Caspase-dependent and -independent Activation of Acid Sphingomyelinase Signaling*

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Sphingomyelinase Signaling*


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Recent evidence suggests clustering of plasma membrane rafts into ceramide-enriched platforms serves as a transmembrane signaling mechanism for a subset of cell surface receptors and environmental stresses (Grassme, H., Jekle, A., Riehle, A., Schwarz, H., Berger, J., Sandhoff, K., Kolesnick, R., and Gulbins, E. (2001) J. Biol. Chem. 276, 20589–20596; Cremesti, A., Paris, F., Grassme, H., Holler, N., Tschopp, J., Fuks, Z., Gulbins, E., and Kolesnick, R. (2001) J. Biol. Chem. 276, 23954–23961). Translocation of the secretory form of acid sphingomyelinase (ASMase) into microscopic rafts generates therein the ceramide that drives raft coalescence. This process serves to feed forward Fas activation, with −2% of full caspase 8 activation sufficient for maximal ASMase translocation, leading to death-inducing signaling complex formation within ceramide-rich platforms, and apoptosis. Here we report that treatment of Jurkat T cells with UV-C also induces ASMase translocation into rafts within 1 min, catalyzing sphingomyelin hydrolysis to ceramide and raft clustering. In contrast to Fas, UV-induced ASMase translocation and activation were caspase-independent. Nonetheless, ceramide-rich platforms promoted UV-C-induced death signaling, because ASMase inhibition or raft disruption inhibited apoptosis, improving clonogenic cell survival. These studies thus define two distinct mechanisms for biologically relevant ASMase activation within rafts: a Fas-mediated mechanism dependent upon caspase 8 and FADD, and a UV-induced mechanism independent of caspase activation. Consistent with this notion, genetic depletion or pharmacologic inhibition of caspase 8 or FADD, which render Jurkat cells incapable of sphingolipid signaling and apoptosis upon Fas ligation, did not impair these events upon UV-C stimulation.

Rafts are distinct plasma membrane microdomains, comprised of cholesterol tightly packed with sphingolipids, in particular sphingomyelin, creating a liquid-ordered domain within the liquid-disordered bulk plasma membrane (1). An emerging body of evidence recognizes rafts as sites of signal transduction (1–10). One mode by which rafts transmit signals involves their coalescence into large platforms into which signaling proteins translocate, concentrate, and multimerize (11). Several groups have recently reported that ceramide, a lipid second messenger for diverse stresses, provides the driving force for coalescence of rafts into platforms (4, 6, 7, 9, 12–16). Specifically, ceramide addition to mammalian cells or model membrane bilayers is alone sufficient for platform formation (17–20). This fusagenic property of ceramide appears to derive from its unique biophysical capacity to self-associate via hydrogen bonding and van der Waal forces (12).

Ceramide may be rapidly generated by sphingomyelinase-catalyzed hydrolysis of sphingomyelin at the plasma membrane (21), or in a more prolonged fashion via de novo synthesis in the endoplasmic reticulum or mitochondria by ceramide synthases (22, 23). Acidic sphingomyelinase (ASMase), a sphingomyelin-specific phospholipase C (sphingomyelin phosphodiesterase) that exists in two forms, a lysosomal and secretory form, initiates a rapid stress response in many cell types (24–28). Recent data show that ASMase translocates onto the outer leaflet of the plasma membrane from intracellular, presumably vesicular stores, to release ceramide within the raft-associated sphingomyelin pool, generating the ceramide therein required for raft clustering. Despite extensive studies on the downstream effects of ASMase translocation and activation (4, 7–9, 12, 25–34), little is known of the initiating events mediating its translocation onto the outer plasma membrane.

The role of ceramide-mediated raft clustering in signaling is best defined for Fas stimulation of Jurkat T cells (14, 16, 31, 36–40). In these cells, engagement of pre-trimerized Fas receptors (41) activates, within seconds, a small percentage (1–2% of maximum) of procaspase 8 and the adaptor protein FADD (15), sufficient for maximal translocation of ASMase into membrane rafts, ceramide generation, and raft coalescence into ceramide-rich platforms (14, 15). Fas oligomerizes within these ceramide-rich platforms, facilitating formation of the death-inducing signaling complex, an event essential for the other 98% of Fas-induced FADD and caspase 8 activation, and apoptosis of these cells (15). Six independent groups have published together that they all detect Fas clustering within seconds in Jurkat cells and in other cells, including SKW 6.4 and JY B cell lymphoma, H9 T cell lymphoma, Chang human conjunctiva epithelial, and mouse granulosa cells, and in primary murine sponocytes and hepatocytes (14). Consistent with

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1 The abbreviations used are: ASMase, acid sphingomyelinase; FADD, Fas-associated death domain; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-related kinase; FcRI, Fc receptor II; TUNEL, terminal dUTP nick-end labeling; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethyl ketone; GM1, Galβ1-3GalNAcβ1-4[sialic acid2-8][Galβ1-4-Glcβ1-]ceramide.
this phenomenon as relevant to overall outcome, pharmacologic inhibition of raft clustering by cholesterol extraction or chelation, inhibition of ASMase activation, or sequestration of ceramide with anti-ceramide antibody renders Jurkat and some of the above-mentioned cells largely resistant to Fas-induced apoptosis (6, 16, 31, 38, 42, 43).

Ceramide-mediated raft clustering also mediates stress responses for stimuli other than Fas (3, 4, 6–10). In this context, ceramide-enriched platforms transmit signals for CD40-induced interleukin-12 secretion and CD-Jun kinase phosphorylation in JY B cells (4), Pseudomonas aeruginosa internalization and activation of the innate immune response in lung (7), Rituximab-induced CD20 clustering and ERK phosphorylation in Daudi and RL lymphoma cells (10), FcγRII clustering and phosphorylation in U937 human monocytic cells (3, 44), and resveratrol-, cisplatin-, and reactive oxygen species-induced apoptosis in HT29 human colon carcinoma cells and neutrophils (6, 8, 9). Hence, ceramide-mediated raft clustering into macromdomains appears to represent a generic mechanism for transmembrane signaling, rather than a specific mechanism for apoptosis induction.

The present studies address the mechanism of ASMase translocation into rafts. Jurkat cells display two distinct mechanisms, the caspase-dependent mechanism used by Fas and a previously unrecognized caspase-independent mechanism utilized by UV-C. In both instances, the process of translocation is ceramide generation in rafts, raft coalescence into platforms, and apoptosis. However, in the case of UV-C, although apoptosis is dependent on ceramide generation, it is independent of Fas activation or death-inducing signaling complex-mediated initiator caspase activation. These data indicate that generation of ceramide, rather than the mechanism of ASMase activation, regulates platform formation and stress-induced apoptosis.

MATERIALS AND METHODS

Cell Culture and Stimulation—Wild-type (clone E6–1), caspase 8<sup>−/−</sup> (clone I9.2) and FADD<sup>−/−</sup> (clone I2.1) Jurkat T lymphocytes were obtained from the ATCC (Rockville, MD). Cells were grown in a 5% CO<sub>2</sub> incubator at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 10 mM Hepes (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, 100 μg/ml penicillin, and 10 μg/ml streptomycin. Prior to stimulation with UV-C or α-Fas, cells were resuspended in fresh medium to acclimate for 30 min. Cells were treated with 10 ng/ml of α-Fas CH-11 activating antibody (Upstate Biotechnology, Lake Placid, NY) or 50 J/m<sup>2</sup> UV-C using an FB-UVXL-1000 cross-linker (Fisher Biotech, Pittsburgh, PA), unless otherwise indicated. For platform studies, cells were incubated with CH-11 at 4 °C for 20 min to ensure uniform receptor engagement and warmed to 37 °C to initiate stimulation.

Where indicated, cells were preincubated with 10 μM z-VAD-fmk (Calbiochem, La Jolla, CA), 30 μg/ml nystatin (Sigma-Aldrich), 50 μM imipramine (Sigma-Aldrich), or 1 μg/ml mouse monoclonal α-ceramide antibody MID15B4 (Alexis Biochemicals, San Diego, CA). Nystatin, imipramine, and α-ceramide studies were performed in RPMI containing 0.5% lipid-free fetal bovine serum (HyClone, Logan UT). In each study, an aliquot of cells was stained with trypan blue to assess viability.

Apoptosis Quantitation—Apoptosis was assessed by two different techniques. Staining for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling was performed on cells permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate at 4 °C for 5 min, according to the manufacturer’s instructions (Roche Applied Science). Alternately, stained cells were fixed with 2% paraformaldehyde, washed with phosphate-buffered saline (PBS), and stained with 100 μl of 24 μg/ml bisbenzimide trihydrochloride solution (Hoechst #33258, Sigma-Aldrich) for 10 min. Morphologic changes of nuclear apoptosis, including chromatin condensation, segmentation, and compaction along the periphery of the nucleus or the appearance of apoptotic bodies were quantified using an Axiovert S-100 Zeiss fluorescence microscope as previously described (16). A minimum of 200 cells was examined per point.

Cleonogenic Assay— Colony formation following UV-C or α-Fas CH-11 treatment was evaluated using a soft agar cloning assay as described previously (45). Briefly, cells were preincubated with nystatin and α-ceramide monoclonal antibody, or vehicle and control IgM in RPMI plus 0.5% lipid-free fetal bovine serum, and stimulated with increasing doses of UV-C or α-Fas. After 4 h, cells were suspended in RPMI medium containing 20% fetal bovine serum, 20 mM L-glutamine, and 40% methylcellulose medium, and plated in triplicate. After 14–16 days of incubation, colonies were scored and colony formation for each condition was calculated in relation to values obtained for untreated control cells. Colony survival curves were calculated by least square regression analysis, using a modification of the program FIT (46). The program fits the curves by iteratively weight least squares to each set of dose-survival data, estimates the covariates of the survival curve parameters and the corresponding confidence regions, and plots the survival curve. It also derives curve parameters, such as the D<sub>0</sub> (the central of the slope on the exponential portion of the curve, representing the level of radiosensitivity) and the N<sub>0</sub> (number (measuring the size of the shoulder)).

Platform Detection—Platforms were detected as previously described. Briefly, 1 × 10<sup>6</sup> Jurkat cells were stimulated with UV-C or α-Fas, fixed with 2% paraformaldehyde at the indicated times, blocked in PBS containing 1% fetal bovine serum, and then washed with PBS. Cells were permeabilized with 0.1% Triton X-100 with the local, sequestrated cholera toxin β-subunit (2 μg/ml, Sigma-Aldrich) for 45 min at 4 °C, washed twice in PBS containing 0.1% Triton X.100, and mounted in fluorescent mounting medium (Dako, Carpenteria, CA). Fluorescence was detected using an Axiosvert S-100 Zeiss fluorescence microscope equipped with a SPOT digital camera. The percentage of cells containing platforms, i.e., those in which the fluorescence condenses onto <i>50%<sub>50</sub></i> of the cell cytoplasmic face, was determined by counting 150–200 cells per point. Alternately, platforms were identified using a mouse monoclonal α-ceramide antibody MID 15B4 IgM (1:5 dilution, Alexis Biochemicals), mouse monoclonal α-Fas CH-11 IgM (1:500 dilution, Upstate Biotechnology) or polyclonal rabbit α-ASMase antibody 1598 (1:100 dilution) and detected using Cy3-conjugated α-mouse or α-rabbit IgG (1:500 dilution, Roche Applied Science), respectively. Rabbit polyclonal α-Fas (Santa Cruz Biotechnology, CA) and a rabbit polyclonal Ab directed against ceramide (Enzo Lifesciences) was used as a negative control. In some studies, confocal images were obtained using a Leica TCS SP2 upright confocal microscope.

The rabbit polyclonal anti-ASMase antibody #1598 was generated against full-length FLAG-tagged human ASMase protein. Anti-sera was purified over a Bio-Rad T-Gel column to obtain an IgG fraction that displays specific immunoreactivity by immunoblot assay at a concentration of 100 ng/ml toward 100 ng of purified recombinant human ASMase or ASMase from 25 μg of Jurkat cell lysates. At a concentration of 200 μg/μl, #1598 quantitatively immunoprecipitates ASMase activity from 100 ng of purified ASMase and at a concentration of 200 ng/ml detects cell surface expression of ASMase by flow cytometry or confocal immunofluorescence microscopy.

Western Blot Analysis—Jurkat cells stimulated with UV-C or CH-11, were incubated for the indicated times at 37 °C. Stimulation was terminated by the addition of 2 ml of chloroform:methanol:HCl (100:100:1, v/v), and ceramide was quantified by the diacylglycerol kinase assay method.

FACS Analysis of ASMase Surface Expression—To detect surface ASMase by FACS, Jurkat cells were stimulated with 50 J/m<sup>2</sup> UV-C or 50 ng/ml CH-11 at 37 °C. Stimulation was terminated with ice-cold PBS, and cells were lysed in radioimmunoprecipitation assay buffer (25 mM HEpes (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 100 mM NaCl, 10 mM NaF, 10 mM Na<sub>2</sub>PO<sub>4</sub>, 10 mM EDTA, and 10 μg/ml each of aprotonin and leupeptin). Samples were centrifuged at 14,000 × g, and the supernatants were added to 4× SDS-sample buffer. Lysates were separated on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes. Caspase cleavage was detected using rabbit polyclonal antibodies against caspase 3 (BD Pharmingen, San Diego, CA), caspase 8 (BD Pharmingen), or caspase 9 (Cell Signaling Technology, Beverly, MA).

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ASMase Activity Assay—ASMase activity was measured using a fluorescence-based, high performance liquid chromatographic assay (48). Briefly, 5 × 10⁵ Jurkat cells were stimulated with 50 J/m² UV-C or 50 ng/ml CH-11 at 37 °C, and at the indicated times washed with ice-cold PBS and lysed on ice in Nonidet P-40 buffer (150 mM NaCl, 25 mM Tris HCl, pH 7.5, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA, 0.1 mM diithiothreitol supplemented with phenylmethylsulfonyl fluoride, leupeptin, and protease inhibitor mixture). ASMase activity was measured by incubating an equal volume of lysate in assay buffer (500 μM BODIPY-C₁₂ sphingomyelin (Molecular Probes, Eugene OR), 0.1 mM ZnCl₂, 0.1 mM sodium acetate, pH 5.0, and 0.6% Triton X-100) for 60 min at 37 °C. Thereafter, the reaction was stopped by 10× dilution in ethanol, and 5 μl of the assay mixture was sampled by a WIPS 712 (Waters Corp., Milford, MA) auto-sampler equipped with a 20 × 4 mm reverse-phase Aquasil C₁₈ column (Keystone Scientific, Bellefonte, PA). The reaction product, BODIPY-C₁₂ ceramide, was specifically separated from substrate within 0.4–0.5 min by isocratic elution with 95% MeOH at a flow rate of 1.2 ml/min. Fluorescence was quantified using a Waters 474 (Waters Corp.) fluorescence detector set to excitation and emission wavelengths of 505 and 540 nm, respectively. The amount of product generated was calculated using a regression equation derived from a standard curve established for known amounts of BODIPY-C₁₂ ceramide standard. Alternatively, ASMase activity was quantified by radioactive assay using [N-methyl-¹⁴C]sphingomyelin (Amersham Biosciences) as substrate, as described (49). Briefly, Jurkat cells were lysed in PBS containing 0.2% Triton X-100 at the indicated times after 50 J/m² UV-C or 50 ng/ml CH-11 stimulation. Post nuclear supernatant was used for activity in 0.1 mM sodium acetate, pH 5.0, supplemented with 0.1 mM ZnCl₂, 1 mM EDTA, and 0.1% Triton X-100 in the presence of substrate. Reactions were terminated after 1 h with CHCl₃:MeOH:HCl (100:100:1, v/v/v), and the product was quantified by using a scintillation counter. Thus, both assays yielded identical fold increases after UV-C or Fas stimulation, these data were collated. Because both assays yielded identical results, we used the fluorescence-based assay for the rest of this study. ASMase activity was measured. ASMase specific activity rapidly increased within 0.4–0.5 min of 50 J/m² UV-C or 50 ng/ml α-Fas activating antibody, cell surface platforms enriched in ceramide and ASMase (Fig. 1D) were observed, which also contained GM1 (Fig. 1E and data not shown). Platform generation, as measured by GM1 clustering (Fig. 1F), was detected as early as 15 s post stimulation with 50 J/m² UV-C or 50 ng/ml α-Fas CH-11 and peaked within 30 s at 52.0 ± 4.4 and 55.6 ± 2.6% of the total population, respectively, compared with 6.4 ± 1.2% in unstimulated controls (p < 0.05 each).

Platform formation was dose-dependent at 1 min (data not shown), reaching a maximum at 50 J/m² UV-C (p < 0.05), with an ED₅₀ identical to UV-C induced ceramide generation (Fig. 1A, right panel) of 10 J/m². ASMase translocation into platforms was specific (Fig. 1E, upper panel), because the non-raft transmembrane glycoprotein CD46, involved in regulating the complement cascade, did not cluster in response to UV-C (Fig. 1E, lower panel) or α-Fas (data not shown).

Ceramide-rich Platforms Are Obligate for UV-C and Fas-induced Apoptosis—Because previous experiments identified ceramide as obligatory for Fas-induced apoptosis in some cell types (14–16, 21, 31, 34, 37, 47, 50, 51), the requirement for UV-C-induced ceramide generation in initiating the apoptotic response was investigated. These studies used imipramine to inhibit ASMase activity, likely by increasing ASMase proteolytic degradation (21, 30). Pre-treatment of Jurkat cells with 50 μM imipramine for 30 min decreased baseline ASMase activity, abrogated UV-C and Fas-induced ASMase activation at 1 min post stimulation (Fig. 2A) and ceramide generation at 2 min (data not shown), and attenuated apoptosis at 4 h post-stimulation (Fig. 2B). These data suggest that ASMase activation is indispensable for optimal Fas- or UV-C-induced apoptosis, although they do not define the role of ceramide in this response.

To elucidate the role of ceramide-mediated raft clustering in UV-C-induced apoptotic signaling, ceramide neutralization using α-ceramide monoclonal antibody was combined with cholesterol depletion. Ceramide neutralization and cholesterol depletion have each been successfully employed in Jurkat cells to afford protection against Fas-induced platform generation and apoptosis (31), however in the present studies greater protection was achieved by using these reagents in combination rather than individually. Preincubation of cells with α-ceramide in combination with nystatin inhibited raft clustering 1 min post 50 J/m² UV-C- or 50 ng/ml α-Fas stimulation (Fig. 2C), as determined by fluorescence microscopy using FITC-conjugated cholera toxin β-subunit. Furthermore, inhibiting raft clustering by α-ceramide and nystatin combination treatment attenuated UV-C (5–50 J/m²) and α-Fas (1–50 ng/ml CH-11)-induced apoptosis 4 h post stimulation (Fig. 2D) and enhanced cell viability by 2.46- and 2.42-fold, respectively, 7 days post
FIG. 1. UV-C- and Fas-activated ASMase mediates raft clustering into ceramide-rich platforms. A, UV-C rapidly induces ceramide generation in a time- and dose-dependent manner. Cells stimulated with 200 J/m² for the indicated times or increasing doses of UV-C for 1 min were analyzed for ceramide content by DG kinase assay as described under “Materials and Methods.” Data (mean ± S.E.) are compiled from three independent experiments performed in triplicate. B, Fas and UV-C rapidly increase surface ASMase expression. FACS analysis was performed on Jurkat cells stained with a polyclonal α-ASMase Ab 1 min post α-Fas or UV-C stimulation. Data are from one representative of three independent studies. C, Fas and UV-C elevate ASMase activity in a time-dependent manner. Cells were stimulated with 50 J/m² UV-C or 50 ng/ml α-Fas CH-11
stimulation with 50 J/m² UV-C or 50 ng of α-Fas. Furthermore, α-ceramide and nystatin pretreatment yielded an approximate 1-log increase in clonogenic cell survival compared with vehicle controls after 5–50 J/m² UV-C (Fig. 2E and not shown) or α-Fas stimulation. Plotting these clonogenic survival data according to the single-hit multig targetType model (52) revealed that pretreatment with α-ceramide and nystatin increased the D₀ of the dose-response curve from 1.6 ± 0.7 to 3.6 ± 1.1 J/m², indicating significant (p < 0.05) protection against the reproductive mode of UV-induced cell death, with a dose-modifying value of 2.32 at the 10% survival level. Taken together, these results suggest that ceramide-mediated raft clustering at the surface of Jurkat cells is obligate for apoptotic transmembrane signal transduction induced by UV-C and that such protection is biologically relevant as evidenced by improved clonogenic survival.

UV-C-induced Platform Generation Occurs Independent of Caspases—In Jurkat cells, only 2% of caspase 8 and FADD are initially recruited by ligated Fas to induce ASMase translocation into plasma membrane rafts (53, 54), generating ceramide-enriched platforms that activate the remaining 98% of caspase 8 and FADD (31, 37). To assess whether UV-C similarly activates a small percentage of caspase 8 to trigger ASMase translocation, procaspase 8 cleavage was assessed by Western blot after 200 J/m² UV-C irradiation. Within minutes of Fas engagement, and in the same time frame as Fas-induced ASMase translocation and ceramide generation, the p43/41 and p20 active cleavage products of procaspase 8 were readily detectable by Western blot analysis (Fig. 3A). In contrast, procaspase 8 processing was not detectable for at least 30 min upon UV-C treatment (Fig. 3A), suggesting that UV-C-induced ceramide generation and platform formation occurred prior to, and thus independent of early processing of caspase 8.

To confirm that caspase 8 activation was not required for UV-C-induced raft coalescence, the effect of caspase inhibition on clustering of rafts into ceramide-rich platforms was measured. Generation of platforms by α-Fas stimulation was completely inhibited by nanomolar quantities of the pan caspase inhibitor z-VAD-fmk, whereas UV-C induced platform formation was resistant to caspase inhibition (Fig. 3B). Taken together with the Western blot analysis, these data indicate that UV-C activates platform generation by a caspase-independent mechanism, and suggest the existence of caspase-dependent and -independent mechanisms for ASMase activation.

UV-C-induced Apoptosis Occurs Independent of Caspase 8—To clarify the role of caspases in UV-C-induced cell death of Jurkat T lymphocytes, apoptosis was quantified after treatment of cells with increasing concentrations of the pan caspase inhibitor z-VAD-fmk. Although pharmacologic caspase inhibition did not impact UV-C induced formation of ceramide-rich platforms, it blocked both UV-C- and α-Fas-mediated apoptosis in a dose-dependent manner (Fig. 4A), confirming that UV-C, like α-Fas, requires cysteine-aspartate proteases for execution of apoptosis. To further examine whether caspase 8 and FADD play a role in apoptosis activated by UV-C, Jurkat T lymphocyte clones containing point mutations in the genes encoding procaspase 8 or FADD were utilized. Caspase 8⁻/⁻ and FADD⁻/⁻ Jurkat cells were stimulated with increasing doses of α-Fas or UV-C irradiation. At 16 h, Fas-induced apoptosis was absent in both caspase 8⁻/⁻ and FADD⁻/⁻ cells, even at doses that induced maximal apoptosis in wild-type cells (Fig. 4B, left panel). In contrast, UV-C-induced apoptosis was unaltered in the mutant lines compared with wild-type cells (Fig. 4B, right panel). Furthermore, equal processing of caspases 3 and 9 occurred in wild-type, caspase 8⁻/⁻ and FADD⁻/⁻ Jurkat cells at 4 h post UV-C irradiation, whereas processing of these caspases was absent in the mutant cell lines post Fas engagement (Fig. 4C). These data suggest that UV-C-induced apoptosis in Jurkat cells occurs independent of Fas stimulation and death-inducing signaling complex-mediated caspase 8 initiation.

UV-C-induced ASMase Surface Expression and Ceramide Generation Are Caspase-independent—To confirm the existence of both caspase-dependent and -independent ASMase activation, wild-type, caspase 8⁻/⁻, and FADD⁻/⁻ Jurkat cells were stimulated with 50 ng/ml α-Fas or 50 J/m² UV-C. Fas and UV-C each initiated rapid ASMase translocation from an intracellular pool to the cell surface of wild-type Jurkat cells, as evidenced by an increase in surface stain by FACS analysis (Fig. 1B). However, caspase 8⁻/⁻ and FADD⁻/⁻ Jurkat cells were unable to recruit ASMase to the membrane upon Fas engagement (Fig. 5A), whereas UV-C induced increase in α-ASMase surface stain was not impaired in these mutant cell lines (compare Figs. 1B and 5A). Consistent with these observations, caspase 8⁻/⁻ and FADD⁻/⁻ cells were defective in generation of ceramide (Fig. 5B) and ceramide-enriched platforms (Fig. 5C, left panel) upon Fas engagement yet retained their capacity to respond to UV-C irradiation by generating ceramide (Fig. 5B) and forming such platforms (Fig. 5C, right panel). These data indicate that UV-C-induced ASMase translocation, ceramide generation, and platform formation occur independent of caspases and that both caspase-dependent and -independent mechanisms for initiation of ASMase signaling exist. Consistent with these observations, z-VAD-fmk blocked α-Fas but not UV-C-induced ceramide generation (Fig. 5B) and platform formation (Fig. 3B).

**DISCUSSION**

Although numerous studies document the role of ASMase in signal transduction of apoptosis (9, 16, 31, 39, 40), senescence (55), and proliferation (56) in response to diverse stimuli, little is known of the mechanism of ASMase translocation and activation. The present study details a previously unrecognized caspase-independent pathway initiating ASMase signaling. These studies corroborate prior studies that identify caspase-independent ceramide generation in response to IgM in WEHI 231 B cells (57), hypoxia in PC12 cells (58), and dexamethasone in thymocytes (59). These prior investigations were based primarily on the use of caspase inhibitors, and the role of ASMase in these events was not investigated. The current study specifically provides information that irradiation of Jurkat cells with antibody, and ASMase activity was determined using either BODIPY-C₂₅ sphingomyelin or [³⁴C]sphingomyelin as substrate and collated as under “Materials and Methods.” Data (mean ± S.E.) are compiled from three independent experiments performed in triplicate. D and E, UV-C- and Fas-induced platform formation. Colocalization of ASMase with ceramide (D) or the lipid raft marker GM1 (E, upper panel) in platforms on the outer leaflet of the plasma membrane of Jurkat cells identified 1 min post α-Fas or UV-C stimulation by reconstructive confocal microscopy after staining with Cy-3-labeled α-ASMase Ab and FITC-labeled α-ceramide Ab or FITC-conjugated cholera toxin β-subunit, respectively. These images are representative of over 50% of cells from three experiments in which 100 cells were analyzed. Exclusion of CD46 from platforms (E, lower panel) was identified by confocal microscopy after staining with Cy-3-labeled α-CD46 Ab and FITC-conjugated cholera toxin β-subunit. F, time-dependent generation of platforms after UV-C irradiation or Fas engagement. Staining of GM1 using FITC-conjugated cholera toxin β-subunit shows 50 J/m² UV-C irradiation or 50 ng/ml Fas stimulation induce raft clustering into platforms by fluorescence microscopy. Data (mean ± S.E.) are collated from three experiments in which 200 cells were analyzed per point.

³ J. Rotolo and R. Kolesnick, unpublished observation.

⁴ R. Kolesnick, manuscript in preparation.
FIG. 2. Ceramide-rich platforms are obligate for UV-C- and Fas-induced apoptosis. A, imipramine inhibits Fas- and UV-C-induced activation of ASMase. Jurkat cells pretreated with 50 µM imipramine were left untreated or treated with 50 J/m² UV-C or 50 ng/ml α-Fas CH-11 antibody for 1 min. ASMase activity was analyzed as in Fig. 1C. Data (mean ± S.E.) are compiled from three experiments performed in triplicate. B, imipramine inhibits Fas- and UV-C-induced apoptosis. Apoptosis was quantified morphologically 4 h after UV-C or α-Fas CH-11 antibody stimulation by bisbenzimide staining. Data (mean ± S.E.) represent three experiments in which 200 cells were analyzed per point. C, ceramide sequestration inhibits UV-C- and Fas-induced platform formation. Staining of GM1 with FITC-conjugated cholera toxin β-subunit was performed on Jurkat cells pretreated with a combination of 30 µg/ml nystatin and 1 µg/ml α-ceramide Ab followed by stimulation with 50 J/m² UV-C or 50 ng/ml α-Fas CH-11 antibody for 1 min. Data (mean ± S.E.) are collated from three experiments in which 200 cells were analyzed per point. D, platform formation is obligate for UV-C- and Fas-induced apoptosis and E, clonogenic cell death. Jurkat cells pretreated with a combination of 30 µg/ml nystatin and 1 µg/ml α-ceramide Ab or vehicle and irrelevant antibody control were stimulated with increasing doses of UV-C or α-Fas CH-11 antibody and apoptosis quantified after 4 h as in B. Colony formation was quantified by clonogenic assay as described under “Materials and Methods.” Apoptosis data (mean ± S.E.) represent three experiments performed in triplicate in which 200 cells were analyzed per point, and clonogenic data (mean ± S.E.) were compiled from three independent experiments performed in triplicate.
UV-C, an environmental stress requiring both ASMase activation (25, 26, 28, 33, 60) and ceramide generation (32, 61–63) for apoptosis induction in some cells, activates the sphingomyelin signaling pathway in the absence of functional caspase 8 or in the presence of a pan caspase inhibitor. UV-C-induced ceramide generation drives the clustering of sphingolipid rafts into platforms, an event obligate for efficient apoptosis signaling. Consistent with this paradigm, antagonism of ceramide action by sphingomyelinase inhibition, sequestration of surface ceramide with α-ceramide antibody, or cholesterol depletion inhibit UV-C-induced platform formation and apoptosis, improving cell survival as measured by the clonogenic assay.
The evidence suggests that the conversion of microscopic rafts into ceramide-rich macrodomains serves as a feed-forward mechanism for transmembrane signaling. Although ASMase is not a direct target for caspase 8, ASMase surface translocation activated by Fas, and perhaps other tumor necrosis factor superfamily receptors (64), nonetheless requires minimal caspase 8 and FADD activation. In fact, <2% of maximal caspase 8 or FADD are necessary for 100% of ASMase activation, ceramide generation, platform formation, and Fas recruitment into ceramide-rich platforms in Jurkat.
cells. Fas oligomerizes within these platforms, activating the other 98% of caspase 8 and FADD necessary for optimal apoptosis induction. Whether Fas oligomerization is exclusively ceramide-mediated or requires concomitant reduction of sphingomyelin or cholesterol levels (65, 66) is currently unknown. As with Fas, we anticipate that UV-C similarly recruits a subset of surface proteins and downstream effector molecules into ceramide-rich platforms to transmit its signal across the plasma membrane. Whether the eventual outcome of UV-C action in Jurkat cells or other cell types requires an additional signal from damaged DNA, as suggested by the clonogenic data, is currently unknown.

Although the exact mechanism of activation of the secretory form of ASMase is uncertain, transmission electron microscopy reveals that ASMase exists in vesicles abutting the inner plasma membrane that, upon activation, appear to fuse with the membrane and empty their content onto the extracellular surface (37). This process likely requires reorganization of microtubules and the actin cytoskeleton, as inhibition with nocodazole, a microtubule-stabilizing drug, and cytochalasin D, an actin cytoskeleton-disrupting agent, abrogates ASMase translocation (15, 67). In contrast, raft disruption does not interfere with ASMase surface translocation, although it does interfere with subsequent ceramide generation, suggesting that enzymatic activity requires intact rafts for substrate recognition. Whether association of ASMase with rafts requires a membrane docking protein, analogous to that of the secretory phospholipases A2, which also target substrate on the exoplasmic membrane, is currently unknown (68).

Although more than 25 reports provide genetic and pharmacologic evidence that ceramide-rich platforms are obligatory for transmembrane signal transduction in response to tumor necrosis factor-superfamily (4, 8, 9, 14–16, 31, 37, 42, 69–71) and FcRII (3) receptors, pathogens such as Pseudomonas aeruginosa and Staphylococcus aureus (7), and some chemotherapeutics, including Rituximab (10) and cisplatin (6), there is disagreement regarding their role in Fas-mediated death. Specifically, Algeciras-Schimnich et al. (72) did not detect early formation of Fas-containing platforms in SKW6.4, K50, or H9 lymphoid cell lines. Six independent groups working together were nonetheless able to detect Fas-containing platforms in Jurkat cells within the first 5 min of stimulation, and in 11 other cell lines of different origins. The inability of Algeciras-Schimnich et al. to detect platforms appears related to a staining-fixing regimen in which rapid Fas clustering was undetectable due to nonspecific antibody binding to unprotected Fc receptors (14). The results of Muppidi and co-workers (73) also differed from that of the six independent groups that published together.

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REFERENCES


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Caspase-dependent and -independent Activation of Acid Sphingomyelinase Signaling
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