are involved in the architecture or DNA replicative components of the cell, GRP94 and Bcl-2 represent a new class of cellular targets for the apoptotic regulatory proteases. This study provides the first evidence that a Ca\(^{2+}\)-binding ER protein with protective functions against Ca\(^{2+}\)-induced apoptosis is a substrate for a Ca\(^{2+}\)-activated protease. Further studies will determine whether specific elimination of subsets of cellular proteins that exhibit general antiapoptotic properties is a critical step in the execution of a selective cell death program.

**FIG. 10. Cytotoxicity assays in etoposide-treated Jurkat cells.** A Jurkat cells were co-transfected with 5 μg of the β-galactosidase reporter plasmid and various amounts of the CMV-empty vector in all the samples. Parallel sets of transfected cells were either untreated or treated with 30 μM etoposide for 10 h and assayed for β-galactosidase activity. The percentage of cell viability of the transfected cells was plotted against the amount of the Bcl-2 expression vector used in the co-transfection experiments. B, various amounts of CMV-grp94 antisense vector (grp94 AS) were co-transfected with the β-galactosidase reporter plasmid into Jurkat cells. The percentage of cell viability of the transfected cells was plotted against the amount of grp94 AS used. The mean and range were as indicated. Total cell lysate was prepared from cells transiently transfected with the empty CMV-vector (v) or the grp94 antisense vector (AS). The inset in B shows Western blot of the relative amounts of GRP94 (94), with β-actin (Ac) as loading control.
Acknowledgments—We thank Drs. Thomas Rudel and Gary Bokoch for the gift of recombinant CPP32, Drs. Peter Danenberg and Axel Schonthal for providing cell lines, Dr. Charles Zacharchuk for the Bcl-2 expression vector, and Dr. Dwight Warren for the use of the imaging densitometer. The confocal laser scanning microscopy was performed at the Electron Microscopy Core Facility at the Doheny Eye Institute, USC, supported by NEI, National Institutes of Health, Grant EY03040.

REFERENCES

1. Vaux, D. L., and Strasser, A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2239–2244
2. Rao, L., Debbas, M., Sabbatini, P., Hockenbery, D., Korsmeyer, S., and White, E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7742–7746
3. Crook, N. E., Clem, R. J., and Miller, L. K. (1993) J. Virol. 67, 2168–2174
4. Nunez, G., and Clarke, M. F. (1994) J. Cell Biol. 125, 217–232
5. Lindquist, S. (1986) Annu. Rev. Biochem. 55, 6001–6005
6. Sugawara, S., Takeda, K., Lee, A., and Dennert, G. (1993) Mol. Cell. Biol. 13, 6690–6694
7. Morimoto, R. I., Sarge, K. D., and Abravaya, K. (1992) J. Biol. Chem. 267, 21987–21990
8. Lee, A. S. (1987) Trends Biochem. Sci. 12, 20–23
9. Watsowich, S. S., and Morimoto, R. I. (1988) Mol. Cell. Biol. 8, 393–405
10. Li, X. A., and Lee, A. S. (1991) Mol. Cell. Biol. 11, 3446–3453
11. Saatstala, M., and Wissing, D. (1993) J. Exp. Med. 177, 231–236
12. Sugawara, T., Takeda, K., Lee, A., and Dennert, G. (1993) Cancer Res. 53, 6001–6005
13. Little, E., Ramakrishnan, M., Roy, B., Gazit, G., and Lee, A. S. (1994) Crit. Rev. Eukaryotic Gene Expression 4, 1–18
14. Mehnlen, P., Schulze-Osthoff, K., and Miller, L. K. (1993) J. Biol. Chem. 268, 4327–4334
15. Zhou, Y., and Lee, A. S. (1998) Oncogene 17, 1069–1078
16. McMillan, D. R., Xiao, X., Shao, L., Graves, K., and Benjamin, I. J. (1998) J. Biol. Chem. 273, 7523–7528
17. Hightower, L. E. (1991) Cell 66, 191–197
18. Koch, G., Smith, M., Maier, D., Webster, P., and Murtaza, R. (1986) J. Cell Sci. 86, 217–232
19. Lievremont, J. P., Rixrutz, R., Hendershot, L., and Meldolesi, J. (1997) J. Biol. Chem. 272, 30873–30879
20. Little, E., and Lee, A. S. (1995) J. Biol. Chem. 270, 9526–9534
21. James, C., Dentert, G., and Lee, A. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7690–7694
22. Liu, H., Bowers, R. C., III, van de Water, B., Silencce, C., Nagelkerke, J. F., and Stevens, J. J. (1997) J. Biol. Chem. 272, 21751–21759
23. McCormick, T. S., McColl, K. S., and Distelhorst, C. W. (1997) J. Biol. Chem. 272, 6087–6092
24. Morris, J. A., Dorner, A. J., Edwards, C. A., Hendershot, L. M., and Kaufman, R. J. (1997) J. Biol. Chem. 272, 4327–4334
25. Reed, J. C. (1997) Cell 91, 559–562
26. Porter, A. G., Ng, P., and Janicke, R. U. (1997) BioEssays 19, 501–507
27. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) Cell 91, 479–489
28. Ubeda, M., and Habener, J. F. (1997) J. Biol. Chem. 272, 19562–19568
29. Cheng, E. H., Kirsch, D. G., Clem, R. J., Ravi, R., Kastan, M. B., Bedi, A., Ueno, K., and Hardwick, J. M. (1997) Science 278, 1966–1968
30. Croall, D. E., and DeMartino, G. N. (1991) Physiol. Rev. 71, 813–847
31. Carafoli, E., and Molinari, M. (1998) Biochem. Biophys. Res. Commun. 247, 193–203
32. Kawasaki, H., and Kawashima, S. (1996) Mol. Membr. Biol. 13, 217–224
33. Squirer, M. K., and Cohen, J. J. (1997) J. Immunol. 158, 3690–3697
34. Kubbutat, M. H., and Vousden, K. H. (1997) Mol. Membr. Biol. 14, 460–466
35. Wood, D. E., Thomas, A., Devi, L. A., Berman, Y., Beavisa, R. C., Reed, J. C., and Newcomb, E. W. (1998) Oncogene 17, 231–236
36. Banerjee, D., Lenz, H. J., Schnieders, B., Manno, D. J., Ju, J. F., Spears, C. P., Hochhauser, D., Danenberg, K., Danenberg, P., and Bertino, J. R. (1995) Cell Growth Differ. 6, 1405–1413
37. Lee, A. S. (1981) J. Cell. Physiol. 106, 119–125
38. Zhou, Y., and Lee, A. S. (1998) J. Natl. Cancer Inst. 90, 381–388
39. Ramakrishnan, M., Schonenthal, A. H., and Lee, A. S. (1997) J. Cell. Physiol. 170, 115–129
40. Rudel, T., and Bokoch, G. M. (1997) Science 276, 1571–1574
41. Kang, H. S., and Welch, W. J. (1991) J. Biol. Chem. 266, 5643–5649
42. Memon, S. A., Petrak, D., Moreno, M. B., and Zacharchuk, C. M. (1995) J. Immunol. Methods 180, 15–24
43. Erhardt, P., and Cooper, G. M. (1996) J. Biol. Chem. 271, 17601–17604
44. Salvesen, G. S., and Dixit, V. M. (1997) Cell 91, 443–446
45. Prong, G. J., Ramer, K., Amiri, P., and Williams, L. T. (1996) Science 271, 808–810
46. Mazzarella, R. A., and Green, M. (1987) J. Biol. Chem. 262, 8875–8883
47. Garcia, P. D. Ou, J.-H., Rutter, W. J., and Walter, P. (1988) J. Cell. Biol. 106, 1093–1104
48. Van, P. N., Peter, F., and Soling, H. D. (1989) J. Biol. Chem. 264, 17494–17501
49. Csermely, P., Schnaidner, T., Stol, C., Prohaszka, Z., and Nardai, G. (1998) Pharmacol. Ther. 79, 129–168
50. Hoshino, T., Wang, J., Devetten, M. P., Iwata, N., Kajigaya, S., Wise, R. J., Liu, J. M., and Youssoufian, H. (1998) Blood 91, 4367–4369
51. Hiller, M. M., Finger, A., Schweiger, M., and Wolf, D. H. (1996) Science 273, 1725–1728
52. Wang, K. R., Villalobos, A., and Roufogalis, B. D. (1989) Biochem. J. 252, 693–706