A switchable ceramide transfer protein for dissecting the mechanism of ceramide-induced mitochondrial apoptosis

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Mitochondrial translocation of ceramides triggers Bax-dependent apoptosis. To elucidate how ceramides activate Bax and commit cells to death, we developed a switchable version of the ceramide transfer protein CERT, sCERT. Upon its drug-induced recruitment to mitochondria, sCERT retains the ability to bind VAP proteins in the ER and catalyzes mitochondrial import of externally added fluorescent ceramides. Mitochondrial recruitment of sCERT also triggers mitochondrial translocation of Bax. The ability of mitochondria-bound sCERT to mediate ceramide import and Bax translocation requires both its START domain and ongoing ceramide biosynthesis. These data extend our previous finding that mistargeting of ER ceramides to mitochondria specifically activates Bax and establish sCERT as a novel tool to dissect the underlying mechanism in a time-resolved manner.

Keywords: Bcl2 proteins; ceramide transfer protein; chemical dimerization technology; mitochondrial apoptosis; sphingolipids

During mitochondrial apoptosis, the most common form of programmed cell death, multiple pathways converge on mitochondria to induce mitochondrial outer membrane permeabilization (MOMP) and subsequent release of intermembrane space proteins like cytochrome c (Cyto c) into the cytosol. Release of Cyto c damages the bioenergetics function of mitochondria and initiates the caspase cascade that eventually dismantles the cell [1,2]. MOMP is controlled by members of the B-cell lymphoma-2 (Bcl-2) family of proteins, which have been classified in pro-survival and pro-apoptotic family members [3]. The principal pro-apoptotic Bcl-2 protein in mammals is Bax, which is a soluble monomeric protein that constitutively shuttles between the cytosol and mitochondria of healthy cells [4,5]. In response to DNA damage and other types of cellular stress, Bax is activated and accumulates on the mitochondrial surface, where it undergoes major conformational changes to form oligomers in the outer mitochondrial membrane (OMM) [6–8]. A widely supported model of MOMP is that Bax oligomerization creates pores that allow Cyto c and other apoptotic factors to escape to the cytosol. Indeed, recent studies revealed ring-like Bax structures on apoptotic mitochondria by super-resolution fluorescence microscopy [9,10]. Pro-survival Bcl-2 proteins inhibit Bax via direct interactions or by sequestering Bax-activating BH3-only Bcl2 proteins [3]. Such regulatory interactions are only observed in the presence of mitochondria or liposomes, suggesting that mitochondrial apoptosis signaling involves membrane-embedded proteins [11]. When expressed in cells lacking all known Bcl-2 protein family members, Bax and Bak spontaneously associate with the OMM and assemble

Abbreviations
Bcl-2, B-cell lymphoma-2; CHO, Chinese hamster ovary; Cyto c, cytochrome c; FRB, FKBP-/rapamycin-binding; MOMP, mitochondrial outer membrane permeabilization; OMM, outer mitochondrial membrane; PH, pleckstrin homology; SM, sphingomyelin.
into oligomers, indicating that the OMM itself acts as a direct activator of Bax and Bak [12]. Interestingly, voltage-dependent anion channel VDAC2 serves a critical role as mitochondrial platform for Bax/Bak translocation [13,14], enabling Bax to mediate apoptosis and suppress tumor development [15].

Several reports have indicated that ceramides, the essential building blocks of sphingolipids, actively participate in mitochondrial apoptosis. For instance, a rise in mitochondrial ceramide levels has been observed to precede MOMP for a variety of distinct stresses [16–19]. In experiments with isolated mitochondria, exogenously added ceramides and recombinant Bax were found to act coordinately to induce the release of Cyto c [20]. Moreover, ceramides have been reported to induce an activating conformational change in Bax in mitochondrial membranes [21]. Other work revealed that ceramides can form stable channels in planar lipid bilayers as well as in the outer membrane of isolated mitochondria that allow passage of Cyto c [22,23]. The pore-forming activity of ceramides does not seem to require any auxiliary proteins but can be blocked by anti-apoptotic Bcl-2 proteins [24].

Yet, other studies indicated that ceramides influence mitochondrial integrity indirectly, as precursors of two other signaling molecules, sphingosine-1-phosphate and hexadecenal, which co-operate specifically with Bax and Bax to promote MOMP [25]. Hence, although in vitro studies indicate that ceramides initiate cell death by acting directly on mitochondria, their actual contribution to the apoptotic response in living cells is not well understood. In this respect, our recent finding that VDAC2 binds ceramide and directly participates in ceramide-mediated apoptosis offers a promising new direction [26].

Ceramides are produced de novo by N-acylation of sphingoid long-chain bases on the cytosolic surface of the ER. In mammals, the bulk of newly synthesized ceramides is converted to sphingomyelin (SM) by SM synthase SMS1 in the lumen of the trans-Golgi [27–29]. Efficient delivery of ER ceramides to the site of SM production requires the cytosolic ceramide transfer protein CERT [30]. CERT contains an N-terminal pleckstrin homology (PH) domain that binds phosphatidylinositol-4-phosphate, a lipid enriched on the cytosolic surface of the trans-Golgi. Additionally, CERT possesses a central diphenylalanine-in-an-acidic tract (FFAT) motif that binds the ER-resident membrane proteins VAP-A and VAP-B, and a StAR-related lipid transfer (START) domain that binds and transfers ceramides [31,32]. Because of its dual targeting motifs, CERT has been proposed to shuttle ceramides across the narrow cytosolic gap at membrane contact sites between the ER and trans-Golgi [33]. Directional transport is achieved by ongoing SM production in the trans-Golgi, which serves as a metabolic trap for newly synthesized ceramides. Cloning of the responsible enzyme, SMS1, led to identification of an SMS1-related protein, SMSr (also known as sterile α-motif domain-containing protein SAMD8) [27]. SMSr catalyzes production of the SM analogue ceramide phosphoethanolamine in the lumen of the ER. Acute disruption of SMSr catalytic activity in mammalian cells causes an accumulation of ER ceramides and their mislocalization to mitochondria, triggering a mitochondrial pathway of apoptosis [34,35]. Apoptosis induction was prevented by blocking de novo ceramide biosynthesis or targeting a bacterial ceramidase to mitochondria, as evidenced by cytosolic release of Cyto c and activation of caspase 9 [36].

To further resolve the mechanism by which ceramides activate Bax and trigger apoptotic cell death in real time, we here developed a drug-inducible version of mitoCERT using FKB-FRBP chemical dimerization technology. Application of this ‘switchable’ CERT variant revealed that an acute mistargeting of ER ceramides to mitochondria specifically activates Bax, enabling us to monitor a shift in its steady state distribution from the cytosol to mitochondria over time. Thus, our data establish a valuable new tool for probing the compartment-specific functions of ceramides as critical determinants of cell fate.

Materials and methods

DNA constructs

A DNA insert encoding human CERT with a C-terminal FLAG tag (DYKDDDDK) was created by PCR and inserted in the NotI and XbaI restriction sites of mammalian expression vector pcDNA3.1(+). The first 117 N-terminal residues of CERT were substituted for an
FKBP-/rapamycin-binding (FRB) domain-encoding sequence with monomeric eGFP fused to its N terminus to create GFP-FRB-CERT or switchable CERT (sCERT). Deletion of the START domain (residues 346–597) yielded sCERT\_START. The FK506 binding protein (FKBP) domain with a N-terminal OMM anchor (corresponding to residues 34–63 of mouse AKAPI) was PCR amplified from DNA construct OMM-FKBP-RFP [37], kindly provided by G. Hajnóczky, Thomas Jefferson University, Philadelphia) and inserted into the EcoRI and EcoRV restriction sites of pSEMS-mCherry and pSEMS-HALO expression vectors (Covalys Biosciences, Witterswil, Switzerland), yielding OMM-FKBP-mCherry and OMM-FKBP-HALO. The OMM anchor sequence in these constructs was swapped for the N-terminal palmitoylation/myristoylation signal of the Lyn protein (MGCGKSKGKDSAGA) [38] by PCR, yielding PM-FKBP-mCherry and PM-FKBP-HALO. A cDNA encoding human VAP-A (kindly provided by Neale Ridgway, Dalhousie University, Halifax, Canada) was PCR amplified and inserted into the Xhol and SacI restriction sites of pSEMS-mCherry to generate mCherry-VAP-A.

Cell culture and transfection

HeLa cells (ATCC-CCL2) were grown in high glucose DMEM supplemented with 2 mM t-glutamine and 10% FBS. Chinese hamster ovary CHO-LYA cells (a kind gift from K. Hanada, Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan) were cultured in Ham’s F12 MEM with 10% FBS. Twenty-four hours prior to transfection, cells were seeded on glass coverslips coated with poly-l-lysine-polyethylene-arginine-glycine-aspartate (PLL-PEG-RGD) [39]. Transfection of DNA constructs was performed using Effectene transfection reagent (Qiagen, Hilden, Germany) according to instructions of the manufacturer. Z-VAD-fmk (10 μM, Calbiochem, San Diego, CA, USA) and myriocin (30 μM; Sigma-Aldrich, St. Louis, MO, USA) were added to cells at the time of transfection (mitoCERT constructs) or upon rapamycin treatment (sCERT constructs). Sixteen hours post-transfection with sCERT constructs, cells were treated with 50 nM rapamycin (Sigma-Aldrich) for 30 min, washed, and then incubated for 0, 4, 8 or 12 h in culture medium supplemented with z-VAD-fmk before immunofluorescence analysis. Cells were treated with staurosporine (1 μM; Sigma-Aldrich) in the presence of z-VAD-fmk for 90 min before immunofluorescence analysis.

Immunofluorescence microscopy

Transfected cells treated as outlined above were fixed with 3% paraformaldehyde in PBS for 10 min, washed in PBS, and then quenched in 50 mM NH4Cl in PBS for 10 min at RT. Cells were permeabilized in PM buffer (0.1% saponin and 0.2% BSA in PBS), immunolabeled with the following primary antibodies: rabbit polyclonal anti-FLAG (cat. no. 2368, 1 : 1000; Cell Signaling, Danvers, MA, USA); rabbit monoclonal anti-Bax (cat. no. 5023, 1 : 1000; Cell Signaling); mouse monoclonal antimitochondrial surface protein p60-Mito (cat. no. MAB1273, 1 : 1000; Millipore). Next, cells were incubated with Cy5/Cy3-conjugated donkey anti-rabbit or donkey anti-mouse secondary antibodies (cat. no. 711-175/152/150, 711-165-152/150, 1 : 400 each; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells expressing HALO-tagged proteins were labeled with 50 nM HaloTag Ligand-tetramethylrhodamine (HTL-TMR) [40]. After counter staining with DAPI (300 nM in PBS), cells were mounted in Prolong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and sealed with nail varnish. Images were captured at RT with a DeltaVision Elite Imaging System (GE Healthcare, Chicago, IL, USA) built on an inverted Olympus IX-71 microscope, a 60x1.42 NA Plan ApoN UIS2 objective, a sCMOS camera (PCO, Kelheim, Germany), an InsightSSI illumination system and SOFTWORX, 6.0, BETA27 software (Applied Precision, Issaquah, WA, USA). Fluorochromes used were DAPI, λex = 390 nm and λem = 435 nm, FITC/GFP, λex = 475 nm and λem = 523 nm; mCherry/TRITC, λex = 542 nm, and λem = 594/45 nm; Cy5, λex = 632, and λem = 676. Z-stacks of 0.2 μm in 20 optical slices to cover a total of 4 μm were acquired, followed by deconvolution using SOFTWORX software. Images were further processed using Fiji software (National Institute of Health, Bethesda, MD, USA). Individual cells were analyzed for co-localization using the JACoP plugin in Fiji. Box and whisker graphs were created using GRAPHPAD PRISM Version 7 (Graph Pad Software, La Jolla, CA, USA).

In vivo ceramide transfer assay

CHO-LYA cells grown on glass coverslips were co-transfected with sCERT or sCERT\_START and OMM-FKBP-HALO. Twenty-four hours post-transfection, cells were incubated with 0.5 μM Bodipy-TR-labeled C5-ceramide (Bodipy-Cer; Thermo Fisher Scientific) complexed to bovine serum albumin at 4 °C for 20 min and then washed twice in ice-cold Hanks’ buffered saline solution (HBSS). After addition of fresh culture medium, cells were chased for 10 min and then incubated in the absence or presence of 50 nm rapamycin at 37 °C while visualized in real time by confocal fluorescence microscopy. Fluorescent images were captured 20–25 min after the chase using an Olympus LSM FV1000 confocal microscope equipped with a temperature-controlled stage-top incubator and UPLSAPO 60X NA 1.35 oil immersion objective (Olympus, Tokyo, Japan). Fluorochromes used were eGFP λex = 488 nm and λem = 515 nm; Bodipy-TR λex = 589 nm and λem = 617 nm. Images were processed using Fiji software (National Institute of Health).
Results

Design of switchable ceramide transfer protein sCERT

We previously demonstrated that translocation of ER ceramides to mitochondria triggers a Bax-dependent pathway of apoptosis [36]. This was accomplished by expressing a ceramide transfer protein equipped with an OMM anchor, mitoCERT. To help elucidate the mechanism by which ceramides commit cells to death in real time, we designed a switchable version of mitoCERT using the rapamycin-inducible FKBP/FRB heterodimerization system [41]. To this end, the N-terminal Golgi-targeting PH domain of CERT was replaced by meGFP and a FRB domain, yielding meGFP-FRB-CERT or switchable CERT (sCERT, Fig. 1). A version of sCERT lacking the ceramide transfer or START domain, sCERT\textsubscript{D\textsubscript{START}}, served as control. To allow rapamycin-induced recruitment of sCERT to mitochondria, the OMM anchor sequence of A-kinase anchor protein 1 (mAKAP1) was fused to a FKBP domain and mCherry or HALO-tag as fluorescent marker, yielding OMM-FKBP-mCherry and OMM-FKBP-HALO, respectively. To direct sCERT to the plasma membrane, we created two additional constructs, PM-FKBP-mCherry and PM-FKBP-HALO, in which the OMM anchor sequence was replaced by the N-terminal palmitoylation/myristoylation signal of the Lyn protein, which serves as plasma membrane targeting sequence [38]. Next, these multidomain constructs were subjected to a detailed functional analysis.

sCERT undergoes drug-induced recruitment to specific organelles

To check whether sCERT can be recruited to mitochondria in response to rapamycin, HeLa cells were co-transfected with sCERT and OMM-FKBP-mCherry, treated with 50 nM rapamycin for 30 min and then analyzed by fluorescence microscopy. In untreated cells, sCERT was distributed throughout the cytosol while OMM-FKBP-mCherry localized to mitochondria. However, upon addition of rapamycin, the bulk of cytosolic sCERT was translocated to mitochondria, as evidenced by extensive co-localization of the protein with OMM-FKBP-mCherry (Fig. 2A). On the other hand, addition of rapamycin to cells co-expressing sCERT and PM-FKBP-mCherry led to a massive redistribution of sCERT from the cytosol to the plasma membrane, but not mitochondria (Fig. 2B). Similar results were obtained when analyzing the impact of rapamycin on the subcellular distribution of sCERT\textsubscript{D\textsubscript{START}} in cells expressing OMM-FKBP-mCherry or PM-FKBP-mCherry (Fig. 2A, B). sCERT and sCERT\textsubscript{D\textsubscript{START}} translocation from the

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![Design principle of sCERT.](image-url)
cytosol to mitochondria or the plasma membrane was complete within the first 5 min of rapamycin treatment and the proteins remained largely associated with these organelles when rapamycin-treated cells were washed and incubated for an additional 16 h in the absence of the drug (data not shown). From this we conclude that a short and transient exposure of cells to rapamycin suffices to induce an efficient recruitment of sCERT and sCERT ΔSTART to their target organelle.

**sCERT interacts with ER-resident VAP-A**

CERT contains a short peptide sequence or FFAT motif, which is recognized by the ER-resident tail-anchored proteins VAP-A and VAP-B. The interaction between CERT and VAPs is crucial for efficient ER-to-Golgi transfer of ceramide [31]. Moreover, the apoptotic activity of mitoCERT critically relies on an intact FFAT motif and the ability of the protein to function as a VAP-dependent ER-mitochondrial tether [36]. This led us to investigate whether sCERT is able to interact with VAPs upon its rapamycin-induced recruitment to mitochondria. To this end, HeLa cells were co-transfected with sCERT, OMM-FKBP-HALO and mCherry-tagged VAP-A (VAP-A-mCherry). In the absence of rapamycin, sCERT was distributed throughout the cytosol whereas VAP-A-mCherry displayed a reticular staining reminiscent of the ER that showed little overlap with the tubular mitochondrial network marked by OMM-FKBP-HALO (Fig. 3). By contrast,
upon 30 min exposure to 50 nM rapamycin, the bulk of sCERT redistributed to mitochondria. Importantly, mitochondrial translocation of sCERT was accompanied by an increased overlap between VAP-A-mCherry and OMM-FKBP-HALO-positive networks. Similar drug-mediated changes were observed in cells co-expressing sCERT\textsuperscript{ΔSTART}, OMM-FKBP-HALO and VAP-A-mCherry (Fig. 3). These results indicate that the rapamycin-induced translocation of sCERT and sCERT\textsuperscript{ΔSTART} to mitochondria does not interfere with the ability of these proteins to interact with VAP receptors in the ER. Hence, analogous to mitoCERT, both sCERT and sCERT\textsuperscript{ΔSTART} seem to act as VAP-dependent ER-mitochondria tethers in OMM-FKBP-expressing cells when exposed to rapamycin.

sCERT mediates ceramide delivery to mitochondria in drug-treated cells

We next addressed whether sCERT can mediate ceramide transport to mitochondria when translocated to these organelles in rapamycin-treated cells. To this end, we monitored the intracellular movement of a fluorescent analogue of ceramide (C5-Bodipy-Cer) in Chinese hamster ovary (CHO) LY-A cells. In these cells, ER-to-Golgi transport of C5-Bodipy-Cer is disrupted by a loss-of-function mutation in the CERT-encoding gene[30]. As shown in Fig. 4, LY-A cells co-transfected with sCERT and OMM-FKBP-HALO readily internalized externally added C5-Bodipy-Cer, distributing the analogue throughout the cytosol. However, when cells were treated with rapamycin, C5-Bodipy-Cer accumulated in sCERT-positive mitochondria. By contrast, in cells co-transfected with sCERT\textsuperscript{ΔSTART} and OMM-FKBP-HALO, C5-Bodipy-Cer remained largely associated with the plasma membrane upon rapamycin treatment (Fig. 4). These results indicate that sCERT is able to bind to ceramides and can mediate their transport to mitochondria upon its rapamycin-induced recruitment to these organelles.

mitoCERT expression triggers mitochondrial translocation of Bax

Our previous work revealed that cells expressing mitoCERT import ER-derived ceramides into mitochondria and undergo Bax-dependent apoptosis[36]. Bax is a pro-apoptotic member of the Bcl2 protein family that actively participates in the release of Cyto c from mitochondria, which results in caspase activation[2]. In healthy cells, Bax is a soluble monomer that primarily resides in the cytosol. Upon exposure to pro-apoptotic stimuli, Bax undergoes a conformational change that frees up a C-terminal membrane anchor, which eventually inserts in the OMM to tether the protein to mitochondria[7,8]. Subsequent oligomerization of the OMM-associated protein marks a crucial step in the creation of the proteolipid pores responsible for Cyto c release[9,10].

To further elucidate the mechanism by which ceramides commit cells to death, we monitored the mitochondrial translocation of Bax in HeLa cells transfected with mitoCERT by immunofluorescence microscopy. To uncouple this step from downstream processes mediated by caspases, cells were incubated with the broad-spectrum caspase inhibitor z-VAD-fmk during the onset apoptosis. We first analyzed the
subcellular distribution of Bax in control and staurosporine-treated cells expressing GFP-tagged Tom20 as mitochondrial marker. Immunostaining of control cells with a monoclonal anti-Bax antibody yielded a grainy staining pattern throughout the cytosol and nucleus that showed little overlap with mitochondria. In contrast, in cells treated with staurosporine for 90 min, the bulk of immunostained Bax concentrated in small dots outside of the nucleus that displayed extensive co-localization with mitochondria (Fig. 5A).

We then addressed the impact of mitoCERT on Bax localization. Expression of mitoCERT caused a marked redistribution of Bax from the cytosol to mitochondria, very similar to that observed in staurosporine-treated cells (Fig. 5A). Contrary to mitoCERT, expression of mitoCERTSTART failed to trigger mitochondrial recruitment of Bax. Moreover, mitochondrial translocation of Bax was largely abolished when mitoCERT-expressing cells were treated with myriocin, a specific inhibitor of long-chain base synthase (Fig. 5C). These results indicate that mitoCERT-induced Bax recruitment to mitochondria requires de novo ceramide biosynthesis as well as a ceramide transfer-competent form of mitoCERT. This is in line with our previous finding that apoptosis in mitoCERT-expressing cells is triggered by the translocation of ER ceramides to mitochondria and strictly dependent on Bax [36].

**Recruitment of sCERT to mitochondria triggers Bax translocation**

We next employed sCERT to analyze the impact of mistargeting ER ceramides to mitochondria on the subcellular distribution of Bax in a time-resolved manner. When immunostained against Bax, HeLa cells co-expressing sCERT and OMM-FKBP-mCherry displayed a grainy staining pattern throughout the cytosol and nucleus that showed little overlap with mitochondria. However, when cells were exposed to rapamycin for 30 min, washed and then incubated for 8 h in the absence of the drug, Bax immunostaining was primarily found in small dots outside of the nucleus that co-localized extensively with mitochondria (Fig. 6A), analogous to the subcellular distribution of Bax in staurosporine-treated or mitoCERT-expressing cells (Fig. 5A, B). Thus, rapamycin-induced recruitment of sCERT to mitochondria triggers mitochondrial translocation of Bax. Importantly, this was not observed when the experiment was repeated with cells...
expressing a ceramide transfer-defective form of sCERT, sCERTΔSTART (Fig. 6A,B), hence ruling out that rapamycin is directly responsible for triggering mitochondrial translocation of Bax. Moreover, Bax translocation did not occur when rapamycin-treated, sCERT-expressing cells were cultured in
Fig. 5. mitoCERT triggers a ceramide-dependent translocation of Bax to mitochondria. (A) HeLa cells transfected with eGFP-tagged Tom20 were incubated for 90 min in the absence (control) or presence of 1 μM staurosporine in medium supplemented with 10 μM z-VAD-fmk. Next, cells were fixed, stained with a monoclonal anti-Bax antibody and visualized by DeltaVision fluorescence microscopy. Note that the overlap between Bax (magenta) and Tom20-associated fluorescence (green) increased dramatically upon staurosporine treatment. Bar, 10 μM. (B) HeLa cells were co-transfected with eGFP-tagged Tom20 and empty vector, Flag-tagged mitoCERT or Flag-tagged mitoCERTSTART and cultured in medium supplemented with 10 μM z-VAD-fmk. At 24 h post-transfection, cells were fixed, double-stained with a monoclonal anti-Bax antibody and polyclonal anti-Flag antibody, counterstained with DAPI (blue), and then visualized by DeltaVision fluorescence microscopy. Note that expression of mitoCERT greatly enhanced the overlap between Tom20 (green) and Bax-associated fluorescence (magenta), an effect abolished by removal of the START domain. Bar, 10 μM. (C) Pearson correlation coefficients between Tom20 and Bax determined in HeLa cells co-transfected with eGFP-tagged Tom20 and empty vector, Flag-tagged mitoCERT or Flag-tagged mitoCERTSTART and incubated in the absence or presence of 30 μM myriocin. At 24 h post-transfection, cells were processed as in (A). For each boxplot, the middle line denotes the median while the top and bottom of the box indicate the 75th and 25th percentile. Whiskers denote the minimum and maximum values. ****P < 0.0001 by two-tailed unpaired Student’s t-test.

the presence of myriocin (Fig. 6B). Collectively, these results indicate that, analogous to mitoCERT, sCERT triggers a ceramide-dependent mitochondrial translocation of Bax, but only in response to rapamycin.

To monitor ceramide-induced translocation of Bax over time, HeLa cells were co-transfected with sCERT and OMM-FKBP-mCherry, treated with rapamycin for 30 min, washed, cultured for different time periods, and then processed for immunostaining against Bax. This revealed that mitochondrial translocation of Bax is initiated within 4 h after rapamycin treatment (Fig. 6C). The mitochondrial pool of Bax then gradually increased within 12 h after rapamycin treatment. In contrast, no Bax translocation was observed in rapamycin-treated cells expressing sCERTSTART. Based on these findings, we conclude that sCERT is a suitable tool for unraveling the mechanism by which mitochondrial ceramides commit cells to death and for monitoring this process in real time.

Discussion

Mitochondrial translocation of ER ceramides activates a Bax-dependent pathway of apoptosis, but the molecular principles by which this occurs remain to be established. To resolve the sequence of events by which mitochondrial ceramides trigger apoptotic cell death in real time, we here developed a rapamycin-inducible version of the ceramide transfer protein CERT, termed ‘switchable’ CERT or sCERT. We show that a short and transient exposure of cells to rapamycin suffices to induce efficient recruitment of cytosolic sCERT to its target organelle. Upon its drug-induced recruitment to mitochondria, sCERT retains the ability to bind VAP protein receptors in the ER and mediates mitochondrial transport of externally added fluorescent ceramides. Analogous to cells treated with the mitochondrial apoptosis-inducing agent staurosporine, sCERT-expressing cells accumulate Bax onto their mitochondria within 4 h after rapamycin exposure. The ability of mitochondrial ceramide import and Bax translocation is abolished upon removal of its ceramide transfer or START domain. Moreover, sCERT fails to initiate Bax translocation upon blocking de novo ceramide biosynthesis, indicating that activation of Bax is triggered by sCERT-mediated delivery of ER ceramides to mitochondria. Bax translocation occurred in the presence of a broad-spectrum caspase inhibitor. This implies that ceramide-induced Bax recruitment ensues independently of a caspase-mediated amplification loop, hence in contrast to Bax-mediated pathways triggered by death receptors and paclitaxel [42,43]. Besides corroborating our previous finding that mistargeting of ER ceramides to mitochondria specifically activates Bax-mediated apoptosis [36], the present data establish sCERT as a novel tool to dissect the underlying mechanism in a time-resolved manner.

Bax and Bak are pro-apoptotic members of the Bcl-2 protein family that, upon activation, commit cells to death by forming oligomeric pores in the OMM [9,10,44,45]. Both proteins are regulated by a constant translocation to mitochondria and retro-translocation back into the cytosol, with pro-survival Bcl-2 proteins promoting retro-translocation [4,5]. In healthy cells, a fast retro-translocation of Bax leads to its steady state accumulation in the cytosol. While Bak is also present in the cytosol, its low retro-translocation rate causes its predominant mitochondrial localization. Swapping the C-terminal OMM anchors of Bax and Bak reverses their subcellular distribution. Moreover, reducing Bax retro-translocation to the level of Bak shuttling renders Bak cytotoxic, resulting in apoptosis induction in the absence of any stress signal [5]. Thus, ceramides may exert their pro-apoptotic activity on mitochondria by interfering with efficient Bax retro-translocation from...
mitochondria into the cytosol. Experiments with isolated mitochondria revealed that retro-translocation of endogenous Bax also occurs in vitro [14] and that the pore-forming activity of externally added Bax is enhanced significantly in the presence of ceramides [20]. Voltage-dependent anion channel VDAC2 enables Bax to mediate apoptosis [15] and concomitantly ensures Bax inhibition by mediating its retro-translocation into the cytosol [14]. Moreover, VDAC2 binds ceramides and cell death induced by a rise in mitochondrial ceramides requires a ceramide-binding competent form of VDAC2 [26]. Collectively, these findings

Fig. 6. Mitochondrial recruitment of sCERT triggers mitochondrial translocation of Bax. (A) HeLa cells co-transfected with sCERT or sCERTΔSTART and OMM-FKBP-mCherry (OMM-FKBP) were incubated in the absence (−rapa) or presence of 50 nM rapamycin for 30 min (+rapa), washed and then cultured for another 8 h in medium supplemented with 10 μM z-VAD-fmk. Next, cells were fixed, stained with a monoclonal antibody against Bax, counterstained with DAPI (blue) and visualized by DeltaVision fluorescence microscopy. Note that the overlap between OMM-FKBP-positive mitochondria (green) and Bax (magenta) increased dramatically upon rapamycin treatment of sCERT-expressing cells, an effect abolished by removal of the START domain. Bar, 10 μm. (B) HeLa cells co-transfected with sCERT or sCERTΔSTART and OMM-FKBP-mCherry (OMM-FKBP) were incubated with 50 nM rapamycin for 30 min in the presence or absence of 30 μM myriocin, washed, and then cultured for another 12 h with or without myriocin in medium supplemented with 10 μM z-VAD-fmk. Next, cells were processed as in (A) to determine Pearson correlation coefficients between OMM-FKBP and Bax. (C) HeLa cells co-transfected with sCERT or sCERTΔSTART and OMM-FKBP-mCherry (OMM-FKBP) were incubated with 50 nM rapamycin for 30 min, washed and then cultured for 0, 4, 8 and 12 h in medium supplemented with 10 μM z-VAD-fmk. Next, cells were processed as in (A) to determine Pearson correlation coefficients between OMM-FKBP and Bax. For each boxplot, the middle line denotes the median while the top and bottom of the box indicate the 75th and 25th percentile. Whiskers denote the minimum and maximum values. *P < 0.05 and ****P < 0.0001 by two-tailed unpaired Student’s t-test.
support a role of VDAC2 as a direct and critical effector of ceramide-mediated apoptosis. Experimental validation of the idea that the apoptogenic activity of mitochondrial ceramides relies on their ability to bind VDAC2 and block VDAC2-mediated retro-translocation of Bax warrants a detailed spatiotemporal analysis of Bax dynamics in live cells upon induction of ceramide-mediated apoptosis. To this end, our ongoing efforts are aimed at the development of assays for real-time imaging of Bax, VDAC2 and other components of the mitochondrial apoptotic machinery in conjunction with the application of sCERT.

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Author contributions

JCMH conceptualized the study and wrote the manuscript, with critical input from AJ and SD. AJ and SD designed and performed the transfection studies and wrote the script, with critical input from AJ and SD. The study was organized by ProjektDEAL.

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