BAD Enables Ceramide to Signal Apoptosis via Ras and Raf-1*

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Prior investigations document that proliferative signaling cascades, under some circumstances, initiate apoptosis, although mechanisms that dictate the final outcome are largely unknown. In COS-7 cells, ceramide signals Raf-1 activation through Ras (Zhang, Y., Yao, B., Delikat, S., Bayoumy, S., Lin, X. H., Basu, S., McGinley, M., Chan-Hui, P. Y., Lichtenstein, H., and Kolesnick, R. (1997) Cell 89, 63–72), but not apoptosis. However, expression of small amounts of the pro-apoptotic Bcl-2 family member, BAD, conferred ceramide-induced apoptosis onto COS-7 cells. Ceramide signaled apoptosis in BAD-expressing cells by a pathway involving sequentially kinase suppressor of Ras (KSR)/ceramide-activated protein kinase, Ras, c-Raf-1, and MEK1. Downstream, this pathway linked to BAD dephosphorylation at serine 136 by prolonged inactivation of Akt/PKB. Further, mutation of BAD at serine 136 abrogated ceramide signaling of apoptosis. The present study indicates that when ceramide signals through the Ras/Raf cascade, the availability of a single target, BAD, may dictate an apoptotic outcome.

It is generally accepted that a family of evolutionarily conserved cysteine aspartate proteases or caspases serve as effectors of the apoptotic response (1). The upstream elements that lead to this committed stage are presently the focus of intensive investigation. For cytokine receptors of the tumor necrosis factor (TNF) receptor superfamily, a death domain adaptor protein such as Fas ligand or TNFα, that mediate the death response (6, 10). Alternately, ceramide induces apoptosis directly through a mechanism inhibitable by anti-apoptotic Bcl-2 family members (11–13). This transcriptionally independent pathway for induction of apoptosis has been observed in cytoplasts (14) and recently reconstituted in a cell-free system (15). The target for ceramide and the signaling system involved in this latter death paradigm are unknown.

Recent investigations have begun to elucidate a pathway for induction of apoptosis through the pro-apoptotic Bcl-2 family member BAD (16–19). Bcl-2, originally discovered at the chromosomal breakpoint of t(14;18)-bearing follicular B cell lymphomas, is overexpressed in many tumors, and inhibits apoptosis induced by a variety of stimuli (20–22). Different mechanisms for the anti-apoptotic action of Bcl-2 family members have been proposed, including the regulation of the opening of the inner mitochondrial pore necessary for permeability transition (23), cytochrome c release required for caspase 9 activation (24, 25), binding of the Ced4 homolog Apaf-1 (26), and electron transport and reactive oxygen species generation (27). Whether some or all of these mechanisms predominate is presently unclear. It is established, however, that Bcl-2 family members homo- and heterodimerize, and that this regulates the tendency to undergo apoptosis upon exposure to stress stimuli. Pro-apoptotic Bcl-2 family members may act by more than one mechanism. For activation of BAD, phosphorylation at two sites, serine 112 and 136, determines heterodimerization, proliferation or senescence depending on the cell type. Very often, however, the outcome of signaling through this pathway is apoptosis.

Several lines of evidence support the role of ceramide as a signal for the apoptotic response. In a variety of cell types, ceramide generation precedes morphological and biochemical manifestations of apoptosis. Further, specific inhibitors of ceramide synthase, such as the fungal toxin fumonisin B1, antagonize apoptosis mediated by de novo ceramide synthesis (4). Perhaps most convincingly, in systems where the acid sphingomyelinase appears to regulate ceramide signaling of apoptosis such as in peripheral B cells or endothelium, genetic deficiency of acid sphingomyelinase compromises ceramide generation and apoptosis (5, 6). Recent studies in Saccharomyces cerevisiae revealed that the heat stress response is mediated by synthesis of sphingolipids (7–9). The development of thermotolerance is defective in mutants incapable of synthesizing ceramide, and sphingolipid analogs bypass the defect, restoring growth at elevated temperature. These data indicate that ceramide signaling is evolutionarily older than the caspase/apoptotic death response system, and hence these two pathways must have been linked later in development.

Ceramide-induced apoptosis occurs via two independent mechanisms. Ceramide signals, perhaps through the Jun kinase cascade, the transcriptional regulation of gene products, such as Fas ligand or TNFα, that mediate the death response (6, 10). Alternately, ceramide induces apoptosis directly through a mechanism inhibitable by anti-apoptotic Bcl-2 family members (11–13). This transcriptionally independent pathway for induction of apoptosis has been observed in cytoplasts (14) and recently reconstituted in a cell-free system (15). The target for ceramide and the signaling system involved in this latter death paradigm are unknown.

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Ceramide Signals Apoptosis through BAD

kinase which phosphorylates serine 136 of BAD in primary neurons is Akt/PKB (18). The kinase selective for serine 112 has not been identified. The cycle of phosphorylation/dephosphorylation appears relevant to the mechanism by which interleukin-3 depletion signals, and interleukin-3 repulsion inhibits, induction of apoptosis in FL5.12 lymphoid progenitor cells (17, 19). Further, the mechanism by which other pro-apoptotic Bcl-2 family members such as BAK or BAX signal apoptosis is fundamentally different from BAD since phosphorylation sites analogous to serine 112 and serine 136 are not present in these proteins.

The present studies link phosphorylation of BAD to ceramide signaling of apoptosis. We show that the pro-apoptotic Bcl-2 family member BAD confers ceramide-induced apoptosis onto COS-7 cells, which are ordinarily resistant. Ceramide signals apoptosis in BAD-expressing cells by a pathway involving sequential kinase suppression of Ras (KSR)/ceramide-activated protein kinase (CAPK), Raf, Raf-1, and MEK1, which links distally to BAD through Akt/PKB. These investigations show that the availability of a single target, in this case BAD, converts the mitogen-activated protein kinase (MAPK) cascade, which is usually proliferative and/or anti-apoptotic, into a pro-apoptotic signaling pathway.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells were grown in high glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Life Technologies, Inc.), penicillin, and streptomycin at 37°C in a 5% CO2 atmosphere, as described (28).

Mammalian Expression Vectors—The plasmids pCDNA3-KSR, pCDNA3-KI-KSR, pCDNA3-Flag-Raf, pCDNA3-Flag-KSR, and pCDNA3-Flag-KI-KSR were generated as described previously (28). The plasmids pFLAG-CMV2-BAD, pCDNA3-BAK and pCDNA3-BAX were provided by Dr. John Reed. The Raf constructs pBSV-BBX Raf and pBSV-C4 Raf were kindly provided by Dr. Joseph Brudner. The Ras constructs pZip H-Ras17N, pZip H-Ras17N69N, and pZip H-Ras17N/1865 were kindly provided by Dr. Lawrence A. Quilliam. The BAD constructs pSFFVBAD, pSFFVBADS112A, pSFFVBADS136A, and pSFFVBADS112AS136A were kindly provided by Dr. Stanley Korsmeyer. The phosphotyrosinolostil (P) I kinase construct pCG-p10(10,10)N and Akt construct pCr.I/Myr-AKT-1A were contributed by Dr. Morris Birnbaum.

Apoptosis Assays—COS-7 cells (2 × 10⁴) were seeded in six-well plates and after 24 h transiently transfected using LipofectAMINE (Life Technologies, Inc.) with the indicated quantities of plasmid according to the manufacturer's instructions. Cells were incubated in transfection media for 20–12 h and switched back to growth media (Dulbecco's Technologies, Inc.), penicillin, and streptomycin at 37°C in a 5% CO2 atmosphere, as described (28).

Immune Complex Kinase Assays—To determine Ras dependence of Raf-1 activation by KSR/CAPK, COS-7 cells (3 × 10⁶) were seeded in 150-mm plates and transiently transfected as above with 10 μg each of KSR (pCDNA3-KSR), Raf-1 (pCDNA3-Flag-Raf), RasN17 (pZip H-Ras17N), RasN17N69 (pZip H-Ras17N69N), or RasN17S186 (pZip H-Ras17N1865), as indicated. Cells were harvested 48 h after transfection in Nonidet P-40 lysis buffer (25 mM Tris (pH 7.5), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% deoxycholate, 0.1% SDS, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin/sodium trypsin inhibitor, 1 mM NaVO₄). 200 μg of post-nuclear supernatants were resolved by 12% SDS-PAGE. Western analysis was performed with either anti-BAD a9-943 (Santa Cruz) or anti-BAD136 pAG (kindly provided by Dr. Michael Greenberg) as primary antibodies.

RESULTS

BAD Enables Ceramide to Signal Apoptosis—Ceramide is a potent activator of apoptosis in a variety of cell systems (2, 3). In contrast, as much as 100 μM C₂₇-ceramide failed to induce apoptosis in COS-7 cells at any time from 0–24 h (Fig. 1B and data not shown). Since Bcl-2 blocks ceramide-induced apoptosis in many systems (11–13), we examined the levels of Bcl-2 family members in COS-7 cells. Western analysis revealed significant levels of endogenous Bcl-2, Bcl-XL, and Bax in COS-7 cells, while the pro-apoptotic Bcl-2 family member BAD was absent (data not shown). Overexpression of BAD induced dose- and time-dependent apoptosis (Fig. 1A and B). For these studies, 2 × 10⁴ COS-7 cells were transfected with pFLAG-CMV2-BAD and after 16 h switched to growth medium. Transfection with as little as 0.01 μg of the BAD expression plasmid increased apoptosis from a basal level of 4–10% within 6 h after removing the transfection media (p < 0.001) and 0.1 μg of the pFLAG-CMV2-BAD construct increased apoptosis to 18% of the total population (Fig. 1A). Qualitatively similar results were obtained between 6 and 12 h after removing the transfection media. Upon transfection of 0.1 μg of pFLAG-CMV2-BAD, elevated apoptosis was detected as early as 2 h after removing the transfection media (Fig. 1B), and was statistically significant by 4 h (p < 0.001). Although ceramide itself was ineffective (Fig. 1B), BAD enabled ceramide to increase apoptosis nearly 3-fold at all transfected doses from 0.01 to 0.1 μg (Fig. 1A) (p < 0.001 at all doses of BAD greater than 0.01 μg). The optimal time for this effect of ceramide was 6 h after changing of the transfection media (Fig. 1B). Immunofluorescence studies revealed that apoptosis was restricted to cells staining positive for BAD (data not shown). BAD also conferred ceramide-induced caspase activation onto COS-7 cells as measured using a fluorogenic caspase substrate Z-DEVD-APC (data not shown) (31). Apoptosis in BAD-expressing cells was not significantly affected by adding 10 μM cycloheximide 1 h prior to ceramide (data not shown). Similarly, expression of low amounts of BAD enabled ceramide to signal apoptosis in NIH 3T3 cells (data not shown), a cell line in which ceramide normally induces a proliferative response (32).

For all subsequent studies, 0.1 μg of pFLAG-CMV2-BAD was arbitrarily selected as it generated a readily detectable baseline level of apoptosis and permitted consistent ceramide enhancement. In contrast to the natural analog of ceramide, C₂₇-ceramide, the natural analog of dihydroceramide, DH-C₂₇-ceramide, did not promote apoptosis (Fig. 1C). These studies indicate an absolute requirement for the trans-double bond of
BAD Enables KSR/CAPK and Raf-1 to Signal Apoptosis—

The next set of experiments examined the effects of KSR/CAPK, a putative target for ceramide, on BAD-mediated apoptosis. KSR was originally defined in genetic screens as downstream of Ras and either upstream or parallel to Raf-1 (33–35). Recent studies from our laboratory provided evidence that CAPK (36) is the mammalian counterpart of KSR (28). We showed that KSR/CAPK phosphorylates c-Raf-1 on Thr269 increasing its activity toward MEK1, leading to increased signaling through the MAPK cascade. Ceramide, but not other lipid second messengers enhanced KSR/CAPK activity in vitro and in vivo. For the present studies, the KSR construct pCDNA3-KSR, which is constitutively active when expressed in COS-7 cells (28), was transfected with or without BAD. At all doses of pCDNA3-KSR up to 3 μg/2 × 10⁵ cells, KSR, like ceramide, failed to initiate apoptosis (Fig. 2A). However, BAD enabled KSR to signal apoptosis in a dose-dependent manner (p < 0.001 at all doses of KSR greater than 1 μg). Co-expression of the kinase-inactive KSR (KI-KSR) construct pCDNA3-KSR(D683A/D700A), generated by substitution of alanine residues for conserved aspartates, failed to stimulate apoptosis (Fig. 2B). In fact, KI-KSR blocked ceramide-induced apoptosis in BAD-expressing cells (C₂-ceramide+KI-KSR+BAD p < 0.001 versus C₂-ceramide+BAD). Neither KSR construct affected BAD expression (data not shown). Thus, KI-KSR serves dominant-negative function during ceramide signaling of apoptosis mediated by BAD. These studies place KSR/CAPK downstream of ceramide in a pathway leading to apoptosis.

Similarly, dominant-positive Raf-BXB (37) alone failed to initiate apoptosis in COS-7 cells, but signaled apoptosis when co-expressed with BAD (Fig. 2C) (Raf-BXB+BAD p < 0.001 versus BAD alone). Further, expression of dominant-negative Raf-C4 (37) failed to signal apoptosis in BAD-expressing cells, yet blocked the effect of C₂-ceramide (Fig. 2D) (C₂-ceramide+Raf-C4+BAD p < 0.001 versus C₂-ceramide+BAD). Neither Raf construct affected BAD expression (data not shown). Additional studies were performed to molecularly order KSR and Raf-1 in signaling of the apoptotic response. Table I shows that dominant-negative KSR failed to block dominant-positive Raf-BXB-induced apoptosis, whereas dominant-negative Raf-C4 blocked constitutively active KSR-induced apoptosis (BAD+KSR+Raf-C4 p < 0.001 versus BAD+KSR). Hence, the target for ceramide, KSR/CAPK, as anticipated, is ordered upstream of Raf-1 in the signaling of apoptosis in BAD-expressing COS-7 cells. Furthermore, the specific chemical inhibitor of MEK1, PD98059 (38), completely blocked ceramide-induced apoptosis in BAD-expressing COS-7 cells.

The sphingoid base backbone for the induction of apoptosis by ceramide. The effect of ceramide to signal apoptosis in BAD-expressing cells was dose-dependent; as little as 30 μM C₂-ceramide induced significant apoptosis (p < 0.001 versus BAD alone) and a maximal effect occurred with 100 μM C₂-ceramide (Fig. 1D).

BAD Enables Ceramide to Signal Apoptosis in COS-7 Cells—

BAD enables ceramide to signal apoptosis in COS-7 cells. A, BAD induces dose-dependent apoptosis which is increased by ceramide in COS-7 cells. 2 × 10⁵ COS-7 cells were transiently transfected as detailed under "Experimental Procedures" with the indicated amounts of the Flag-BAD construct (pFLAG-CMV2-BAD). After 16 h, transfection medium was replaced with growth media containing 1% Me₂SO (control) or 100 μM C₂-ceramide. After 6 h, cells were harvested and 600 cells scored for apoptosis by staining with bisbenzamide, as described. This experiment is representative of three independent experiments. B, time course of ceramide-induced apoptosis in COS-7 cells expressing BAD. COS-7 cells, transiently transfected with 0.1 μg of Flag-BAD or empty vector (pFLAG-CMV2), were incubated with Me₂SO or the indicated amounts of C₂-ceramide and apoptosis quantified after 6 h as in A. This experiment is representative of three independent experiments. C, ceramide but not dihydroceramide signals apoptosis in COS-7 cells expressing BAD. COS-7 cells, handled as in B, were incubated with Me₂SO or the indicated amounts of C₂-ceramide or C₂-dihydroceramide and apoptosis was quantified after 6 h as in A. This experiment is representative of three independent experiments. D, ceramide induces dose-dependent apoptosis in COS-7 cells expressing BAD. COS-7 cells, handled as in B, were incubated with the indicated amounts of C₂-ceramide and apoptosis was quantified after 6 h as in A. This experiment is representative of three independent experiments.
apoptosis (n = 2, data not shown). In contrast, dominant negative SEK-1 failed to prevent ceramide-induced apoptosis (data not shown).

Ceramide Signaling through KSR/CAPK to Raf-1 Depends on Ras—To evaluate a requirement for Ras in executing apoptosis through BAD, initial studies determined whether Ras was necessary for KSR/CAPK signaling. For these studies, Raf-1 was co-expressed with the appropriate empty vectors, KSR, and/or Ras constructs for 48 h, and then Raf-1 was immunoprecipitated, and an immune complex kinase assay performed with kinase-inactive MEK1 as substrate (Fig. 3A). As described previously (28), co-expression of KSR with Raf-1 resulted in marked Raf-1 activation. This effect was blocked by dominant-negative RasN17 (39), but not by RasN17N69 (40) or RasN17S186 (41), mutants which interfere with the dominant negative function of RasN17 and serve as its controls. Similar results were obtained when Raf-1 activity was assessed by reconstituting the entire MAPK cascade (data not shown). These studies indicate that Ras is required for KSR/CAPK activation of Raf-1. Moreover, RasN17, but not RasN17N69 or RasN17S186, blocked ceramide-induced apoptosis in BAD-expressing COS-7 cells (Fig. 3B) (RasN17 + BAD + C2-ceramide p < 0.001 versus BAD + C2-ceramide). These findings indicate that Ras regulates ceramide signaling of apoptosis through the MAPK cascade.

Ceramide Signaling of Apoptosis Is Specific for BAD and Depends on Serine 136—Phosphorylation of BAD on serine 112 or 136, canonical 14-3-3 recognition sites, results in dissociation of BAD-Bcl-2/Bcl-XL heterodimers, BAD inactivation by sequestration as a cytoplasmic complex with 14-3-3, and an anti-apoptotic state (16, 17). A reciprocal set of events reportedly signals apoptosis. Other pro-apoptotic Bcl-2 family members, such as BAK (42, 43) and BAX (44), do not contain 14-3-3 recognition sites, and thus are regulated by different signaling
mechanisms than BAD. To determine if the ceramide-mediated effects described above were unique to BAD, COS-7 cells were transfected with BAK and BAX. Fig. 4A shows that BAK and BAX, like BAD, induced dose-dependent apoptosis when expressed in COS-7 cells. In contrast to BAD, however, BAK and BAX failed to confer ceramide signaling of apoptosis. These findings indicate that neither BAK nor BAX are intermediates in the ceramide-induced signaling cascade leading to apoptosis in COS-7 cells. Moreover, they led us to hypothesize that serine 112 or 136 of BAD might be targeted to regulate this form of ceramide-induced apoptosis.

To elucidate the function of these sites, the serine substitution mutants BAD112A and BAD136A (17) were expressed in COS-7 cells (Fig. 4B). BAD112A and BAD136A, which lack negative regulatory phosphorylation sites, were more effective in inducing apoptosis than wild type BAD. Transfection of $2 \times 10^5$ COS-7 cells with 0.1–1.3 $\mu$g of pSFFVBAD yielded 9–17% apoptosis, whereas 0.1–1.0 $\mu$g of pSFFVBAD136A yielded 13–23% apoptosis and 0.075–1.0 $\mu$g of pSFFVBADS136A generated 14–37% apoptosis (data not shown). Thus, removal of serine 136 increased apoptosis 2–3-fold in COS-7 cells whereas substitution of serine 112 had a modest effect. It should be noted that transfection of equal quantities of the different BAD constructs yielded similar levels of expression. For subsequent studies, the doses of BAD112A and BAD136A were adjusted to yield a comparable base-line level of apoptosis of approximately 17% (Fig. 4B). Whereas BAD112A mimicked the effect of wild type BAD, confering ceramide signaling of apoptosis, BAD136A was ineffective (Fig. 4B). BAD112A136A also induced dose-dependent apoptosis but failed to confer ceramide sensitivity (data not shown). When equal amounts of pSFFVBAD and pSFFVBADS136A were transfected, at each dose within the range of 0.1–1.0 $\mu$g/$2 \times 10^5$ COS-7 cells, the level of apoptosis in cells expressing BAD136A was similar to that observed in wild type BAD-expressing cells treated with ceramide (data not shown). These studies implicate the serine 136 site of BAD as necessary for ceramide signaling of apoptosis through BAD. Further, they suggest that ceramide might induce dephosphorylation of BAD to allow for signaling of apoptosis.

To evaluate this possibility, we examined the phosphorylation state of BAD before and after ceramide stimulation. Similar to what has been reported in FL5.12 cells (17), BAD resolves as a doublet in resting COS-7 cells, as evidenced by Western analysis using an anti-BAD antibody (Fig. 4C). The upper band is phosphorlated on serine 136 since a polyclonal antibody specific for phosphoBAD136, anti-BAD136 pAb (18), recognized only this band (data not shown). Further, treatment of cells with ceramide results in loss of the phosphorylated form of BAD induced by the selective loss of the upper band in Fig. 4C, and accumulation of the dephosphorylated lower band. As previously demonstrated by Greenberg and co-workers (18), the upper band could be further resolved on a large gel as two phophoBAD136 forms by Western analysis using anti-BAD136 pAb (Fig. 4D). Both forms of phosphoBAD136 were depleted by C$_2$-ceramide treatment. The reduction in phosphoBAD136 was detected within 2 h of ceramide treatment (data not shown). Pretreatment of COS-7 cells with 100 $\mu$g MEK1 inhibitor PD980590 (similar results were seen with 50 $\mu$g), which blocked apoptosis, also prevented dephosphorylation of BAD in response to ceramide, indicating that ceramide signals this event via the MAPK cascade (Fig. 4D). Consistent with this observation, KI-KSR and Raf-C4 prevented C$_2$-ceramide-induced phosphoBAD136 depletion (data not shown).

Ceramide Induces Suppression of Akt/PKB—Recent investigations showed that Akt/PKB, which suppresses apoptosis induced by various stimuli in many cell types (45), phosphorylates BAD at serine 136, blocking BAD-induced apoptosis (18, 19). To evaluate whether the upstream signaling initiated via ceramide integrates into the Akt/PKB pathway, endogenous Akt/PKB was immunoprecipitated from COS-7 cells and an immunocomplex kinase assay performed using histone 2B as a substrate. Fig. 5A shows that ceramide treatment leads to a marked decrease in Akt/PKB activity. This effect was detected as early as 2 h after ceramide treatment (data not shown). To ascertain whether the upstream pathway initiated by ceramide acts at the level of the Akt/PKB activator PI 3-kinase or at Akt/PKB itself, a constitutively active mutant of Akt lacking the pleckstrin homology domain, ΔPH-AKT (46), or a constitutively active PI 3-kinase, p110*, generated by appending the
p85 inter-Src homology 2 regulatory region of PI 3-kinase to the p110 catalytic unit (47), were cotransfected with BAD in COS-7 cells. While DPH-AKT blocked ceramide-induced apoptosis, p110* failed to do so (Fig. 5B), despite marked expression of p110* by Western blot. Even higher doses of p110*, which resulted in a decrease in base-line BAD-induced apoptosis, did not affect the fold increase in apoptosis in response to ceramide (data not shown). These studies support the premise that ceramide signals apoptosis by inactivating Akt/PKB, thereby promoting accumulation of dephosphorylated BAD.

**DISCUSSION**

The present studies link signaling through Ras and Raf-1 to apoptosis via dephosphorylation of the pro-apoptotic Bcl-2 fam-

Fig. 4. Ceramide signals apoptosis specific for BAD and requires serine 136. A, ceramide does not signal apoptosis through BAK or BAX. COS-7 cells, transiently co-transfected as in Fig. 1 with the indicated amounts of either BAK (pcDNA3-BAK) or BAX (pcDNA3-BAX), were incubated with 0.2% MeSO or 100 μM C₂-ceramide and apoptosis quantified as in Fig. 1. This experiment is representative of three independent experiments. B, serine 136 of BAD is necessary for ceramide to signal apoptosis. COS-7 cells, transiently transfected as in Fig. 1 with either 1.3 μg of wild type (wt) BAD (pSFFVBAD), 0.7 μg of BAD112A (pSFFVBAD112A) or 0.1 μg of BAD36A (pSFFVBAD36A) to yield a base-line level of apoptosis of approximately 17%, were incubated with 0.2% MeSO or 100 μM C₂-ceramide and apoptosis quantified as in Fig. 1. This experiment is representative of three independent experiments. C, ceramide signals dephosphorylation of BAD. 1.5 × 10⁶ COS-7 cells transiently transfected as in Fig. 1 with 0.4 μg of Flag-BAD, were incubated with 0.2% MeSO or 100 μM C₂-ceramide for 6 h and harvested in RIPA lysis buffer. 200 μg of post-nuclear supernatants were resolved by 12% SDS-PAGE and analyzed by Western blotting employing a C-terminal specific anti-BAD antibody. This experiment is representative of three independent experiments. D, the MEK1 inhibitor PD98059 protects against phosphoBAD136 depletion. COS-7 cells were handled as in C except for pre-incubation in 100 μM PD98059 or 0.2% MeSO for 1 h prior to treatment with 0.2% MeSO or 100 μM C₂-ceramide. PhosphoBAD136 was resolved by 12% SDS-PAGE and identified by Western analysis using anti-BAD136 pAb. This experiment is representative of three independent experiments.

Fig. 5. Ceramide signals suppression of Akt/PKB activity. A, ceramide induces a decrease in Akt/PKB activity. COS-7 cells, handled as in Fig. 4C, were incubated with 0.2% MeSO or 100 μM C₂-ceramide for 3–4 h and harvested in RIPA lysis buffer as detailed under “Experimental Procedures.” Endogenous Akt/PKB was immunoprecipitated from post-nuclear supernatants and an immune complex kinase assay using histone 2B as substrate was performed as described under “Experimental Procedures.” This experiment is representative of three independent experiments. B, ceramide signals through Akt/PKB but not PI 3-kinase. COS-7 cells, transiently co-transfected as in Fig. 1 with 0.1 μg of Flag-BAD, and 2 μg of constitutively active PI 3-kinase p110* (pCG-p110*myc) or constitutively active Akt ΔPH-AKT (pCr3.1/Myr-AKT-HA), were incubated with 0.2% MeSO or 100 μM C₂-ceramide, and apoptosis quantified as in Fig. 1. This experiment is representative of three independent experiments.
ceramide signaling of apoptosis through BAD. These studies support the notion that the availability of a single target, in this case BAD, converts a proliferative signaling cascade to an apoptotic response.

These findings are consistent with the known regulation of ceramide signaling of apoptosis through Bcl-2 family members. Kroemer and co-workers (49) have shown that ceramide signals permeability transition, release of apoptogenic factors and generation of reactive oxygen species in cytoplasts, indicating these events are transcriptionally independent. Further support for this notion is derived from studies in a cell-free system, which show that ceramide-induced mitochondrial dysfunction in intact cells, cytoplasts, and the cell-free system was inhibited by Bcl-2 (14, 15, 49). Similarly, ceramide signaling of apoptosis inhibition in naive mitochondria, leading to apoptosis of naive nuclei (15). Ceramide-induced mitochondrial dysfunction from cytoplasts and the cell-free system was inhibited by Bcl-2 (14, 15, 49). Similarly, ceramide signaling of apoptosis in BAD-expressing COS-7 cells was not inhibited by cycloheximide, indicating the connection between the MAPK cascade and BAD does not require new protein synthesis. Ceramide signaling of BAD dephosphorylation appears to occur through inactivation of Akt/PKB. Ceramide markedly reduced endogenous Akt/PKB activity in BAD-expressing cells, and constitutively active Akt/PKB, but not PI 3-kinase, abolished ceramide-induced apoptosis. This latter observation argues that it is unlikely that activation of a phosphatase primarily mediates this process. Zhou et al. have similarly observed that ceramide inactivates Akt/PKB in HMN1 motor neurons by a mechanism independent of PI 3-kinase, suggesting that this pathway may operate in cells other than COS-7 cells.2 It should be noted that BAD has a limited tissue distribution (50) whereas ceramide-induced apoptosis is more generalized (2, 3). Hence, this mechanism is not likely to mediate all of ceramide signaling of apoptosis, unless another Bcl-2 homolog is found that serves the same function. This suggests that ceramide, like Akt/PKB (45), must link to other effectors of the apoptotic process. BAX and BAK are not likely candidates, as neither supported ceramide-induced apoptosis in COS-7 cells.

In many systems, activation of the Ras, Raf-1 and MAPK pathway is mitogenic and anti-apoptotic (51, 52). However, recent investigations have begun to define situations in which Ras signals apoptosis. Gulbins and co-workers (53, 54) showed that activation of Ras/G1C95 stimulated Ras-GDP/GTP exchange in Jurkat T cells, and that dominant interfering mutants of Ras blocked Ras-induced death. Guillot et al. (55) extended this concept to include a requirement for MAPKs in Ras-induced death of a neuroblastoma cell line. Trent et al. (56) reported similar results in L929 cells treated with TNFa. Although the present studies do not address whether resting or stimulated Ras activity is required for ceramide-induced death, ceramide is capable of activating Ras, by an unknown mechanism, in Jurkat cells (53, 54).

Ras signaling appears context dependent, as even within the same cell type apoptosis or mitogenesis may result, depending on the genetic and environmental milieu. For instance, v-Ras and v-Raf induce transformation of p53(−/−) mouse embryo fibroblasts, whereas they induce apoptosis of p53(+/+) mouse embryo fibroblasts (57). Similarly, transfection of v-Ras rendered Jurkat cells susceptible to apoptosis once protein kinase C was down-regulated or pharmacologically inhibited (58). Further, oncogenic Ras induced apoptosis in NIH3T3 cells if activation of NF-kB was prevented by co-expression of a superrepressor of IkBa (59). In some instances, the decision to die may reflect gene dosage. For example, expression of small amounts of Raf-1 correlates with mitogenesis of MCF-7 breast cancer cells whereas high expression correlates with induction of apoptosis (60).

A study by Evan and co-workers (61) addressed the issue of which of the known downstream Ras effector systems might mediate apoptosis in a model of c-Myc-induced death. In rat-1 fibroblasts in which c-Myc is expressed conditionally through an estrogen promoter, co-expression of oncogenic V12Ras enhanced c-Myc-induced apoptosis. By employing partial loss of function mutants of V12Ras, these investigators showed that Ras-induced apoptosis was mediated through Raf-1, Raf.GDS was without effect on apoptosis, and PI 3-kinase signaled anti-apoptosis. These studies predict that the eventual outcome of signaling through Ras will be determined at least in part by the relative strength of these downstream effector systems. In some of the above studies, Ras/Raf-1-mediated apoptosis, like ceramide-stimulated apoptosis, was inhibitable by Bcl-2 (55, 58, 62), suggesting the possibility that Akt/BAD might be involved.

The present studies extend our prior investigations into ceramide signaling through KSR/CAPK (36, 63–66). Based primarily on the ability of ceramide but not other lipid messengers to activate KSR in vitro and in vivo, and a requirement for the CAPK recognition site Thr269 of Raf-1 for KSR transactivation, we proposed that KSR and CAPK are identical (28). In the present studies, constitutively active KSR mimicked the effect of ceramide to signal apoptosis through Ras and Raf-1, and KI-KSR was ineffective, indicating that the kinase activity of KSR was required. Further, KI-KSR blocked ceramide-induced apoptosis through BAD, consistent with the proposed role of KSR as a target for ceramide.

Other groups have arrived at different conclusions as to the mechanism by which KSR acts. Morrison and co-workers (67), and Muslin and co-workers (68), like ourselves, find that KSR binds to and activates Raf-1, enhancing signaling through the MAPK cascade. This interaction is reported as required for Xenopus laevis oocyte maturation, cellular transformation, and Drosophila eye development, although there is disagreement as to whether the kinase domain of KSR is obligatory (69). In contrast, Williams and co-workers (70) as well as Eychene and co-workers (71) report that KSR binds to and functionally inactivates MEK1, blocking signaling through MAPK, and attenuating Ras-induced transformation and serum-induced mitogenesis. Perhaps the discrepancies in these data reflect induction of apoptosis. Karim and Rubin (72) have recently shown that overexpression of V12Ras at low gene doses induces hyperproliferation of cells in Drosophila wing and eye discs, whereas higher levels of expression induce apoptosis. Both events require functional KSR, Raf, MEK, and MAPK as loss of

2 M. J. Birnbaum, personal communication.
function mutants suppress lethality and disc overgrowth. In some of the systems described above, inadvertent apoptosis may account for the reduced transformation/proliferation observed. Apoptosis, which occurs early after transfection of KSR into COS-7 cells, might select for a population in which alternative actions of KSR predominate.

The present studies define one form of transcriptionally independent apoptosis in response to ceramide requiring signaling through Ras/Raf-1 and the MAPK cascade. Numerous previous investigations have documented interrogation of proliferative and apoptotic signaling cascades (55, 57, 61). The present study suggests that the availability of a single target may dictate the final outcome.

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