The Marine Product Cephalostatin 1 Activates an Endoplasmic Reticulum Stress-specific and Apoptosome-independent Apoptotic Signaling Pathway

Received for publication, August 17, 2006, and in revised form, August 30, 2006 Published, JBC Papers in Press, August 31, 2006 DOI 10.1074/jbc.M607904200

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Cephalostatin 1, a bis-steroidal marine natural product, has been reported to induce apoptosis without the requirement of an active caspase-8 or mitochondrial cytochrome c release and apoptosis formation. Here we show that despite the absence of these events, caspase-9 activation is essential for cephalostatin 1-induced apoptosis. Cephalostatin 1 initiates a rapid endoplasmic reticulum stress response characterized by phosphorylation of eukaryotic initiation factor-2 α-subunit and increased expression of the chaperone immunoglobulin heavy chain-binding protein GRP78 as well as the transcription factor C/EBP homologous protein (CHOP)/GADD153. Cephalostatin 1 activates apoptosis signal-regulating kinase 1 and c-Jun N-terminal kinase (JNK). However, this pathway does not play a major role in cephalostatin 1-induced apoptosis, as assessed by stable expression of a dominant negative apoptosis signal-regulating kinase 1. Importantly, the endoplasmic reticulum-associated caspase-4 is required and as shown by biochemical and genetic inhibition experiments, acts upstream of caspase-9 in cephalostatin-induced apoptosis.

Apoptosis dysregulation represents an important contribution to the development of cancer as well as chemoresistance. Moreover, a wide range of chemotherapeutic drugs induces death in malignant cells by inducing apoptosis (1, 2). Two major pathways leading to apoptosis have been elucidated: one triggered by tumor necrosis factor (TNF)†/Fas family receptors and the other mediated by mitochondrial release of cytochrome c and other proteins (1, 3). However, damage or stress in many organelles (besides mitochondria) may trigger apoptosis through mechanisms, which remain partially unclear (4). In this respect, a pathway of apoptosis induction has been linked to stress in the endoplasmic reticulum (ER) (5–8). ER stress leads to the activation of genes possessing an unfolded protein response (UPR) element, which controls the levels of molecular chaperones, such as M, 78,000 glucose-regulated stress protein (BiP/GRP78) involved in protein folding in the ER (9). Furthermore, the eukaryotic initiation factor-2 (eIF2) is phosphorylated by the PKR-like ER-localized eIF2α kinase (PERK) in response to ER stress leading to an attenuation of translational initiation and protein synthesis (8). When these stress modulators are unable to rescue cells, various apoptotic pathways are activated. Recruitment of TNF receptor-associated factor 2 (TRAF2) to activated stress sensor proteins, called IREs, induces the apoptosis signal-regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK) cascade (6). In addition, ER-specific caspases, such as caspase-12 in the murine system, seem to aggregate at the ER membrane surface through TRAF2 proteins resulting in their cleavage and activation (7). In humans, caspase-4 has been proposed to play a role as ER stress-specific caspase similar to caspase-12 (10). Furthermore, the UPR increases the transcription of the transcription factor C/EBP homologous protein (CHOP), which is closely associated with cell death (11, 12). ER stress pathways linked to apoptosis have been reported in pathological states such as ischemia-reperfusion injury and diabetes as well as in neurodegenerative diseases such as Alzheimer and Parkinson, where abnormalities in protein folding have been identified (8, 9, 13). The role of ER stress in tumor development and therapy is unclear at present, although supposedly ER stress response is important for regulating the balance between tumor cell death and its growth as well as for the sensitivity to chemotherapeutic agents (14).

Because most of the cancer chemotherapeutic drugs signal through mitochondrial cytochrome c release, it is very difficult to distinguish as to whether the ER may play a role as stress sensor for chemotherapeutics that reroute the signal directly through mitochondria and the apoptosome or whether the ER is able to activate unfolded protein response; Z, benzyloxy carbonyl; FMK, fluoromethyl ketone.
its own death pathway (2–4, 8). In this respect, it is important to characterize specific elements of the ER stress response because they could represent novel targets for the development of new cancer chemotherapeutic strategies.

We recently characterized an experimental anticancer agent, cephalostatin 1, that showed promise to be a helpful tool in this respect. Cephalostatin 1 is a bis-steroidal marine natural product, which induces a novel pathway of apoptosis in leukemia T cells. Cephalostatin 1 triggers cell death in a CD95- and caspase-8-independent manner. Even more intriguingly, without triggering cytochrome c release from mitochondria and apoptosis formation, cephalostatin-induced apoptosis is accompanied by caspase-9 activation (15). Apoptosome-independent cell death induction has been described in the literature before (16, 17), but in these cases, apoptosis occurred independently of caspase-9 activation.

In search of the mechanisms responsible for apoptosome-independent caspase-9 activation, we hypothesized that cephalostatin 1 induces ER stress. Thus, we investigated several markers of ER stress induced by cephalostatin 1 and identified ER-specific molecular players such as caspase-4 and the ASK1/JNK cascade being involved in apoptosome-independent execution of cell death.

**EXPERIMENTAL PROCEDURES**

**Compounds**—Cephalostatin 1 was isolated from the marine worm *Cephalodiscus gilchristi* as described previously (18). Purity of the compound was 98% as judged by high-performance liquid chromatography. Before application, cephalostatin 1 was dissolved and further diluted in Me2SO. Final Me2SO concentration did not exceed 1%, a concentration verified not to interfere with the experiments performed. Propidium iodide, thapsigargin (TG), tunicamycin (TM), and human TNF-α were from Sigma (Deisenhofen, Germany); etoposide (ETO) and the specific JNK inhibitor SP600125 were from Calbiochem; and etoposide (ETO), etoposide (ETO), bisbenzoxazoyl carbonyl-LEVD-fluoromethyl ketone (Z-LEVD-fmk) was from MBL (Woburn, MA).

**Cell Culture**—Human leukemia Jurkat T cells (J16) (kindly provided by P.H. Krammer and H. Walczak, Heidelberg, Germany), Jurkat T cells lacking caspase-9 (Casp.9−/−), deficient cells stably retransfected with full-length caspase-9 (Casp.9+/−) (19), Bak-deficient Jurkat cells (Bak−/−, JCaM1.6), deficient cells reconstituted with Bak (Bak+/+) (20) as well as a Jurkat cell line expressing an inactive form of ASK1 (ASK1-DN, clones A2–1 and A2–3) (21) were cultured (37 °C and 5% CO2) in RPMI 1640 medium containing 2 mM L-glutamine (PAN Biotech, Aidenbach, Germany) supplemented with 10% fetal calf serum (FCS) (PAA Laboratories, Colbe, Germany) and 1% pyruvate (Merck, Darmstadt, Germany). Medium of ASK1-DN transfected cells was supplemented with 1 mg/ml G418 (PAA Laboratories, Colbe, Germany) every fifth passage.

**Quantification of Apoptosis**—Quantification of apoptosis was performed according to Nicoletti et al. (22). Briefly, cells were incubated in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 μg/ml propidium iodide) overnight at 4 °C and analyzed by flow cytometry on a FACSCalibur (Becton

DICKINSON, Heidelberg, Germany). Nuclei to the left of the G1 peak containing hypodiploid DNA were considered apoptotic.

**Western Blot Analysis**—Cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline, and lysed for 30 min either in 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, and 30 mM Tris-HCl, pH 7.5 with the protease inhibitor Complete™ (Roche, Mannheim, Germany) (for Apaf-1, BiP, CHOP, and caspases); in 2 mM EDTA, 137 mM NaCl, 10% glycerol, 2 mM tetrasodium pyrophosphate, 20 mM Tris, 1% Triton X-100, 20 mM sodium glycerophosphate hydrate, 10 mM NaF, 2 mM sodium orthovanadate, and 1 mM phenylmethylsulfonyl fluoride supplemented with Complete™ (for p-JNK, p-Elf2α, and JNK); or in 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM tetrasodium pyrophosphate, 2 mM sodium orthovanadate, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 10 mM Tris, pH 7.4 with Complete™ (for p-ASK1 and ASK1). Lysates were homogenized with an ultrasonic device and centrifuged at 10,000 × g for 10 min at 4 °C. Equal amounts of protein were separated by SDS-PAGE (7.5% for Apaf-1 and ASK1; 10% for JNK; 12% for BiP, CHOP, p-Elf2α, and caspases) and transferred to nitrocellulose membranes (Hybond™ ECL™; Amersham Biosciences). Membranes were blocked with 5% fat-free milk powder in phosphate-buffered saline containing 0.05% Tween 20 (1 h) and incubated with specific antibodies against Apaf-1 (mouse IgG1; BD Transduction Laboratories), Bak (mouse IgG2a; Calbiochem), BiP (mouse IgG2a; BD Transduction Laboratories), CHOP (rabbit polyclonal antibody; Sigma), p-Elf2α (rabbit polyclonal antibody; Cell Signaling, Frankfurt, Germany), p-JNK (Thr-183/Tyr-185) (mouse IgG1; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories), BiP (mouse IgG2a; BD Transduction Laboratories) and incubated with specific antibodies against Apaf-1 (mouse IgG1; BD Transduction Laboratories), Bak (mouse IgG2a; Calbiochem), BiP (mouse IgG2a; BD Transduction Laboratories), CHOP (rabbit polyclonal antibody; Sigma), p-Elf2α (rabbit polyclonal antibody; Cell Signaling, Frankfurt, Germany), p-JNK (Thr-183/Tyr-185) (mouse IgG1 monoclonal antibody; Cell Signaling), JNK (rabbit polyclonal antibody; Cell Signaling), caspase-4 (mouse IgG1, clone 4B9; MBL), or caspase-9 (rabbit polyclonal antibody; Cell Signaling) overnight at 4 °C. Specific proteins were visualized by secondary antibodies conjugated to horseradish peroxidase and the ECL Plus™ Western blotting detection reagent (Amersham Biosciences). Membranes were exposed to x-ray film for the appropriate time periods and subsequently developed in a tabletop film processor (Curix 60; Agfa, Cologne, Germany). Equal protein loading was controlled by Ponceau S staining of membranes.

**Apaf-1 siRNA**—Sense and antisense small interfering RNA (siRNA) oligonucleotides corresponding to nucleotides 978–998 of Apaf-1 (AATTGGTGCACTTTTACGTGA)(23) and oligonucleotides corresponding to a scramble sequence were purchased from biomers.net GmbH (Ulm, Germany) and annealed to create the double-stranded siRNAs. Jurkat cells were transfected with 3 μg of scramble or Apaf-1 siRNAs using the Nucleofector™ II (Amaxa, Cologne, Germany) according to the manufacturer’s instructions.

**Clonogenic Assay**—Caspase-9-deficient Jurkat cells and cells retransfected with full-length caspase-9 were left untreated or treated with cephalostatin 1 for 2 h. Subsequently, cells were washed with phosphate-buffered saline and resuspended in culture medium (5 × 104 cells/ml). Cell suspensions were diluted 1:10 with methylcellulose (0.52%) medium containing 40% fetal calf serum. Cells were seeded in 96-well plates (100 μl), and colonies were scored after 7 days of culture.
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Caspase-9 Activity Assay—Caspase-9 activity was measured using the Caspase-Glo™ 9 assay from Promega GmbH (Mannheim, Germany) following the manufacturer’s protocol. Jurkat T cells grown in 96-well plates were left untreated or treated with cefalostatin 1 for 4 h with or without preaddition of caspase-4 inhibitor. After stimulation, Caspase-Glo™ 9 reagent was added to each well and gently mixed. Luminescence was measured immediately in a plate-reading multifunction photometer (SPECTRAFluor Plus; Tecan, Crailsheim, Germany) every 10 min for 2 h. Background luminescence corresponding to the culture medium was subtracted from experimental values.

Plasmid Construction—A siRNA-expressing sequence for targeting the caspase-4 gene was cloned into a psiRNA-h7SKneo G1, expression vector system (InvivoGen, San Diego, CA) to generate siRNA. Forward target sequences of the caspase-4 siRNA hairpin transcripts were 5’-acctcAAGTG-GCCTTTCACAGTCATcaagagATGACTGTGAAGG-CGCTTTt-3’ and 5’-acctcAAGATTTCCACTGTGTGGTTT-CaagagAACCCAGTGAGGAAATCTTtt-3’, respectively (uppercase letters denote the double-stranded region corresponding to the caspase-4 sequence targeted). Oligonucleotides encoding a scramble sequence (universal control from InvivoGen) were used as a control. After hybridization, oligonucleotides were cloned into the BbsI site of psiRNA-h7SKneo, and the plasmids were sequenced and amplified. The recombinant plasmid was transformed into Escherichia coli LyoComp GT116 strain, and the resultant cells were cultured in LB-kanamycin-containing medium. The recombinant plasmids in the selected colonies were extracted, digested with Spel, and run on 0.8% agarose gel to extract the plasmids containing psiRNA-h7SKneo G1 caspase-4 siRNA plasmids.

Plasmid Transfection—Jurkat cells were transfected by electroporation with the Nucleofector™ II (Amaxa) according to the manufacturer’s protocol. 4 × 10⁶ Jurkat T cells in exponential growing phase were transfected with 3 μg of psiRNA-h7SKneo, 3 μg of psiRNA-h7SKnScr (a control plasmid containing a scramble sequence), or 3–4 μg of a 1:1 mixture of the siRNA constructs. Efficiency of RNA interference was checked by Western blot analysis using antibodies against caspase-4.

To study the influence of caspase-4 down-regulation in apoptosis, caspase-4 siRNA-generating plasmids were transfected into Jurkat cells as described above, and the cells were incubated for 24 h before induction of apoptosis. To investigate caspase-9 activation in cells with silenced caspase-4 expression, 1 mg/ml etoposide that use the intrinsic mitochondrial pathway hallmark was added to the culture medium 2 days after transfection, and experiments were performed 48 h later. In both experiments, cells containing psiRNA-h7SKneo and psiRNA-h7SKnScr vectors were used as controls.

Statistical Analysis—All experiments were performed at least three times in triplicate. Results are expressed as mean value ± S.E. Student’s unpaired two-tailed t test was performed using GraphPad Prism version 3.0 (GraphPad Software, San Diego, CA). p values <0.05 were considered significant.

RESULTS

Caspase-9 Is Essential in Apoptosome-independent Apoptosis Induced by Cephalostatin 1—Cephalostatin 1 activates caspase-9 and induces apoptosis without cytochrome c release and apoptosis formation (15). To further support this unique finding two strategies were used: 1) Bak-deficient Jurkat cells (Bak⁻/⁻) were exposed to cefalostatin 1 as well as etoposide as positive control. Because Jurkat cells do not express Bax (24), the Bak⁻/⁻ Jurkat cells are resistant to apoptotic stimuli such as etoposide that use the intrinsic mitochondrial pathway hallmark by release of cytochrome c (Fig. 1A). Importantly, Bak⁻/⁻ cells are equally sensitive to cefalostatin as Bak-reconstituted control cells (Fig. 1A), further pointing to a cytochrome c-independent signaling pathway. 2) To confirm cefalostatin 1 induction of apoptosis without apoptosome formation, Apaf-1 was silenced via siRNA in Jurkat cells. Cefalostatin 1 killed cells transfected with Apaf-1 siRNA to the same extent as cells transfected with a scramble siRNA sequence (Fig. 1B, left). The apoptotic response to etoposide, however, was significantly blunted in Apaf-1-silenced cells. Moreover, caspase-9 was activated to the same extent in Apaf-1 as in scramble siRNA-transfected cells upon treatment with cefalostatin 1 (Fig. 1B, right), confirming that activation of caspase-9 occurred independently of the apoptosome complex. Apoptosis induction and more intriguingly, activation of caspase-9 independently from the classical mitochondrial signaling raises the question of the role and activation pathway of caspase-9 in cefalostatin 1-induced apoptosis. For this purpose, we used a Jurkat cell line deficient in caspase-9. Interestingly, apoptosis induced by cefalostatin 1 was almost completely inhibited in caspase-9-deficient cells, whereas cells stably retransfected with full-length caspase-9 died normally when exposed to cefalostatin 1 (Fig. 1C, left). Moreover, significant differences between both cell lines were still observed in a 7-day clonogenic assay (Fig. 1C, right). These data ask for clarifying the mechanism underlying the apoptosome-independent activation of caspase-9 by cefalostatin 1.

Cefalostatin 1 Induces ER Stress in Jurkat Leukemia T Cells—In search of the initial event leading to caspase-9 activation and apoptosis, cefalostatin 1 was hypothesized to induce ER stress. In fact, the expressions of an ER stress sensor (BiP/GRP78) and an ER stress-induced cell death modulator (CHOP/GADD153) were affected by cefalostatin 1. As shown in Fig. 2, cefalostatin 1 (1 μM) increased expression of the ER chaperone BiP/GRP78 and the transcription factor CHOP/GADD153 as did the known ER stress-inducer tunicamycin. The increase of BiP showed a biphasic pattern with an early increase (30 min to 1 h) followed by a plateau and a second strong elevation after 16–24 h. CHOP protein level increased rapidly (15 min to 1 h) and decreased slowly thereafter. The eukaryotic initiation factor-2 is phosphorylated by the PKR-like eIF2α kinase in response to ER stress leading to an attenuation of translational initiation and protein synthesis and activation of pathways leading to cell death or survival (8). Cefalostatin 1 induced a very early and strong phosphorylation of the α-subunit of eIF2α as did the known ER stress-inducer thapsigargin (Fig. 2), further supporting the assump-
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Caspase-9 is essential for apoptosis. A, Bak-deficient (Bak−/−) and Bak-reconstituted (Bak+/+) Jurkat cells were left untreated (CO) or treated with cephalostatin 1 (CPH) or as a control with ETO for 24 h. Apoptotic cells were quantified by flow cytometry as described under "Experimental Procedures." Bars, mean ± S.E. of two independent experiments (left panel). Protein extracts from Bak−/− and Bak+/+ cells were prepared, and Bak levels were analyzed by Western blot. β-Actin was used as loading control (right panel). B, Jurkat T cells were transiently transfected with scramble or Apaf-1 siRNA oligonucleotides. At 48 h post-transfection, cells were stimulated with cephalostatin 1 (CPH; 1 μM) or ETO (2 μM) for 16 h, and apoptosis was quantified by flow cytometry. Apaf-1 protein levels in transfected cells were analyzed by Western blot and are shown below (left panel). Cell lysates from cephalostatin 1 (CPH) and ETO-treated cells were prepared, and caspase-9 activation was analyzed by Western blot (right panel). C, Jurkat cells lacking caspase-9 (Casp.9−/−) and Jurkat cells retransfected with full-length caspase-9 (Casp.9+/+) were left untreated (CO) or treated for 24 h with cephalostatin 1 (CPH; 1 μM). Apoptotic cells were quantified by flow cytometry. Protein extracts from caspase-9−/− and caspase-9+/+ cells were prepared, and caspase-9 protein levels were analyzed by Western blot (left panel). Casp.9+/+ and Casp.9+/+ Jurkat cells were left untreated or stimulated with cephalostatin 1 (CPH; 1 μM), and a clonogenic assay was performed as described under "Experimental Procedures." Results are represented as the number of colonies referred to untreated cells (CO) (right panel). Equal protein loading was controlled by staining membranes with Ponceau S (a representative section of the stained membrane is shown). Bars, mean ± S.E. of three independent experiments performed in triplicate. **, p < 0.01; ***, p < 0.001; n.s., not significant (unpaired two-tailed t test).

Cephalostatin 1 activates the ASK1-JNK pathway, which is involved in cell death. The ASK1 and the downstream JNK cascade are known to be activated under conditions of ER stress-induced apoptosis (6). As observed in Fig. 3A, both kinases were activated very early upon cephalostatin 1 treatment. Phosphorylation of ASK1 at Thr-845, which is correlated with ASK1 activity, was observed after only 15 min and maintained up to at least 2 h of treatment. Its downstream kinase JNK was also already phosphorylated after 15 min, and the intensity of phosphorylation increased further in the course of time.

To evaluate the role of the ASK1-JNK pathway in cephalostatin 1-induced apoptosis, two Jurkat clones expressing a dominant negative ASK1 (ASK1-DN) (21) were employed. In both clones of ASK1-DN cells (A2–1, A2–3), only a partial reduction of cephalostatin 1 (1 μM, 24 h)-induced apoptosis was observed as compared with the parental J16 cells (Fig. 3B). TNF-α (10 ng/ml, 24 h) was used as a positive control because TNF-induced apoptosis also partially depends on the activation of ASK1 (25). To verify the lack of ASK1 activity of our ASK1-DN clones, J16 and A2–3 clones were treated with cephalostatin 1 or TNF-α and investigated for JNK phosphorylation by Western blot. As expected, JNK phosphorylation was severely impaired in A2–3 cells in both cases compared with control J16 cells (Fig. 3C). In summary, Fig. 3 shows that the ASK1-JNK cascade seems not to play a major role in cephalostatin 1-induced apoptosis.

Cephalostatin 1-induced activation of caspase-4 is necessary for apoptosis. In humans caspase-4 seems to play a similar role as caspase-12 in mice being localized predominantly to the ER and involved specifically in ER stress-induced apoptosis (10). Fig. 4A shows that cephalostatin 1 as well as the well-known ER stress inducers tunicamycin and thapsigargin activate caspase-4 time-dependently. A strong reduction of the proform was already observed after 4 h of cephalostatin 1 treatment. Employment of the specific caspase-4 inhibitor Z-LEVD-fmk led to a marked inhibition of cephalostatin 1-induced DNA fragmentation (Fig. 4B), demonstrating a crucial role of caspase-4 in the apoptotic pathway of cephalostatin 1. Caspase-4 contribution to tunicamycin and thapsigargin-induced apoptosis was similar to that seen for cephalostatin 1, further supporting its role in ER stress-induced apoptosis. To further prove the involvement of caspase-4 in cephalostatin 1-induced apoptosis, expression of caspase-4 was inhibited by RNA interference. Using two
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expression constructs with different siRNA sequences, Fig. 4C shows that the expression of caspase-4 was strongly down-regulated 24 h after transfection. DNA fragmentation induced by cephalostatin 1 treatment was significantly reduced in the caspase-4 knockdown cells, which is consistent with the results obtained by the specific caspase-4 inhibitor (Fig. 4B). A crosstalk between JNK and caspase-4 has been very recently described (26). However, examination of the processing of caspase-4 in ASK1-DN cells revealed a similar activation as compared with control cells (Fig. 4D). Furthermore, the JNK inhibitor SP600125 did not impair but even increased activation of caspase-4 upon cephalostatin 1 treatment in Jurkat cells.

Cephalostatin 1-activated Caspase-4 Acts Upstream of Caspase-9—We hypothesized that the ER-stress related caspase-4 might be responsible for the activation of caspase-9 seen after treatment with cephialostatin 1. First evidence is given in Fig. 5A (upper panel), showing a strong and time-dependent processing of procaspase-4 in caspase-9-deficient Jurkat cells. The fact that pretreatment of caspase-9−/− cells with the specific caspase-4 inhibitor Z-LEVD-fmk was not able to reduce the DNA fragmentation induced by cephialostatin 1 (Fig. 5A, lower graph) further supports the hypothesis that caspase-4 is activated upstream of caspase-9 and requires the presence of caspase-9 to induce apoptosis.

Second evidence for an upstream role of caspase-4 is shown in Fig. 5, B and C examining the impact of caspase-4 inhibition on activation of caspase-9. Pretreatment of normal Jurkat cells with the peptidic caspase-4 inhibitor Z-LEVD-fmk completely abrogated caspase-9 activity induced by cephialostatin 1 (Fig. 5B). To verify these results, Jurkat cells with strongly reduced caspase-4 expression were generated by siRNAs (Fig. 5C). Stimulated with cephialostatin 1 for 16 and 24 h, and then analyzed for caspase-9 activation by Western blot. Caspase-4 siRNA-treated cells showed a marked inhibition of the activation of caspase-9 in response to cephialostatin 1, confirming the upstream role of caspase-4 in caspase-9 activation. Reduction of caspase-4 expression also inhibited the activation of caspase-9 by thapsigargin and tunicamycin, pointing to a general activation of caspase-4 upstream of caspase-9 in ER stress-induced apoptosis.

DISCUSSION

Employing cephialostatin 1, an experimental anticancer drug that acts independently of cytochrome c release and apoptosisome formation, we were able to define the impact of distinct elements, especially the caspase-4, in the ER stress response leading to apoptotic cell death.
The endoplasmic reticulum (ER) is the organelle responsible for proper post-translational modification of proteins to obtain their mature conformation. When cytotoxic conditions such as alteration in calcium homeostasis compromise the capacity of the ER, a highly conserved UPR is activated (9). Many aspects of the UPR are cytoprotective giving the cell a chance to correct the environment within the ER. In this respect, evidence is given that activation of the UPR can also favor the survival of tumor cells (14). However, prolonged activation of this response can initiate apoptosis (8). At least three pathways (Fig. 6) are known to be involved in UPR-associated apoptosis (12). All of them deserve attention as potential ER-specific targets for chemotherapeutic strategies. The first is the transcriptional activation of the gene for CHOP. The second is activation of the c-Jun N-terminal kinase pathway, which is mediated by activation of apoptosis signal-regulating kinase 1 (ASK1). The third is activation of ER-associated caspase-12 or caspase-4 in humans, respectively. Cephalostatin 1 activates all three pathways indicating the important role of ER stress response for its chemotherapeutic profile (Fig. 6).

CHOP, also known as growth arrest- and DNA damage-inducible gene 153 (GADD153), is one of the highest inducible genes during ER stress, and studies with CHOP−/− animals clearly indicated a role of CHOP in the induction of cell death under conditions associated with malfunction of the ER (11, 27). Expression of CHOP is mainly regulated at the transcriptional level through ER stress transducers including PKR-like ER-localized eIF2α kinase, ATF6, and IRE1 (9, 12). ER stress transducers are activated when BiP, a sensor for ER stress, dissociates from their luminal domains and binds to unfolded proteins (9, 12, 13). Cephalostatin 1 increases the expression of BiP, induces phosphorylation of the eIF2, and moreover augments the
expression of CHOP. All these effects have been described for agents known to induce ER stress such as tunicamycin or thapsigargin (11, 28, 29). The fact that cephalostatin 1 increases CHOP expression deserves attention. As overexpression of CHOP has clearly shown to lead to cell cycle arrest and/or apoptosis (12, 28); pharmacological or genetical increase of CHOP expression may represent a new anticancer strategy.

The second pathway in ER stress is activation of the c-Jun N-terminal kinase. ER stress activates JNKs through activation of IRE1 that bind TRAF2, an adaptor protein that couples plasma membrane receptors to JNK activation (6). JNK activation can promote cell survival as well as death. However, sustained activation of JNK is known to lead to apoptosis and requires activation of ASK1 (6). It is well known that ASK1 is activated in cells treated with death receptor ligands and oxidative stress (25) but also required for ER stress-induced apoptosis (30). Our experiments employing ASK1 dominant-negative Jurkat cell clones support this notion, although the ASK1/JNK pathway may not play a major role in the ER stress pathway used by cephalostatin 1.

Although cephalostatin 1 activates ASK1 and induces a strong and sustained activation of JNK, apoptosis evoked by cephalostatin 1 was only partially reduced in cells deficient of active ASK1.

The third signaling way in ER stress is activation of caspase-12, a caspase identified in mice but not unequivocally identified in human tissue (7). Functional caspase-12 is lacking in most humans (31) because of a frameshift mutation and a premature stop codon. Caspase-4 has recently been identified as a gene homologous to caspase-12 (7, 10). Caspase-12 and caspase-4 are both localized on the cytoplasmic side of the ER and are suggested to function as mediators of ER stress-induced cell death至少在大脑（10, 32）。In this respect, two recent reports argued against...
the role of caspase-12 and caspase-4 in ER-stress-induced apoptosis (33, 34). Certain cell lines (a murine pro-B cell line and a human myeloma cell line), which do not express caspase-12 and caspase-4, respectively, are shown to respond to ER stress agents via apoptotic cell death, which do not differ from cells retransfected with caspase-12 (33). Furthermore, a neural cell line, which has been depleted of caspase-12 by RNA interference is reported to undergo apoptosis upon tunicamycin-induced ER stress (34). Our data certainly support the role of caspase-4 in ER stress-induced apoptosis in human leukemia T cells because the apoptotic efficiency as well as caspase-9 activation of known ER stress agents was significantly reduced in cells with abrogated caspase-4 activity. Thus, the role of caspase-4 or -12 may depend on the cell type and possibly also on the mechanism of action of the ER stress.

In line with this, several mechanisms for the activation of caspase-12 and possibly caspase-4 have been proposed. For example, calpain, a protease that can be activated by calcium released from ER upon ER stress starts cleavage of caspase-12 (35). Caspase-7 for instance is reported to cleave caspase-12 (36) but is not able to cleave caspase-4 upon sustained ER stress (10). Caspase-12 and possibly caspase-4 could also be activated via TRAF2 (37). A possible role of JNK in caspase-4 activation has also been described (26). The proteasome inhibitor bortezomib induces ER stress and leads to apoptosis via a mechanism where JNK seems to be responsible for caspase-4 activation. However, cephalostatin 1-induced caspase-4 activation was neither affected by overexpression of ASK1-DN nor inhibition of JNK.

Furthermore, caspase-12 activation seems to be independent of the formation of the apoptosome because Apaf-1-deficient cells are able to activate caspase-12 (38, 39). Thus, caspase-12 and most likely caspase-4 serve as mediators of an intrinsic apoptosis pathway that is independent of Apaf-1, cytochrome c, and mitochondria. However, a recent study proposes that apoptosome formation is essential for ER stress-induced apoptosis in at least in neural cells (34). In addition, another group reported that overexpression of a dominant-negative caspase-9 initial role of caspase-4.

In summary, our data point to two important findings. First, the ER stress response evoked by cephalostatin 1 accompanied by caspase-4 activation is able to induce cell death without the requirement to reroute signaling to the classical mitochondria pathway because cephalostatin 1 was shown to induce apoptosis independent of an apoptosome formation. Second, cephalostatin 1 uses the ER stress pathway rather than the classical intrinsic mitochondrial pathway, which might be of advantage in the treatment of chemoresistant tumors because of defects in the mitochondrial pathway.

Acknowledgments—The authors thank Drs. Peter H. Krammer and Henning Walczak (German Cancer Research Center, Heidelberg, Germany) for supplying the used Jurkat T cell clones and Dr. Irina Müller for her Ph.D. work initiating this paper.

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