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Biochemical and Biophysical Research Communications 313 (2004) 193–204
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Complestatin prevents apoptotic cell death: inhibition of a mitochondrial caspase pathway through AKT/PKB activation☆

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Received 25 September 2003

Abstract

Complestatin, a bicyclo hexapeptide from *Streptomyces*, was isolated as a possible regulator of neuronal cell death. In this study, we report an anti-apoptotic activity of complestatin and its underlying molecular mechanism. Complestatin blocked TRAIL (TNF-related apoptosis-inducing ligand)-induced apoptosis and activation of caspase-3 and -8 at micromolar concentration levels without inhibiting the catalytic activities of these caspases. Complestatin potently induced a rapid and sustained AKT/PKB activation and Bad phosphorylation, resulting in inhibition of mitochondrial cytochrome *c* release. These anti-apoptotic activities of complestatin were significantly abrogated in cells expressing dominant negative AKT/PKB. Taken together, our results suggest that complestatin prevents apoptotic cell death via AKT/PKB-dependent inhibition of the mitochondrial apoptosis signal pathway. The novel property of complestatin may be valuable for developing new pharmaceutical means that will control unwanted cell death.

Keywords: Complestatin; Apoptosis; TRAIL; AKT/PKB; Caspases

A balance between cell proliferation and death is critical for tissue homeostasis in multicellular organisms. Programmed cell death, or apoptosis, occurs through an evolutionarily conserved cellular program. It is an active process of cell death that occurs in many important physiological conditions, such as embryonic development and tissue remodeling [1,2]. Many diseases are also associated with either too much or too little apoptosis, including cancer, autoimmunity, sepsis, and neurodegenerative diseases [3]. Understanding the biochemical pathways of apoptosis under pathological conditions and the development of specific modulators of apoptotic cell death are valuable for establishing new therapeutic approaches for the prevention of apoptosis-dependent human diseases.

On the molecular level, the apoptotic process is strictly regulated by various internal and external signals and a large number of molecules. Among these molecules, the caspase family of cysteine proteases is crucially involved both in the initiation and final execution of apoptosis [4]. These caspases are synthesized as proenzymes (zymogens) that are activated by proteolysis in response to a variety of apoptotic stimuli. One of the best-characterized caspase activation cascades is triggered by death receptors (DRs), a subset of cytokine receptors of the tumor necrosis factor (TNF) family. So far, six members of this family are known, namely

☆ *Abbreviations:* TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly(ADP-ribose) polymerase; PP1, 4-amino-5-[4-methylphenyl]-7-[*t*-butyl]pyrazolo[3,4-d]pyrimidine; DD, death domain; DED, death effector domain; DISC, death-inducing signaling complex; DN-AKT, dominant negative AKT/PKB; DR, death receptor; ERK, extracellular signal-regulated kinase; FADD, Fas-associated death domain; HA, hemagglutinin; PI3K, phosphatidylinositol 3′-kinase; CPS, complestatin; SDS, sodium dodecyl sulfate; TtCL, TRAIL-treated cytosolic cell lysate.

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doi:10.1016/j.bbrc.2003.11.104

TNF-R1, CD95, TRAMP, TRAIL-R1, TRAIL-R2, and DR6 [5]. The DRs share a common intracellular domain, the death domain (DD), which confers the ability to induce apoptosis. The DD binds to the intracellular adapter Fas-associated death domain (FADD) [6,7] and, in turn, the death effector domain (DED) of FADD interacts with the DED of caspase-8 to recruit this apoptosis-initiating protease to the death receptors [8,9], leading to the assembly of the death-inducing signaling complex (DISC) [10]. Caspase-8 is activated through trans- and autocatalytical cleavage in the DISC [11] and activated caspase-8 leads to cleavage of downstream effector caspases by sequential reactions, namely cleavage of cytosolic p22 Bid, release of mitochondrial cytochrome *c* into cytosol, and activation of caspase-9 [12]. In turn, caspase-9 cleaves and activates procaspase-3 and ultimately leads to apoptosis [8,9]. Besides this caspase signaling cascade, it has been also shown that activated caspase-8 directly activates caspase-3 [13] and conversely caspase-8 can be active downstream of caspase-3 [14,15].

Recent studies have demonstrated an important role for the serine/threonine kinase AKT/PKB in promoting cell survival. This enzyme is activated in response to many extracellular stimuli including insulin-like growth factor, nerve growth factor, and lysophosphatidic acid [16–19]. Several downstream targets of the AKT/PKB signaling pathway that may underlie the ability of this regulatory cascade to promote survival have been recently identified. These substrates include two components of the intrinsic cell death machinery, Bad and caspase-9, transcription factors of the forkhead family, and a kinase, IKK, that regulates the NF-κB transcription factor [19,20]. In particular, the Bcl-2 family member Bad, which directly interacts with prosurvival Bcl-2 family members such as Bcl-X_L and thus blocks Bcl-X_L-dependent cell survival, is the first component of the apoptotic machinery found to be phosphorylated by AKT/PKB [21–23]. Phosphorylated Bad appears to be the inactive form incapable of binding Bcl-X_L and sequestered in the cytosol [24]. Thus, phosphorylation of Bad by AKT/PKB inactivates its ability to cause cell death and promotes cell survival.

Complestatin, a bicyclo hexapeptide isolated from *Streptomyces*, was originally found to have potent anticomplement activity [25]. Additionally, it is known to be the first gp120-CD4 binding inhibitor of microbial origin [26] and enhance binding of plasminogen to cells and fibrin [27]. Recently, we have demonstrated that complestatin has the ability of protecting neurons from exitotoxicity induced by *N*-methyl-D-aspartate, α-amino-3-hydroxy-5-methyl-4-isoxasolepropionic acid, or kinate [28,29]. Moreover, it protected cortical neurons from prolonged deprivation of oxygen and glucose in vitro and prevented neuronal cell death evolving within the inner nuclear and ganglion cell layers, after transient retinal ischemia [28]. Thus, complestatin is thought to be a novel neuroprotective molecule capable of blocking excitotoxic cell death caused by hypoxic-ischemia and other acute brain injuries. However, molecular mechanisms by which complestatin prevents cell death are poorly understood.

In this study, we sought to investigate anti-apoptotic activity of complestatin and its underlying molecular mechanism.

Materials and methods

Materials. DMEM, penicillin, and streptomycin were purchased from Life Technologies (Grand Island, NY). Benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk), *N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA), *N*-acetyl-Ile-Glu-Thr-Asp-*p*-nitroanilide (Ac-IETD-pNA), and *N*-acetyl-Leu-Glu-His-Asp-*p*-nitroanilide (Ac-LEHD-pNA) were from Alexis (San Diego, CA). [^{35}S]Methionine was purchased from Amersham–Pharmacia Biotech. LY294002, PP1, and U0126 were from BIOMOL (Plymouth Meeting, PA). Cytochrome *c* antibody was obtained from PharMingen (San Diego, CA) and antibodies for caspase-3, caspase-8, caspase-9, p-Bad (S-136), Bad, Bax, Bcl-2, Bid, and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for p-AKT/PKB (S-473) and AKT/PKB were obtained from New England Biolabs (Beverly, MA). All other reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Cells and culture condition. HeLa cells were grown onto a 100 mm culture plate in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a CO_2 incubator (5% CO_2) at 37 °C. To induce apoptosis, serum-free medium was used. To prepare cells overexpressing hemagglutinin (HA)-tagged wild type AKT/PKB (HA-AKT) or kinase-dead form of AKT/PKB (K179M, DN-AKT), HeLa cells were transfected for 12 h in 6-well tissue culture plates with 2 μg of the empty vector (pcDNA3) or vector containing the gene for HA-AKT or DN-AKT using LipofectAMINE plus (Life Technologies). Clonal selection was performed by adding neomycin to the medium at 200 μg/ml final concentration 2 days after the transfection. After 3 weeks, several clones were isolated using cloning rings. Selected clones were then maintained in medium supplemented with neomycin (100 μg/ml), and only low passage number cells ($p < 10$) were used for the experiments.

Cell viability assay. HeLa cells (2×10^5 cells/well) grown in 12-well were exposed to TRAIL (200 ng/ml). Viable cells were stained with crystal violet for HeLa cells followed by spectrophotometric analysis as described previously [30]. In brief, cells were stained with 0.5% crystal violet in 30% ethanol and 3% formaldehyde for 10 min at room temperature, and then plates were washed 4 times with tap water. Cells were solublized with 1% sodium dodecyl sulfate (SDS) solution and dye uptake was measured at 550 nm using a microplate reader.

Assay of caspase activity. The cell pellets were washed with ice-cold PBS and resuspended in 100 mM Hepes buffer, pH 7.4, containing protease inhibitors (5 μg/ml aprotinin and pepstatin, 10 μg/ml leupeptin, and 0.5 mM phenylmethanesulfonyl fluoride). The cell suspension was lysed by three freeze–thaw cycles and the cytosolic fraction was obtained by centrifugation at 12,000*g* for 20 min at 4 °C. DEVDase, IETDase, and LEHDase activities were evaluated by measuring proteolytic cleavage of chromogenic substrates Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, which were used as the substrates for caspase-3, -8, and -9-like proteases, respectively. Briefly, cell lysate (50 μg of protein) was added into the buffer containing 150 μM

Ac-DEVD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA in a final volume of 150 μl in the presence or absence of the caspase inhibitor (100 μM z-VAD-fmk; a pan-caspase inhibitor, 100 μM Ac-DEVD-CHO; a specific caspase-3 inhibitor, and 100 μM Ac-IETD-CHO; a specific caspase-8 inhibitor). The reaction mixture was incubated at 37 °C for 1 h. The increase in absorbance of enzymatically released pNA was measured at 405 nm in a microplate reader every 20 min.

In vitro cleavage of PARP. [^{35}S]Methionine-labeled poly(ADP-ribose) polymerase (PARP) was synthesized using a transcription/translation-coupled transcription and translation system (Promega, Madison, WI). Aliquots (8 μl) of in vitro translated [^{35}S]-labeled PARP were incubated with 50 μg of cytosolic protein in 30 μl of the total reaction volume at 37 °C for 1 h. The reaction was stopped by mixing with an equal volume of 2× SDS–PAGE sample buffer and boiling for 2 min. Cleavage profiles of PARP were examined by electrophoresis on 10% SDS gel and autoradiography.

Detection of cytochrome c release. HeLa cells incubated with TRAIL (200 ng/ml) were harvested and resuspended in ice-cold isolation buffer (20 mM Hepes, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 10 mM phenylmethanesulfonyl fluoride, 10 μM leupeptin, and 250 mM sucrose). After homogenization using a Dounce homogenizer, cell lysate was centrifuged at 12,000*g* for 20 min at 4 °C. The cytosolic fraction was collected and loaded onto a 15% SDS gel. The released cytochrome *c* was detected by immunoblotting.

Western blot analysis. After treatment with different reagents as described in figure legends, cells were collected and lysed in SDS–PAGE sample buffer. The samples were boiled for 10 min and proteins were separated on SDS–PAGE and then transferred onto polyvinylidene difluoride membrane. The blots were blocked for 2 h at room temperature with TBST (10 mM Tris–HCl, 1.4 M NaCl, and 0.1% Tween 20) containing 3% bovine serum albumin for AKT/PKB and p-AKT/PKB, and 5% non-fat dried milk for the others. The blocked membranes were washed three times with TBST, then incubated with secondary antibody for 30 min, and sufficiently washed with TBST, and the immunoreactive bands were visualized using a chemiluminescent substrate.

Data analysis and statistics. All data were expressed as means ± SD of at least three independent experiments. Statistical comparisons between groups were performed using Student's *t* test.

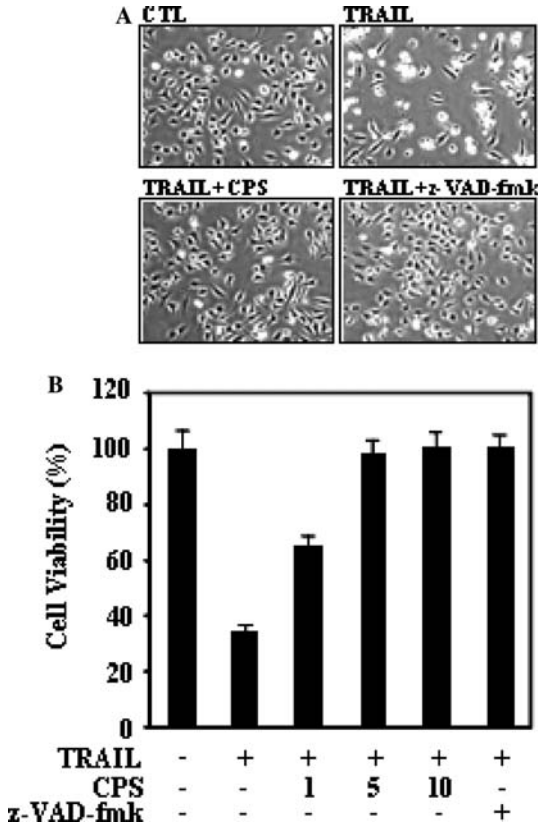

Fig. 1. Complestatin protects HeLa cells from TRAIL-induced apoptosis. (A) HeLa cells were preincubated for 30 min with 10 μM complestatin (CPS) or for 1 h with 100 μM z-VAD-fmk and then exposed to TRAIL. After 8 h TRAIL stimulation, morphological features were analyzed with phase contrast microscopy. (B) HeLa cells were preincubated for 30 min with various concentrations (1–10 μM) of complestatin or for 1 h with 100 μM z-VAD-fmk and then exposed to TRAIL. Viable cells were stained with crystal violet, followed by spectrophotometric analysis. Viability of control cells was set at 100% and viability relative to the control is presented. The data represent means ± SD of three independent experiments.

Results

Complestatin protects cells from TRAIL-induced apoptosis

To investigate anti-apoptotic activity of complestatin, we tested the effect of complestatin on TRAIL-induced HeLa cell apoptosis. Microscopic analysis showed that treatment of HeLa cells with 200 ng/ml TRAIL underwent apoptotic cell death within 8 h, evident by membrane blebbing and blistering (Fig. 1A). This TRAIL-induced apoptosis was completely blocked by pretreatment with 100 μM z-VAD-fmk, a pan-caspase inhibitor, indicating a caspase-dependent cell death. Under the same condition, 10 μM complestatin also inhibited TRAIL-induced apoptosis. The effect of complestatin on cell viability was quantitatively assayed by staining with crystal violet. At a concentration of 5 μM, complestatin almost completely reversed the TRAIL effect (Fig. 1B). These results indicate that complestatin can block apoptotic cell death induced by TRAIL.

Complestatin inhibits TRAIL-induced caspase activation but has no direct effect on the catalytic activity of caspase-3

To further determine mechanisms of anti-apoptotic action of complestatin, we examined the effect of complestatin on DEVDase (caspase-3-like protease) activity. DEVDase activity was measured in the cytosol from HeLa cells by a colorimetric assay using the tetrapeptide substrate Ac-DEVD-pNA. Treatment of HeLa cells with TRAIL increased DEVDase activity by 8-fold at 8 h compared with that of the untreated control cells (Fig. 2A). This increase in DEVDase activity was completely blocked by pretreatment of HeLa cells with either complestatin or z-VAD-fmk prior to TRAIL addition. To determine whether complestatin acts as a direct inhibitor of caspase-3, we examined the effect of complestatin on proteolytic cleavage of Ac-DEVD-pNA, a chromogenic substrate, and PARP, a well-known biosubstrate of caspase-3, by lysates prepared

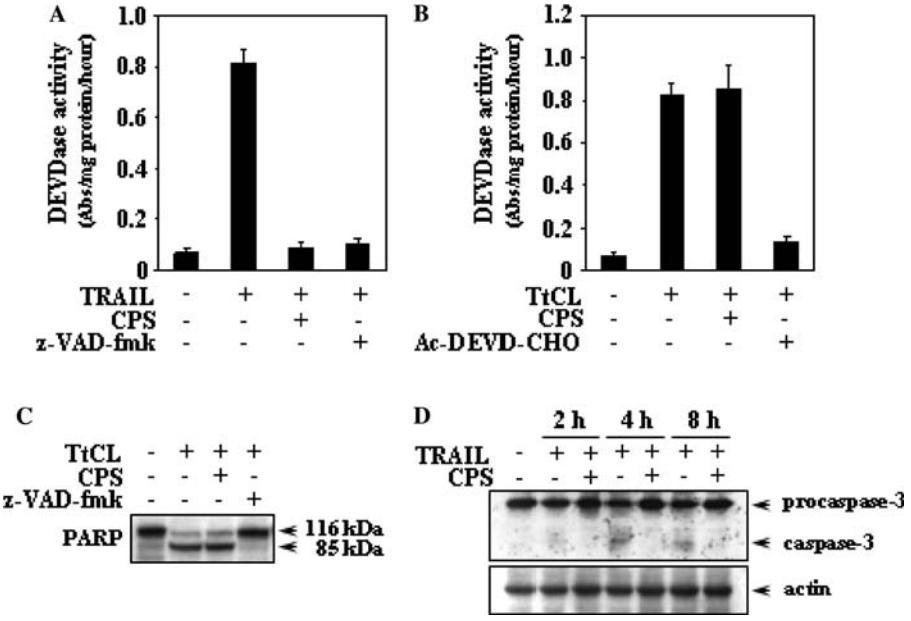

Fig. 2. Complestatin inhibits activation of caspase-3 by TRAIL but has no direct effect on the catalytic activity of caspase-3. (A) HeLa cells cultured in 100-mm plates were treated for 8 h with TRAIL (200 ng/ml) in the presence or absence of 10 μM complestatin (CPS) or 100 μM z-VAD-fmk. Cytosolic cell lysates (50 μg) were prepared and then assayed for DEVDase activity (caspase-3-like activity) as described under "Materials and methods." (B) HeLa cells treated with TRAIL for 8 h and then cytosolic cell lysates were prepared as described under "Materials and methods." Ac-DEVD-CHO (100 μM), a specific caspase-3 inhibitor, or 10 μM complestatin (CPS) was added to TRAIL treated-cytosolic cell lysates (TtCL, 50 μg), and then caspase-3-like enzymatic activity was measured with Ac-DEVD-pNA in a colorimetric assay (means ± SD; $n = 3$). (C) TRAIL treated-cytosolic cell lysates (TtCL, 50 μg) were mixed with 100 μM z-VAD-fmk or 10 μM complestatin (CPS) and then incubated with aliquots (8 μl) of in vitro translated [^{35}S]-labeled PARP in 30 μl of the total reaction volume at 37 °C for 1 h. Cleavage profiles of PARP were examined by electrophoresis on 10% SDS–PAGE and protein was analyzed by autoradiography. The upper band indicates 116 kDa PARP whereas the lower band indicates the 85 kDa apoptosis-related cleavage fragment. (D) HeLa cells were treated for 2, 4, and 8 h with TRAIL in the presence or absence of 10 μM complestatin (CPS). Cell lysates were immunoblotted with antibody against caspase-3. Actin was used as a loading control.

from HeLa cells treated with TRAIL for 8 h. Cleavage of both Ac-DEVD-pNA and PARP was significantly inhibited by the specific caspase-3 inhbitor Ac-DEVD-CHO and z-VAD-fmk but not affected by complestatin (Figs. 2B and C). These results suggest that complestatin does not act as a direct inhibitor of active caspase-3 but does inhibit the upstream components of intracellular caspase-3 activation signaling pathways. Thus, we investigated the effect of complestatin on TRAIL-induced caspase-3 activation by measuring appearance of the active fragment (p17) of caspase-3 by immunoblot analyses. As shown in Fig. 2D, TRAIL induced the cleavage of pro-caspase-3 (p32) into a 17-kDa fragment. This event was suppressed by the addition of complestatin or z-VAD-fmk (Fig. 2D), indicating that complestatin inhibits caspase-3 activation signaling pathway.

Complestatin inhibits caspase-9 activation and cytochrome c release

Previous studies have shown that TRAIL-induced caspase-3 activation is preceded by mitochondrial apoptotic events including release of mitochondrial cytochrome *c* into cytosol and activation of caspase-9 [12]. We therefore sought to examine the activation of caspase-9 and the release of mitochondrial cytochrome *c* in cells treated with TRAIL in the absence or presence of complestatin. As shown in Fig. 3A, the level of procaspase-9 in HeLa cells was significantly reduced by TRAIL treatment and this effect was reversed by complestatin, indicating that complestatin inhibits TRAIL-induced caspase-9 activation. Consistently, TRAIL-induced caspase-9 activity was also blocked by pretreatment of cells with complestatin (Fig. 3B). In addition, TRAIL-induced release of cytochrome *c* to cytosol was suppressed by the addition of complestatin or z-VAD-fmk (Fig. 3C). These results suggest that complestatin inhibits upstream of mitochondria-dependent caspase activation.

Complestatin inhibits TRAIL-induced cleavage of caspase-8 and Bid but has no effect on the catalytic activity of caspase-8

TRAIL-mediated apoptosis requires activation of apical caspase-8 and subsequent cleavages of Bid, which leads to the release of cytochrome *c* from the

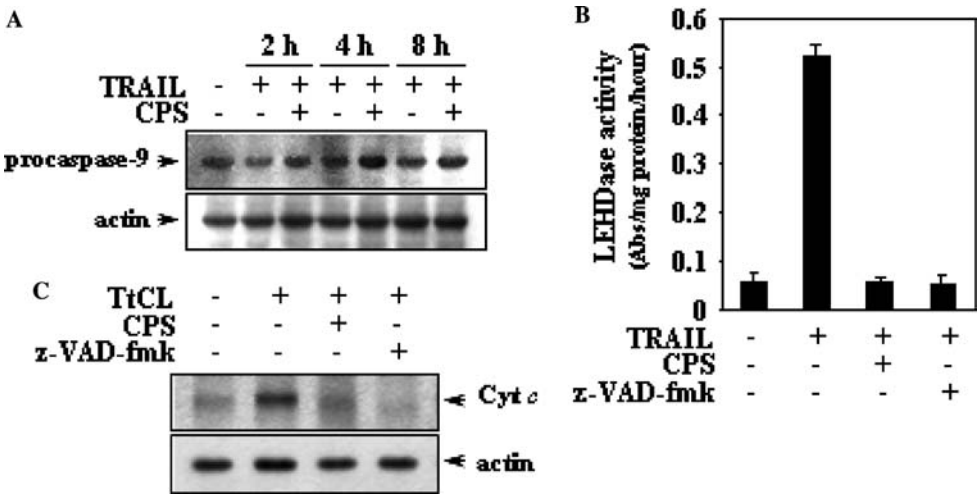

Fig. 3. Complestatin inhibits caspase-9 activation and cytochrome *c* release. (A) HeLa cells were treated with TRAIL (200 ng/ml) for the indicated times in the presence or absence of 10 μM complestatin (CPS). Protein extracts were prepared and subjected to SDS–PAGE. Western blotting with an anti-caspase-9 antibody was performed as described under "Materials and methods." Actin was used as a protein loading control. (B,C) HeLa cells were treated for 8 h with TRAIL in the presence and absence of 10 μM complestatin (CPS) or 100 μM z-VAD-fmk. Cytosolic cell lysates were prepared and 50 μg proteins were assayed for LEHDase activity (caspase-9-like activity) (means ± SD; $n = 3$) (B). Cytochrome *c* release from cytosolic fraction was analyzed as described under "Materials and methods" (C). Actin was used as a protein loading control.

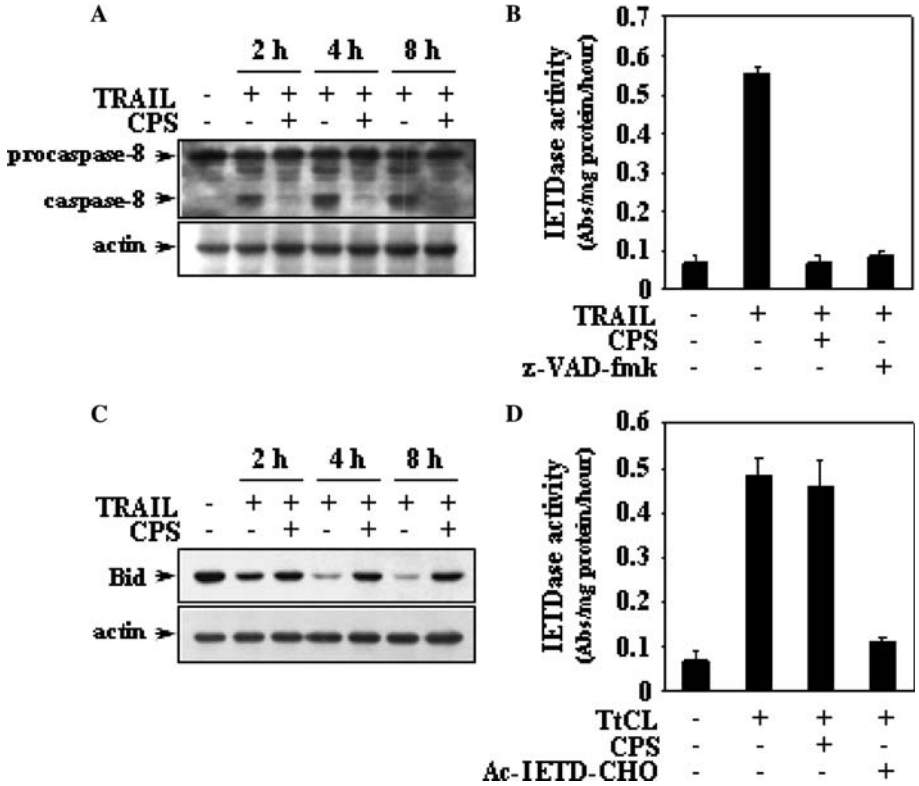

Fig. 4. Complestatin inhibits TRAIL-induced cleavage of caspase-8 and Bid but has no effect on the catalytic activity of caspase-8. HeLa cells were treated for 2, 4, and 8 h with TRAIL in the presence or absence of 10 μM complestatin (CPS). Protein extracts were prepared and subjected to SDS–PAGE. Western blotting with an anti-caspase-8 (A) or anti-Bid antibody (C) was performed as described under "Materials and methods." Actin was used as a protein loading control. (B) HeLa cells that were treated for 8 h with TRAIL in the presence and absence of 10 μM complestatin (CPS) or 100 μM z-VAD-fmk. Cytosolic cell lysates were prepared and 50 μg proteins were assayed for IETDase activity (caspase-8-like activity) as described under "Materials and methods." (D) HeLa cells treated with TRAIL for 8 h and then cytosolic cell lysates were prepared as described under "Materials and methods." Ac-IETD-CHO (100 μM), a specific caspase-8 inhibitor, or 10 μM complestatin (CPS) was added to TRAIL treated-cytosolic cell lysates (TtCL, 50 μg) and then caspase-8-like enzymatic activity was measured with Ac-IETD-pNA in a colorimetric assay (means ± SD; $n = 3$).

mitochondria [12]. We therefore examined the effect of complestatin on activation and catalytic activity of caspase-8. Pretreatment of HeLa cells with complestatin prior to TRAIL stimulation inhibited cleavage of procaspase-8 into its active form (Fig. 4A) and caspase-8 activity was also blocked by pretreatment of cells with complestatin (Fig. 4B). Activated caspase-8 is known to cleave cytosolic p22 Bid at its amino terminus and generate a p15 tBid (truncated Bid) that translocates to the mitochondria [12]. As expected, complestatin blocked TRAIL-induced Bid cleavage in HeLa cells (Fig. 4C).

To determine whether complestatin acts as a direct inhibitor of caspase-8, we sought to examine the effect of complestatin on the enzymatic activity of caspase-8. Lysates prepared from HeLa cells treated with TRAIL for 8 h possessed ~7-fold higher caspase-8 activity compared to that from untreated control cells (Fig. 4D). This caspase activity was significantly inhibited by the specific caspase-8 inhibitor Ac-IETD-CHO but not affected by complestatin (Fig. 4D). Taken together, these results indicate that complestatin suppresses caspase-8 activation but has no effect on the catalytic activity of caspase-8.

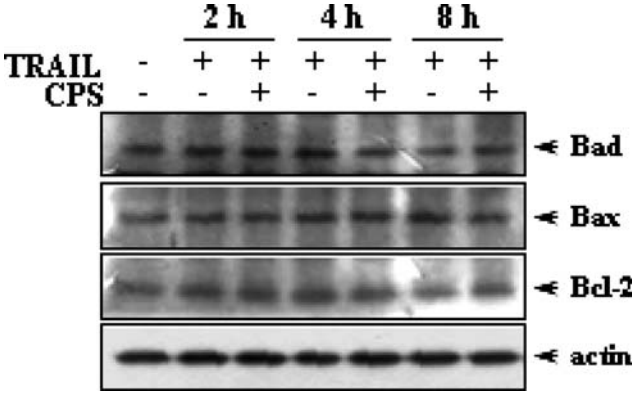

Fig. 5. Complestatin has no significant effect on expression of Bad, Bax, and Bcl-2. HeLa cells were treated with TRAIL (200 ng/ml) for the indicated times in the presence or absence of 10 μM complestatin. Protein extracts were prepared and subjected to SDS–PAGE. Immunoblotting with an anti-Bad, anti-Bax, or anti-Bcl-2 antibody was performed as described under "Materials and methods." Actin was used as a loading control.

Complestatin induces phosphorylation of AKT/PKB and Bad

The Bcl-2 family, comprised of both pro-apoptotic and anti-apoptotic members, constitutes a critical intracellular checkpoint for apoptosis within a common cell death pathway [31]. Thus, a careful balance in gene expression of Bcl-2 family members is crucial in determining the fate of cells that undergo apoptosis. We therefore investigated the effects of TRAIL and complestatin on the expression of Bcl-2 family proteins in HeLa cells treated with TRAIL in the absence or presence of complesatin for various time periods. Immunoblot analyses revealed that both TRAIL and complestatin had no significant effect on the expression

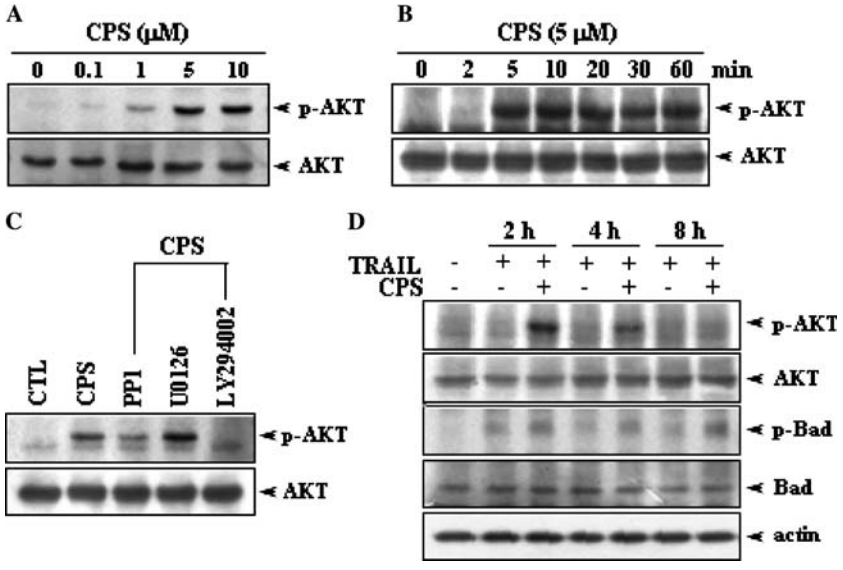

Fig. 6. Complestatin induces phosphorylation of AKT/PKB and Bad. HeLa cells were treated with various concentrations of complestatin for 10 min (A) or with 5 μM complestatin for the indicated times (B). HeLa cells were pretreated for 30 min with or without 10 μM PP1, 5 μM U0126, or 5 μM LY294002 prior to stimulating with complestatin (5 μM) for 10 min (C). Cell lysates were separated by SDS–PAGE and immunoblotted with an anti-phospho-S473 AKT/PKB or anti-AKT/PKB antibody. (D) HeLa cells were treated with TRAIL (200 ng/ml) for the indicated times in the presence or absence of 10 μM complestatin. Cell lysates were electrophoresed and immunoblotted with an anti-phospho-S473 AKT/PKB, anti-AKT/PKB, anti-phospho-Bad, or anti-Bad antibody. Actin was used as a loading control. CTL indicates control.

of Bcl-2 family proteins such as Bad, Bax, and Bcl-2 (Fig. 5).

Previous studies have demonstrated that activation of the serine/threonine kinase AKT/PKB by phosphorylation at Ser-473 plays an important role in survival when cells are exposed to different apoptotic stimuli such as growth factor withdrawal and administration of TRAIL, anti-Fas antibody, or transforming growth factor-β [32–39]. Also, this enzyme is activated in response to a number of different growth factors [19]. Therefore, the involvement of AKT/PKB activation in anti-apoptotic function of complestatin was assessed in HeLa cells exposed to various concentrations of complestatin for 10 min or stimulated with 5 μM complestatin for various time periods. Activation of AKT/PKB was analyzed by immunoblotting using a specific antibody against phosphorylated AKT/PKB at Ser-473. Complestatin induced AKT/PKB phosphorylation in a dose- and time-dependent manner. Maximal activation was observed after 5 min of complestatin stimulation and the effect was sustained for up to 60 min (Figs. 6A and B). Complestatin was also observed to induce AKT/PKB phosphorylation in other cell lines such as human umbilical vein endothelial cells, human embryonic kidney 293 cells, and EC219, rat microvascular endothelial cells (data not shown). To further delineate the complestatin-mediated anti-apoptotic signal pathway in HeLa cells, we were prompted to explore the upstream signaling mechanisms related to activation of AKT/PKB. AKT/PKB is known to be regulated by phosphoinositide 3-kinase (PI3K), which is activated by either receptor tyrosine kinase or non-receptor tyrosine kinase c-Src [16,40]. Pretreatment of HeLa cells with either PP1, an inhibitor for Src family kinases, or LY294002, a PI3K inhibitor, significantly or completely blocked AKT/PKB phosphorylation by complestatin. In addition, genistein, a tyrosine kinase inhibitor, also prevented complestatin-induced AKT/PKB phosphorylation, whereas the MAPK inhibitor U0126 had no effect (data not shown and Fig. 6C). These results indicate that complestatin induces AKT/PKB activation via the tyrosine kinase/PI3K-dependent signaling pathway.

It has been reported that AKT/PKB phosphorylates pro-apoptotic protein Bad specifically at Ser-136 and this phosphorylation connects proximal survival signals to the Bcl-2 family [19]. We investigated the effects of complestatin on Bad phosphorylation by immunoblot analysis using anti-phospho-Ser-136 Bad antibody. As shown in Fig. 6D, phosphorylation of Bad, concurrently with AKT/PKB phosphorylation, was significantly increased in complestatin-treated HeLa cells. Taken together, these results suggest that complestatin may prevent TRAIL-induced cell death via activation of AKT/PKB, which leads to Bad phosphorylation.

AKT/PKB is required for the protective effects of complestatin against apoptosis

Expression of catalytically inactive AKT/PKB (K179M, DN-AKT) has been demonstrated to act as a dominant negative blocking AKT/PKB phosphorylation of its anti-apoptotic substrates in several different cell types [19,43,44]. To further determine the role of

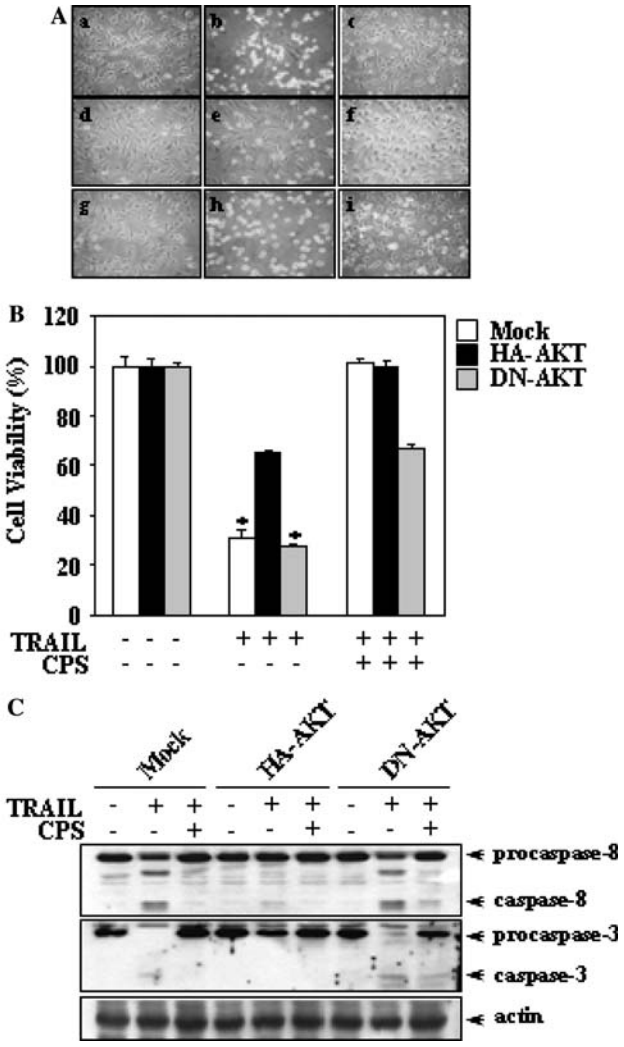

Fig. 7. AKT/PKB is involved in the inhibitory effect of complestatin on apoptosis as well as activation of caspase-8 and -3 induced by TRAIL. (A) HeLa cells transfected with an empty vector (pcDNA3) (Mock, a–c) or HeLa cells overexpressing wild type AKT/PKB (HA-AKT, d–f) or dominant negative AKT/PKB (DN-AKT, g–i) were incubated without (a,d,g) or with TRAIL (200 ng/ml) in the presence (c,f,i) or absence (b,e,h) of 10 μM complestatin. After 8 h, photographs were taken. (B) Viable cells were stained with crystal violet, followed by spectrophotometric analysis. Viability of control cells was set at 100% and viability relative to the control is presented. Data represent means ± SD ($n = 3$), $^*p < 0.01$. (C) Indicated cells were treated for 8 h with TRAIL (200 ng/ml) in the presence or absence of 10 μM complestatin. Protein extracts were prepared and subjected to SDS–PAGE. Western blotting with an anti-caspase-8 or -3 antibody was performed as described under "Materials and methods." Actin was used as a loading control.

AKT/PKB in cell survival signaling elicited by complestatin, the anti-apoptotic effect of complestatin on HeLa cells stably transfected with dominant negative AKT/PKB (DN-AKT) was compared with its effects on control cells transfected with an empty vector (pcDNA3) or cells overexpressing wild type AKT/PKB (HA-AKT). At first, HeLa cells transfected with an empty vector, HA-AKT, or DN-AKT were treated with TRAIL in the absence of complestatin and apoptotic cell death was quantified after 8 h. By comparison to control cells, TRAIL-induced apoptosis was significantly decreased in HA-AKT overexpressing cells, but slightly increased in DN-AKT expressing cells (Figs. 7A and B). These results indicate that the AKT/PKB activity can change the apoptotic potential of HeLa cells in response to TRAIL. If AKT/PKB activation is crucial for the anti-apoptotic effect of complestatin, suppression of AKT/PKB phosphorylation by its dominant negative form will abrogate cell survival mediated by complestatin. As expected, in cells transfected with empty vector or HA-AKT complestatin almost completely blocked TRAIL-induced apoptosis, whereas in cells expressing DN-AKT complestatin only partially reversed the apoptotic response to TRAIL (Figs. 7A and B). These data suggest that AKT/PKB activation is required for complestatin survival response against TRAIL-induced apoptosis.

Classically, caspase-8 is an initiator caspase involved in TRAIL-mediated apoptotic signal cascade [12] and AKT/PKB exerts anti-apoptotic effects through phosphorylation of substrates such as Bad and caspase-9 [41,45], which are downstream molecules of caspase-8. However, the data presented here suggest that complestatin is a strong agonist of AKT/PKB signaling pathway and effectively blocks activation of caspase-8 triggered by TRAIL (Figs. 2–4). The potential role of AKT/PKB pathway in controlling caspase-8 activation is further supported by the additional observation that caspase-8 activation by TRAIL was slightly reduced in HeLa cells overexpressing HA-AKT, in comparison to control cells (Fig. 7C). Furthermore, in comparison to both control and HA-AKT overexpressing cells, the inhibitory effect of complestatin on TRAIL-induced caspase-8 activation was significantly reduced in DN-AKT expressing cells (Fig. 7C). In parallel, the pattern of caspase-3 cleavage in the blot is similar to that of caspase-8 (Fig. 7C). These results further indicate that the AKT/PKB activity negatively regulates activation of caspase-8 and -3 induced by TRAIL.

Discussion

Recently, we have reported that complestatin isolated from *Streptomyces* has the ability of protecting neuronal cell death caused by exitotoxicity as well as prolonged deprivation of oxygen and glucose in vitro and transient retinal ischemia in vivo [28]. However, the mechanisms of cell survival mediated by complestatin have heretofore been uncharacterized. In this study, the anti-apoptotic activity of complestatin and its underlying molecular mechanisms were investigated by employing a TRAIL-mediated apoptotic cell death system. Complestatin was found to almost completely block TRAIL-induced apoptosis of HeLa cells at micromolar concentrations. Complestatin inhibited activation of multiple caspases from their inactive proenzymes, including those involved in the death receptor pathway (caspase-8) and the mitochondrial pathway (caspase-9) as well as the executioner caspase-3. Using in vitro caspase assays, complestatin was shown to have no significant effect on the catalytic activity of caspases. These results indicate that the anti-apoptotic function of complestatin is mediated through inhibition of caspase activation signaling pathway.

TRAIL is known to induce apoptosis by interacting with two cell–surface death receptors—DR4 (or TRAIL-R1) and DR5 (or TRAIL-R2) [46]. Previously, it has been reported that two types of cells exist that differ with respect to their requirement for mitochondria during death receptor-mediated apoptosis [15]. In type I cells, caspase-8 is activated without involvement of mitochondria to a level sufficient to process the effector caspase-3 and death receptor signaling is not blocked by Bcl-2/Bcl-X_L [15]. In contrast, in type II cells a mitochondria-dependent amplification loop is required to fully activate caspase-8 and transduce an apoptotic signal [15]. In these cells, TRAIL-induced apoptotic signaling is transmitted by sequential reactions, activation of caspase-8 in the DISC, cleavage of cytosolic p22 Bid, release of mitochondrial cytochrome *c* into cytosol, activation of caspase-9, and finally the executioner caspase-3 [47]. The data presented here demonstrate that in HeLa cells TRAIL induces caspase-dependent apoptosis and conducts mitochondria-dependent apoptotic signaling cascade, suggesting that HeLa cell is categorized in type II cells. Complestatin suppressed caspase-3 activity by blocking TRAIL-induced cleavage of caspase-3 from its proform (Fig. 2). Indeed, complestatin was found to inhibit signaling events upstream of caspase-3 activation such as mitochondrial cytochrome *c* release and activation of caspase-9. These results indicate that mechanism of the anti-apoptotic activity of complestatin involves, at least in part, prevention of the release of pro-apoptotic factors from the mitochondria.

A number of recent studies have shown that release of pro-apoptotic proteins from mitochondria including cytochrome *c* [48], apoptosis-inducing factor [49], and SMAC/DIABLO [50,51] is modulated by targeting of Bcl-2 family members to the outer mitochondrial membrane [52]. These include proteins that predispose cells to apoptosis, such as Bad and Bax, and proteins

that antagonize apoptosis, such as Bcl-X_L or Bcl-2. The balance between these proteins is important for release of mitochondrial pro-apoptotic proteins. However, the present study showed that the protein levels of Bcl-2 family members were not significantly changed by complestatin. Alternatively, extracellular stimuli-mediated signaling pathways that lead to the MAPK kinase MEK or AKT/PKB have been recently analyzed for their roles in suppressing mitochondria-dependent apoptosis in a wide range of cell types [19,32,53]. Both kinases were found to promote cell survival through phosphorylation and inactivation of the pro-apoptotic protein Bad, which is capable of forming heterodimers with the anti-apoptotic proteins Bcl-X_L or Bcl-2 and antagonizes their anti-apoptotic activity [21]. Whereas Bad can be phosphorylated at either Ser-112 or Ser-136 by extracellular signal-regulated kinase (ERK) [54], AKT/PKB phosphorylates Bad specifically at Ser-136 [41,42]. Therefore, the ability of complestatin to induce Bad phosphorylation at the AKT/PKB site was assessed. In cells treated with complestatin, phosphorylation of Bad at Ser-136 was significantly increased compared to the control cells (Fig. 6D). Consistently, complestatin rapidly induced AKT/PKB activation and this effect was sustained for up to 4 h (Fig. 6D). Using pharmacological approaches, Src and PI3K were also identified as upstream signaling components of AKT/PKB activation in response to complestatin (Fig. 6C). Furthermore, the role of AKT/PKB in anti-apoptotic activity of complestatin was demonstrated using molecular approaches (Fig. 7). However, the survival effect of complestatin was not completely reduced in cells expressing a dominant negative AKT/PKB, implying the contribution of other anti-apoptotic signaling pathways. Indeed, in HeLa cells complestatin was observed to induce a rapid activation of ERK in a Src-dependent manner (data not shown). Since the role of the MAPK pathway in protecting cells from DR-mediated apoptosis has been reported [55], it is suggested that the Src-ERK pathway may in part participate in mediating anti-apoptotic response of complestatin.

Interestingly, the data demonstrated that complestatin markedly suppressed caspase-8 processing in response to TRAIL. In general, caspase-8 is thought to be an initiator caspase triggered by DRs and its activation occurs at the DISC. This activation can be blocked by cFLIP, an inhibitor of the Fas signaling pathway with a structural similarity to caspase-8 [56]. It binds to FADD and interferes with the proper formation of the DISC complex, thus representing a naturally occurring dominant negative form of caspase-8 [56]. The ability of complestatin to induce increases in cFLIP expression was also evaluated. The expression level of cFLIP was not significantly altered by complestatin (data not shown). It has been also known that TRAIL activates caspase-8 and then cleaves cFLIP into intermediate fragments in the DISC [57]. In other studies, TRAIL-induced cleavage of cFLIP was found to be inhibited by complestatin (data not shown), further supporting complestatin's inhibitory effect on caspase-8 activation. Alternatively, the possibility exists that complestatin may suppress feedback activation of caspase-8 via mitochondria-dependent activation of caspase-9 and -3. A schematic model that would account for the observations reported here as well as these other possibilities is presented in Fig. 8.

Recently, it has been demonstrated that caspase-8 can be activated by caspase-3. In type II cells, caspase-8 is

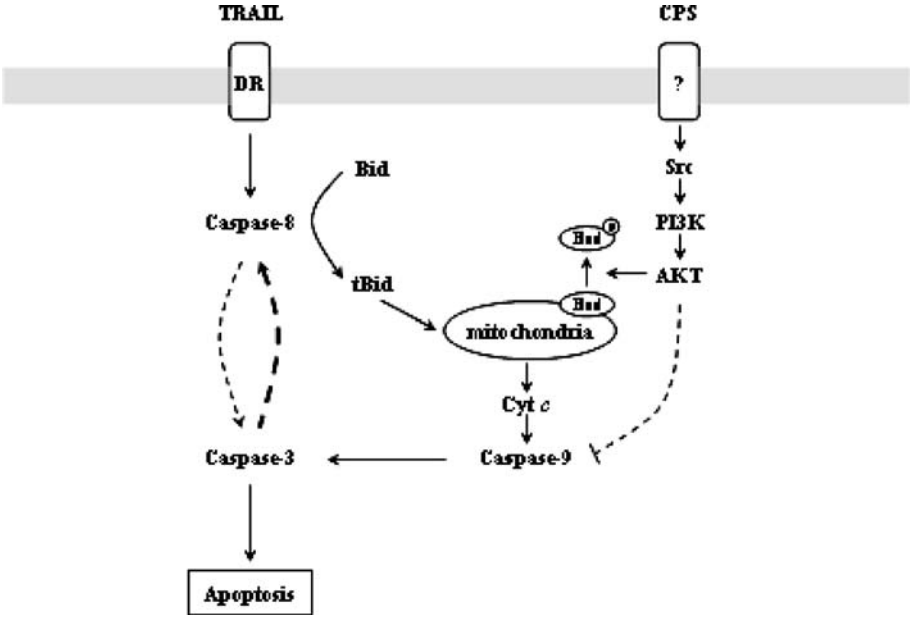

Fig. 8. Model for the mechanisms by which complestatin protects HeLa cells from TRAIL-induced apoptosis.

mainly activated after the mitochondrial amplification loop, when apoptotic signals were triggered by DRs [15]. In iron chelator-induced apoptosis, the chelator tachpyridine sequentially activated caspase-9, -3, and -8 [58]. Activation of caspase-8 was independent of FADD signaling, whereas tachpyridine-mediated cell death was blocked in cells microinjected with Bcl-X_L and completely inhibited in cells microinjected with a dominant-negative caspase-9 expression vector [58]. It has also been shown that caspase-8 activation or apoptosis induction by chemotherapeutics was not blocked by a FADD dominant-negative and that caspase-8 activation can occur downstream of caspase-9 and -3 in response to anti-cancer drugs [59,60]. Therefore, the lack of caspase-8 activation by complestatin hints at a defect in the mitochondrial amplification loop. Consistently, the data reported here showed that overexpression of HA-AKT significantly abrogated apoptosis of HeLa cells induced by TRAIL and concomitantly suppressed activation of caspase-3 as well as caspase-8. Moreover, complestatin suppressed activation of both caspase-8 and -3 in response to TRAIL in HeLa cells and these inhibitory effects of complestatin were significantly abrogated in DN-AKT expressing cells. Given that AKT/PKB blocks mitochondria-dependent apoptosis through Bad phosphorylation, it is suggested that caspases which are activated downstream of mitochondria play an important role in activation of caspase-8 in response to TRAIL. Therefore, complestatin is most likely to inhibit TRAIL-induced caspase-8 activation by impairment of mitochondrial amplification loop that is linked to caspase-3. However, the possibility that complestatin inhibits caspase-8 activation by impairing DISC formation cannot be excluded and is the subject of present investigation.

In conclusion, this study demonstrates that complestatin is a noble anti-apoptotic molecule that is capable of blocking the caspase-dependent signaling cascade. Although a precise molecular target of complestatin is incompletely understood, the results reported here reveal that complestatin induces AKT/PKB activation through the Src and PI3K signaling route. The mechanisms by which complestatin functions as an anti-apoptotic factor are summarized in Fig. 8. This information may be valuable in the future development of therapeutic treatments to prevent human diseases caused by excessive cell death.

Acknowledgments

We extend our sincere thanks to Dr. Timothy R. Billiar (University of Pittsburgh) for providing HA-AKT and DN-AKT expression vector. We thank Dr. James A. Bibb (University of Texas Southwestern) for critical reading of the manuscript. This work was supported by G7 and NRL grants (to I.-D.Y.) and a Vascular System Research Center grant (to Y.-G.K.), Ministry of Science and Technology, Republic of Korea.

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