Acid Sphingomyelinase Is Indispensable for UV Light-induced Bax Conformational Change at the Mitochondrial Membrane*

Received for publication, September 21, 2004, and in revised form, February 23, 2005
Published, JBC Papers in Press, March 1, 2005, DOI 10.1074/jbc.M410869200

Hamid Kashkar§, Katja Wiegmann‡, Benjamin Yazdanpanah†, Dirk Haubert‡, and Martin Krönke‡§

From the ‡Institute for Medical Microbiology, Immunology and Hygiene and §Center for Molecular Medicine Cologne, University of Cologne, 50935 Cologne, Germany

Ultraviolet light-induced apoptosis can be caused by DNA damage but also involves immediate-early cell death cascades characteristic of death receptor signaling. Here we show that the UV-light-induced apoptotic signaling pathway is unique, targeting Bax activation at the mitochondrial membrane independent of caspase-8 or cathepsin D activity. Cells deficient in acid sphingomyelinase (ASMase) do not show UV-light-induced Bax activation, cytochrome c release, or apoptosis. In ASMase-deficient cells, the apoptotic UV light response is restored by stable or transient expression of human ASMase-deficient cells, the apoptotic UV light response is restored by stable or transient expression of human ASMase. Bax conformational change in ASMase−/− cells is also caused by synthetic C16-ceramide acting on intact cells or isolated mitochondria. The results suggest that UV light-triggered ASMase activation is essentially required for Bax conformational change leading to mitochondrial release of pro-apoptotic factors like cytochrome c and Smac.

Ultraviolet light induces a complex transcriptional cellular response that is similar to that of tumor promoters and mitogens yet also includes the induction of growth arrest and apoptosis (1, 2). UV light-induced apoptosis seems to represent a controlled scavenging mechanism that protects cells from malignant transformation. UV light triggers a variety of signaling pathways, including nuclear DNA damage and activation of the tumor suppressor gene p53 (3) or triggering of cell death receptors either directly (4, 5) or by autocrine release of death ligands leading to mitochondrial damage and cytochrome c release (6, 7), which leads to the activation of executioner caspases and eventually to apoptosis.

The primary mechanism of UV light-induced apoptosis was originally thought to be the signaling induced by DNA damage and p53 (3), which could be promoted by UV light-induced increase of reactive oxygen species (8). However, the UV response also entails a number of p53-independent signaling pathways (9–13). For example, exposure to UV light induces clustering and internalization of cell surface death receptors, like TNF-R1. This may result not only in activation of the c-Jun N-terminal protein kinase cascade but also in caspase-8 activation (4, 5). Caspase-8 can cleave Bid to form truncated (t)Bid that then translocates from the cytosol to the mitochondrial membranes causing cytochrome c release (14). Furthermore, like various other apoptotic stimuli, including CD95, UV light activates acid as well as neutral sphingomyelinases (15). Although the activation of sphingomyelinases (SMases) by apoptotic stimuli is well established and evidence has been accumulated for their role in apoptosis (16–19), the precise molecular mechanisms of SMase pro-apoptotic action remained ill-defined and in dispute (20).

Despite this variety of established UV light-induced signaling pathways, there are gaps in our understanding of the rapid induction of mitochondrial pro-apoptotic function that appears to be an important hallmark of the UV light response (21). The pro-apoptotic activity of mitochondria is known to be regulated by the family of Bcl2 proteins (22–23). The Bcl2 family consists of anti-apoptotic members such as Bcl2 but also includes pro-apoptotic members such as Bax. Bax can induce the release of cytochrome c from mitochondria and represents an important relay of the mitochondrial apoptotic pathway and subsequent activation of caspases (24). In apoptotic cells, Bax is translocated from the cytosol to the mitochondria, undergoes a conformational change, oligomerizes, and inserts into the mitochondrial outer membrane by which it triggers the cytochrome c release and gains its pro-apoptotic activity (25–26). The pro-apoptotic action of Bax is antagonized by Bcl2 and Bcl-XL, which inhibit the release of cytochrome c from mitochondria (23). In unstimulated cells, Bax is a soluble monomeric protein present in the cytosol or loosely associated with mitochondria (27). On isolated mitochondria, Bax monomers do not trigger the release of cytochrome c, whereas Bax oligomers do (27). In order to oligomerize, Bax is suggested to undergo first a conformational change, which can be demonstrated in cell lysates using detergents like Triton X-100 (28). Immunoprecipitation and immunofluorescence analysis with conformationally sensitive antibodies revealed conformational change of Bax in apoptotic cells stimulated by different apoptotic agents including CD95 ligand (29), TNF (30), protein kinase C inhibitors like staurosporine (STS) (14, 31), anti-microtubule agents like Epothilon B analogues (32), and fludarabine/dexamethasone (33). Thus, the induction of Bax conformational change is viewed as a key regulatory step initiating the mitochondrial apoptotic pathway. Upon apoptotic induction, Bax conformational change of Bax is viewed as a key regulatory step initiating the mitochondrial apoptotic pathway. Upon apoptotic induction, Bax conformational change of Bax.
tional change takes place as an early event and is suggested to be a result of the action of BH3 (Bcl2 homology)-only pro-apoptotic Bcl2 proteins such as Bim (32), Bid (14), and Bad (34). Although BH3-only pro-apoptotic Bcl2 members induce the conformational change of Bax, anti-apoptotic Bcl2 family proteins like Bcl2, Bcl2 homolog adenosinenucleotide gene products E1B 19K, and Bcl-XL are known to inhibit this process (26).

The precise UV light-mediated pro-apoptotic mechanisms acting upstream of and targeting mitochondria are less well understood. Here we provide evidence that UV light rapidly induces Bax conformational change, which is accompanied by the mitochondrial release of pro-apoptotic factors such as cytochrome c and Smac. We show that the UV light-induced Bax activation pathway can be dissected from that triggered by CD95 ligation or STS. UV light-induced Bax activation is not sensitive to inhibition of either caspases-8 and -3 or caspase-7. By using two types of ASMase-deficient cells from Niemann Pick disease type A patients, we found that ASMase is indispensable for UV light-induced Bax activation. In these genetically defined ASMase-deficient cells, Bax activation is restored by stable or transient expression of human ASMase as well as by exogenous ceramide. The activation of Bax by ceramide is abrogated in Bcl2-overexpressing cells, indicating that ceramide itself does not exert direct mitochondrial release activity. Finally, Bax activation by ASMase and ceramide was observed in isolated mitochondria but not in cytosolic fractions, localizing the critical step of UV light-induced Bax conformational change at the mitochondrial membrane.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials—**HeLa, Bcl2-HeLa, and NPD fibroblasts (provided by Dr. Sandhoff, Bonn, Germany, 35) were cultured in DMEM and Jurkat, MS1418, and ASM-MS1418 lymphoblasts (originally obtained from Dr. D. Green, San Diego) (36); cells were cultured in VLE RPMI 1640 supplemented with 10% fetal calf serum, 2 mM t-glutamine, 100 μg ml−1 streptomycin, and 100 units ml−1 penicillin (Biochrom, Berlin, Germany). Apoptosis was induced by exposing cells to 10 μj cm−2−1 UV light (253–255 nm) or by incubating cells with 0.5 μM STS (Alexis, Grunberg, Germany) or 20 ng ml−1 CD95 antibody (clone CH11) (Upstate Biotechnology, Inc., Eching, Germany). Caspase activity was blocked by a 1-h pretreatment of cells with 20 μM Z-VAD(Ome)FMK and/or 2 μM Z-DEVD-CHO (Alexis, Grunberg, Germany). Cc-ceramide (N-acyethylsphingosine, N-acyethyltroylinosine, N-acyethylsphingosine, N-acyethylsphingosine, N-acyethylsphingosine, N-acyethylsphingosine, N-acyethylsphingosine) was purchased from Biochrom (Germany). Dihydo-Cer-ceramide (N-palmitoyl, N-ehydrosphin-gosine) (dh-C16) was purchased from Sigma. Ceramides were suspended in dodecane/ethanol (2:98, v/v; 0.05% final concentration), and cells were incubated with 0.5 μM ATR (0.5 μM ATR, 1 mM DMSO, 50 μM EDTA, and 2 mg ml−1 saponin in PBS, and then incubated for 30 min with secondary antibodies conjugated with Alexa Fluor 568 and/or Alexa Fluor 488 (Molecular Probes, Leiden, Netherlands). Nuclei were counterstained with Hoechst 33258 (10 μg ml−1 PBS), mounted on glass slides, and examined using a fluorescence microscope.

**SMase Assays—**Activation of neutral and acidic SMase after UV treatment was measured as described recently (39), with minor modifications. Briefly, cells were lysed in two different buffer systems as described, and equal amounts of protein (between 15 and 50 μg) were added to 52.25 μl of reaction buffer (250 μM sodium acetate, 1 mM EDTA (pH 5.0)) for ASMase measurements. [N-methyl-14C]Sphingomyelin (56 μCi mmol−1) was added (1.1 μCi ml−1 final concentration), and the reaction mixtures were incubated at 37 °C for 1 h. SMase activity was quantified by counting released [14C]phosphocholine using a β-counter.

**DNA Constructs and RNA Interference—**Open reading frames of genes encoding human Bcl2 and Smac were amplified by PCR using Bcl2 primers containing HindIII/Apal restriction sites and Smac primers containing XhoI/BamHI restriction sites and subjected to SDS-PAGE and immunoblotted as described above.

**DNA Fragmentation—**Cells were either exposed to UV light (10 μj cm−2−1) or incubated with 0.5 μM STS or ceramide (20 μM) and washed twice with cold PBS. Cells were then fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.1% saponin in PBS for 10 min, and blocked with 3% bovine serum albumin, 0.005% sodium azide, 4 μl ml−1 gelatin (45% Teleolea gelatin), and 0.1% saponin in PBS for 30 min. For immunostaining, cells were incubated with specific primary antibodies, washed with 1% saponin in PBS, and then incubated for 30 min with secondary antibodies conjugated with Alexa Fluor 568 and/or Alexa Fluor 488 (Molecular Probes, Leiden, Netherlands). Nuclei were counterstained with Hoechst 33258 (10 μg ml−1 PBS), mounted on glass slides, and examined using a fluorescence microscope.

**Cell Fractionation and Cell-free Systems—**The subcellular fractionation was performed as described (14, 31). Briefly, cells were washed twice with cold PBS, resuspended in 10 mM Hepes, pH 8.5, 200 mM NaCl, 0.2% (v/v) SDS, 5 mM EDTA, and 2 mg ml−1 proteinase K, and incubated for 30 min at 56 °C. After two successive extractions with phenol/chloroform/isoamyl alcohol (24:24:1, pH 8.8), polynucleotides were precipitated with 1.10 volume of 3 M sodium acetate, pH 5.8, and 2.5 volumes of ethanol. The DNA precipitate was washed once with 70% ethanol (v/v) and air-dried for 5 min and resuspended in 100 μl of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). cDNA was then transcribed to 1×106 cells using Lipofectamine (Invitrogen), according to the manufacturer’s instructions.

**DNA Fragmentation—** For DNA fragmentation assays, 1×107 Jurkat cells were harvested at 1000 × g, resuspended in 200 μl of lysis buffer containing 10 μg ml−1 proteinase K, and incubated for 30 min at 56 °C. After two successive extractions with phenol/chloroform/isoamyl alcohol (24:24:1, pH 8.8), polynucleotides were precipitated with 1.10 volume of 3 M sodium acetate, pH 5.8, and 2.5 volumes of ethanol. The DNA precipitate was washed once with 70% ethanol (v/v) and air-dried for 5 min and resuspended in 100 μl of TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA). cDNA was then transcribed to 1×106 cells using Lipofectamine (Invitrogen), according to the manufacturer’s instructions.
RNAse A and incubation at 37 °C for 1 h. DNA of ~5 × 10⁵ cells was loaded with 10% Ficoll onto a 1.8% agarose gel and visualized with ethidium bromide under UV light.

RESULTS

UV Light Induces Bax Conformational Change in HeLa Cells—The release of cytochrome c from mitochondria has been reported as an early event in the apoptotic process induced by UV light treatment (7, 21). To investigate the mechanisms causing cytochrome c release, the mitochondrial apoptotic signaling cascade induced by UV light was analyzed in HeLa cells (Fig. 1). Bax activation/conformational change was detected as an immediate-early event within 0.5 h following UV light ex-
posure (Fig. 1A). Bax conformational change was detected by combined immunoprecipitation/Western blot analysis of Bax using a conformation-specific antibody (clone 6A7 specific for the N-terminal epitope 12–24 of Bax) that recognizes Bax only in its active conformation (28). The total amount of Bax protein remained unchanged, suggesting that the increasing fraction of active Bax protein does not correspond to newly synthesized protein. Concomitantly, pro-apoptotic proteins like cytochrome c were released from mitochondria into the cytosol after 60 min, which was paralleled by diminished levels of cytochrome c detected in mitochondrial fractions (Fig. 1A).

To determine the precise subcellular site of UV light-induced Bax conformational change, we performed combined immunoprecipitation/Western blot analysis of Bax in cytosolic and mitochondrial fractions. In accordance to previous reports (14), Bax is found in both the cytosolic and mitochondrial fractions of untreated HeLa cells (Fig. 1B). After UV light treatment, Bax translocation from cytosol to mitochondria occurred only after 120 min, indicating that Bax conformational change precedes Bax translocation. Activated Bax, however, could be discerned in the mitochondrial but not in cytosolic fractions 15 min after UV light treatment by 6A7 antibody. These results indicated that UV light induces the conformational change of only that fraction of Bax that is already sequestered by mitochondrial membranes. This notion could be confirmed by immunofluorescence-based co-localization studies. 30 min after UV light exposure, HeLa cells co-stained with Alexa 488-conjugated 6A7 anti-Bax antibody (conformation specific) (green) and Alexa 568-conjugated anti-cytochrome c antibody (red) showed colocalization of activated Bax and cytochrome c in a distinct punctated mitochondrial pattern (Fig. 1C). These results confirm that UV light-induced Bax activation only occurs at mitochondrial membranes but not in the cytosol.

UV Light Induces Bax Conformational Change Independent of Caspases-8 and -3—In general, apoptotic stimuli can activate Bax through caspase-dependent and -independent signaling cascades. CD95-induced Bax conformational change is mediated through tBid previously cleaved by caspase-8 (29), whereas STS activates the mitochondrial apoptotic pathway independent of caspase activity (40). In order to examine the role of caspases in UV light-induced Bax activation, Jurkat cells were stimulated with UV light, STS, and anti-CD95. All three stimuli induced caspase-8 processing, Bid cleavage, Bax conformational change, and efflux of pro-apoptotic proteins such as cytochrome c and Smac into the cytosol (Fig. 2A, lanes 1–4). Pretreatment of Jurkat cells with caspase inhibitors completely blocked both caspase-8 activation and the generation of tBid induced by any of the three stimuli (Fig. 2A, lanes 5–8). Bax conformational change induced by CD95 was abrogated after caspase inhibition. In contrast, UV light- and STS-induced Bax conformational change remained unaffected (Fig. 2A, lanes 6 and 7). Likewise, CD95-induced cytochrome c and Smac release was blocked by caspase inhibition, whereas UV light- and STS-induced release of cytochrome c and Smac was not impaired by Z-VAD-FMK and DEVD-CHO (Fig. 2A, compare lane 8 with lanes 6 and 7). Proper action of caspase inhibitors is demonstrated by the lack of DNA fragmentation in response to all three stimuli (Fig. 2B, compare lanes 1–4 with lanes 5–8). These results indicate that UV light-induced Bax conformational change is a caspase-8 and -3 independent event, and it is not specific to HeLa cells.

UV Light-induced Acid Sphingomyelinase Activity Is Associated with Bax Conformational Change, Cytochrome c Release, and Cell Death—SMases have been suggested to play a role in apoptosis induced by UV light (15). Specifically, an ASMase has been reported to respond to apoptotic stimulation (18) and therefore is a candidate enzyme that could be involved in UV light-induced apoptosis. As shown in Fig. 3A, UV light treatment indeed leads to increased ASMase activity. To investigate the involvement of ASMase in UV light-induced apoptotic response, RNA interference was used to reduce the expression of ASMase. Compared with ASMase activity in control cells or cells transfected with a control siRNA, basal ASMase activity was reduced by ~70% in cells transfected with ASM-siRNAs. In ASM-siRNA2-transfected HeLa cells, UV light treatment led to marginal, if any, enhancement of ASMase activity, which did not even reach basal ASMase activity levels of untreated control cells (Fig. 3A). The down-regulation of ASMase by ASM-siRNA could be confirmed by immunofluorescence analysis. To
monitor the transfection efficiency, ASM-siRNA was co-transfected with an expression plasmid encoding a Smac-GFP fusion protein. GFP was expressed as a Smac fusion protein to localize GFP fluorescence to mitochondria. As shown in Fig. 3B, ASMase was detected in fluorescent HeLa cells, indicating that ASM-siRNA transfection within 48 h leads to complete down-regulation of ASMase protein expression. After UV light treatment, Bax activation (Fig. 3C) and cytochrome c release (Fig. 3D) were only observed in nontransfected HeLa cells or in HeLa cells transfected with scrambled control siRNA. These results show at the single cell level that down-regulation of ASMase by ASM-siRNA abrogates the mitochondrial pro-apo-
potic UV light response. To assess the consequences of ASMase down-regulation for apoptosis, at least 300 transfected HeLa cells were microscopically scrutinized for the presence of activated Bax, release of cytochrome c, and condensed nuclei. As depicted in Fig. 3E, only 40% of ASM-siRNA-transfected HeLa cells showed signs of apoptosis 8 h after UV light exposure, whereas almost all control cells were apoptotic, indicating that down-regulation of ASMase by RNA interference protects HeLa cells from UV light-induced apoptosis.

UV Light Fails to Induce Bax Conformational Change and Cytochrome c Release in ASMase-deficient Cells—To provide more pertinent evidence for the possible involvement of ASMase in UV light-induced Bax activation, we employed genetically determined, ASMase-deficient cells derived from patients suffering inherited Niemann-Pick type A disease (NPD), where a defined splicing site mutation in the ASMase gene causes almost complete loss of ASMase enzymatic activity (35). Epstein-Barr virus-transformed B-lymphoblasts (MS1418) (36) as well as fibroblasts from patients suffering NPD type A were used to examine the apoptotic response to UV light. As shown in Fig. 4A, UV light fails to induce Bax conformational change in ASMase-deficient lymphoblasts. Correspondingly, neither release of cytochrome c nor Smac nor caspase-9 processing were detected in UV light-treated MS1418 lymphoblasts. In contrast, NPD lymphoblasts stably transfected with an ASMase-expressing vector (ASM-MS1418) responded to UV light with Bax conformational change, cytochrome c, Smac release, and caspase-9 processing (Fig. 4A, lanes 3 and 4). Similarly, immunofluorescence analysis of NPD lymphoblasts revealed that UV light induces Bax conformational change, as well as cytochrome c release and fragmentation of nuclei (blue) only in ASMase-transfected cells (ASM-MS1418) but not in ASMase-deficient MS1418 cells (Fig. 4B and C).

To rule out any possible cell type-specific effects secondary to inherited ASMase deficiency, UV light-mediated Bax activation pathways were investigated in ASMase-deficient NPD type A fibroblasts. As shown in Fig. 4D, UV light fails to induce Bax conformational change and release of cytochrome c and Smac in NPD fibroblasts, whereas the STS-induced mitochondrial apoptotic pathway was completely functional. By transient ASMase expression in NPD fibroblasts, the capability of UV light to induce Bax conformational change was restored (Fig. 4E) when NPD fibroblasts were co-transfected with an ASMase expression plasmid along with an eGFP-C3 control vector to visualize transfected cells. UV light-induced Bax conformational change was only observed in transfected NPD fibroblasts. Approximately 80% of UV light-treated fluorescent NPD fibroblasts stained positive for activated Bax (Fig. 4F), indicating that overexpression of ASMase restored the pro-apoptotic UV light response. Accordingly, cytochrome c release was also restored in fluorescent NPD fibroblasts (green cells) (Fig. 4G). The release of cytochrome c is indicated by the change from a punctuated (mitochondrial) staining pattern to a diffuse (cytosolic) staining. Together, these results provide genetic evidence for the essentiality of ASMase in the UV light-induced Bax activation pathway and subsequent pro-apoptotic signaling.

Ceramide Induces Bax Conformational Change and Cytochrome c Release in ASMase-deficient Cells—Ceramide, the reaction product of UV light-induced ASMase, is a well known stimulus of apoptotic cell responses. The next set of experiments was designed to show that ceramide serves as an initiator of Bax conformational change and cytochrome c release from mitochondria. Like UV irradiation, ceramide induces Bax conformational change in HeLa cells as shown by immunofluorescence analysis (Fig. 5A). Furthermore, Western blot analysis revealed that C16-ceramide in a dose-dependent manner induced Bax conformational change, release of cytochrome c/Smac, and processing of caspase-9 (Fig. 5B). In contrast, dh-C16-ceramide at 50 μM failed to initiate any apoptotic response in HeLa cells. Notably, ceramide induced Bax conformational change and cytochrome c/Smac release in ASMase-deficient NPD lymphoblasts (Fig. 5C), indicating that the defective UV light response in ASMase-deficient MS1418 cells can be bypassed by the reaction product of ASMase.

Ceramide Induces Bax Conformational Change at Mitochondrial Membranes in Cell-free Systems—As indicated by Bax activation and release of cytochrome c/Smac, UV light-induced pro-apoptotic signaling clearly targets the mitochondria. Therefore, we next addressed the effects of UV light and ceramide on isolated mitochondria. As shown in Fig. 6A, UV light failed to induce Bax conformational change or cytochrome c release in isolated mitochondria. In contrast, incubation of isolated mitochondria with C16-ceramide but not dh-C16-ceramide led to conformational change of Bax (Fig. 6B). Notably, C16-ceramide did not activate Bax in the cytosol. As expected, C16-ceramide induced Bax conformational change and release of cytochrome c and Smac from mitochondria isolated from ASMase-deficient MS1418 cells (Fig. 6C). The finding that ceramide failed to induce Bax conformational change in the cytosolic fractions of HeLa cells (Fig. 6D, compare lanes 2 and 4), indicating the critical role of mitochondrial membranes or membrane-associated factors for ceramide action on Bax. It is important to note that Bax conformational change was inducible in membrane-free mitochondrial lysates by Triton X-100 (Fig. 6D, lane 6), a nonionic detergent known to induce Bax conformational change (28), indicating the presence of intact, inducible Bax. The observation that C16-ceramide but not dh-C16-ceramide induced Bax conformational change as well as release of cytochrome c/Smac suggested a specific action of ceramide. To provide a second line of evidence for the specificity of ceramide, Bcl2-overexpressing HeLa cells were used for further investigations. Bcl2 is known to localize in mitochondria and to antagonize Bax activation (26). As shown in Fig. 7A, C16-ceramide was unable to induce Bax conformational change or release of cytochrome c/Smac from mitochondria isolated from Bcl2-overexpressing HeLa cells, indicating that ceramide action is not secondary to nonspecific perturbation of the membrane integrity. The amounts of Bax were equal in mitochondrial fractions of HeLa and Bcl2-HeLa cells suggesting that overexpression of Bcl2 does not affect the basal level of Bax associated with mitochondria (Fig. 7A). Similar results were obtained when HeLa cells were transiently transfected with a GFP-Bcl2 fusion protein expressing vector (Fig. 7B). Bcl2 localizes to mitochondria and the endoplasmic reticulum and is known to inhibit Bax and other pro-apoptotic factors interacting with mitochondrial membranes but does not affect signaling events upstream of mitochondria (29, 41). Immunofluorescence analysis revealed that both UV light and ceramide induced Bax activation in untransfected cells but failed to do so in GFP-Bcl2-transfected cells. This suggests that ceramide has no direct mitochondrial releasing activity but rather promotes and acts through Bax conformational change.

DISCUSSION

UV light is known to induce a cell death cascade involving mitochondria, which eventually leads to apoptosis. However, the precise initiating apoptotic mechanisms upstream of mitochondria remained obscure. By using a conformation-specific
anti-Bax antibody that recognizes activated Bax, we elucidated the immediate-early events of UV light-induced apoptosis that target mitochondria for release of cytochrome c and other pro-apoptotic factors. UV-irradiated cells show rapid conformational change of Bax, followed by release of cytochrome c and Smac and eventually by apoptosis. Strikingly, the UV light-induced mitochondrial pro-apoptotic events absolutely require previous activation of ASMase.

Our finding that UV light does not promote Bax activation on isolated mitochondria but rather needs intact cells suggests that an ASMase-dependent signaling cascade upstream of mitochondria is activated by UV light to mediate mitochondrial...
modification within a very short period of time, i.e. 15–30 min after UV light treatment. As for other apoptotic stimuli, Bax conformational change is known to be initiated by BH3-only pro-apoptotic Bcl2 family members (29–34). In addition, it has been reported previously that UV irradiation leads to clumping and internalization of cell surface receptors, including death receptors of the TNF receptor family (4). Thus, on theoretical grounds it can be envisioned that UV light-induced clumping of a death domain-containing member of the TNF receptor family could trigger activation of caspase-8 that in turn may cause cytochrome c release through formation of tBid (29, 30). However, unlike CD95, UV light-induced Bax conformational change in the presence of caspase inhibitors as well as in the absence of tBid, indicating that UV light-induced Bax activation involves a caspase-8- and tBid-independent mechanism. Western blot analysis of other BH3-only pro-apoptotic Bcl2 family members such as Bad, Bim, and Noxa did not indicate any interactions with Bax or mitochondria within 2 h after UV light treatment (data not shown). In addition, examination by specific inhibitors of other established UV light signaling pathways, including reactive oxygen species production or c-Jun N-terminal protein kinase activation, excluded

**Fig. 5.** Ceramide induces Bax conformational change and cytochrome c release. A, HeLa cells were treated with UV light (10 mJ cm⁻²) or with C₁₆-ceramide (50 μM) and incubated for 4 h. Activated Bax was detected by monoclonal 6A7 antibody. B, HeLa cells were treated and incubated with increasing concentrations of C₁₆-ceramide or dh-C₁₆-ceramide for 4 h. Total cell lysates and cytosolic extracts were analyzed for activated Bax and cytochrome c, Smac, and caspase-9 processing, respectively. Reprobing for actin ensured equal loading of cytosolic extracts. C, MS1418 lymphoblasts were treated with C₁₆-ceramide (50 μM) for 4 h. Activated Bax and cytochrome c were detected by specific primary antibodies and Alexa Fluor 568 (red) or Alexa Fluor 488-conjugated (green) secondary goat anti-mouse IgG antibodies, respectively. **IF,** immunofluorescence.

**Fig. 6.** Ceramide induces Bax conformational change in isolated mitochondria. A, HeLa cells or isolated mitochondria were exposed to UV light, and mitochondria were analyzed for activated Bax and cytochrome c release. B, mitochondria isolated from HeLa cells were incubated for 1 h at 30 °C with 2 μM C₁₆-ceramide or 2 μM dh-C₁₆-ceramide. Mitochondria were pelleted, lysed by CHAPS, and analyzed for activated Bax. Supernatants were analyzed for released cytochrome c and Smac and activated Bax. C, mitochondria isolated from MS1418 and ASM-MS1418 lymphoblasts (5 × 10⁷) were incubated for 1 h at 30 °C with 2 μM C₁₆-ceramide. Mitochondria were pelleted, lysed by CHAPS, and analyzed for activated Bax. Supernatants were analyzed for released cytochrome c and Smac. D, cytosolic extracts of HeLa cells and isolated mitochondria as well as membrane-free mitochondrial (CHAPS) lysates were prepared and incubated for 1 h at 30 °C with C₁₆-ceramide or for 1 h on ice with Triton X-100 (1%) and analyzed for activated and total Bax. **IP,** immunoprecipitate; **IB,** immunoblot.
the involvement of these pathways in UV light-induced mitochondrial Bax activation.

Our finding that ASMase is activated by UV light is in line with previous studies indicating that exposure of cells to UV light activates SMases (15, 42), which is followed by a rapid increase in intracellular ceramide significantly above basal levels of untreated cells. Although none of these reports suggested SMase to play a role in UV light-induced apoptosis at the level of mitochondria, we now provide evidence for the essentiality of SMases in UV light-induced Bax activation. The idea that ASMase may play a critical role in UV light-induced Bax activation was corroborated by use of siRNAs targeting ASMase expression in HeLa cells and genetically defined ASMase-deficient cells derived from NPD patients. Whereas STS was able to induce Bax conformational change and cytochrome c release in NPD fibroblasts, UV light completely failed to promote Bax conformational change or any other apoptotic mitochondrial event. Similarly, down-regulation of ASMase expression in HeLa cells by RNA interference reproduced a defective mitochondrial apoptotic pathway, which was observed with cells derived from patients with inherited ASMase deficiency. Transient as well as stable expression of functional ASMase in NPD fibroblasts or NPD lymphoblasts, respectively, restored the capability of UV light to induce Bax conformational change, cytochrome c release, and apoptosis. These data provide genetic evidence for the essential role of ASMase in UV light-triggered apoptotic signaling pathways targeting the mitochondria. In addition, pepstatin A, an inhibitor of cathepsin D (40), did not abrogate UV light-induced cell death (data not shown). As shown by others (40), pepstatin A did inhibit STS-induced Bax activation and cytochrome c release, indicating that STS activates apoptotic signaling mechanisms distinct from those induced by UV light.

How can ASMase activity be linked to pro-apoptotic mitochondrial events? Two forms of ASMase have been described, one showing an endo-lysosomal localization and the other secretory form is targeted to the plasma membrane (43). The secretory form of ASMase has been implicated in stress-associated cell death signaling by producing ceramide in the plasma membrane (44). However, a translocation of ASMase to mitochondria has never been reported. This raises the question of how ceramide produced at the plasma membrane or within the endo-lysosomal compartment may trigger apoptotic mitochondrial events. It has been shown earlier by Lipsky and Pagano (45) that exogenous ceramide exposed to intact cells rapidly distributes from the plasma membrane to different organelles, including mitochondria. Indeed, ceramide can be intracellularly transported by lipid flux routes such as the endocytosis of plasma membrane lipids occurring by vesicle-mediated mechanisms (46) or by the mitochondria-associated endoplasmic reticulum subcompartment (MAM fraction) (47), which provides a network for targeting of lipids. Furthermore, a ceramide transfer protein (CERT) has been described recently (48) that is able to propel ceramide transport between different organelles. These observations suggest that ceramide produced in a specific subcellular site may end up in the mitochondrial membrane through vesicle-mediated mechanisms. This notion is supported by the recent finding that accumulation of ceramide can be detected in mitochondrial membranes after UV light-induced SM hydrolysis (42). The idea that ceramide may rather act directly on mitochondrial membranes to promote Bax activation was addressed by adding synthetic ceramides to either intact cells or isolated mitochondria. Treatment of HeLa cells with ceramide resulted in Bax conformational change,

FIG. 7. Bcl2 controls ceramide action on mitochondria. A, mitochondrial and cytosolic fractions isolated from HeLa and Bcl2-HeLa cells (5 × 10⁷) were incubated for 1 h at 30 °C with 2 μM ceramide analogues. Mitochondria were pelleted and lysed by CHAPS, and Bax and Bcl2 were detected in mitochondrial fractions by specific antibodies. Cytochrome c and Smac were detected in supernatants by Western blot analysis using specific antibodies. B, HeLa cells were transiently transfected with GFP-Bcl2 expression vector and incubated for 24 h. Cells were treated with UV light or C₁₆-ceramide and incubated for 4 h. Activated Bax was detected by primary monoclonal antibody (6A7) and Alexa Fluor 568-conjugated secondary goat anti-mouse IgG antibody (red).
change and subsequent cytochrome c/Smac release without any additional cytosolic component (Fig. 6B). This finding suggests but does not formally prove that ceramide itself rather than a derivative thereof causes alterations of the mitochondrial membrane that promote the activation of Bax. Ceramide-induced Bax conformational change and cytochrome c/Smac release are completely abrogated in mitochondria from Bcl2-HeLa cells, excluding the possibility that ceramide action on Bax and cytochrome c release is secondary to the general perturbation of membrane integrity.

Ceramide was found to induce Bax conformational change in isolated mitochondrial fractions but not in cytosolic fractions or mitochondrial protein lysates, strongly suggesting the need of the mitochondrial membrane to support Bax action (Fig. 6B). Our findings are consistent with a previous report (49) that breaches can also allow transient interactions to occur with the lipid bilayer that facilitate Bax conformational change in a manner-induced remodeling of the membrane by exposing amide-induced residues of activated Bax. Ceramide-membrane lipid microdomains in the outer mitochondrial membrane, for example by generating disturbance of membrane integrity.

We thank J. B. Villmow for the excellent technical assistance.

Acknowledgment—We thank B. Villmow for the excellent technical assistance.

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J. Biol. Chem. 2005, 280:20804-20813.
doi: 10.1074/jbc.M410869200 originally published online March 1, 2005

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