Ceramide Enables Fas to Cap and Kill*

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Recent studies suggest that trimerization of Fas is insufficient for apoptosis induction and indicate that super-aggregation of trimerized Fas might be prerequisite. For many cell surface receptors, cross-linking by multivalent ligands or antibodies induces their lateral segregation within the plasma membrane and co-localization into "caps" on one pole of the cell. In this study, we show that capping of Fas is essential for optimal function and that capping is ceramide-dependent. In Jurkat T lymphocytes and in primary cultures of hepatocytes, ceramide elevation was detected as early as 15–30 s and peaked at 1 min after CH-11 and Jo2 anti-Fas antibody treatment, respectively. Capping was detected 30 s after Fas ligation, peaked at 2 min, and was maintained at a lower level for as long as 30 min in both cell types. Ceramide generation appeared essential for capping. Acid sphingomyelinase−/− hepatocytes were defective in Jo2-induced ceramide generation, capping, and apoptosis, and nanomolar concentrations of C16-ceramide restored these events. To further explore the role of ceramide in capping of Fas, we employed FLAG-tagged soluble Fas ligand (sFasL), which binds trimerized Fas but is unable to induce capping or apoptosis in Jurkat cells. Cross-linking of sFasL with M2 anti-FLAG antibody induced both events. Pretreatment of cells with natural C16-ceramide bypassed the necessity for forced antibody cross-linking and enabled sFasL to cap and kill. The presence of intact sphingolipid-enriched membrane domains may be essential for Fas capping since their disruption with cholesterol-depleting agents abrogated capping and prevented apoptosis. These data suggest that capping is a ceramide-dependent event required for optimal Fas signaling in some cells.

The current model of Fas (CD95 or APO-1) signaling suggests that engagement of Fas by its ligand or anti-Fas antibody leads to receptor trimerization and recruitment of the cytoplasmic adapter protein FADD (MORT-1) and pro-caspase 8 (Flice/Caspase 8) required for binding Fas ligand and triggering cell death. Homotrimer generation in the absence of ligand is mediated by the pre-ligand assembly domain located within the extracellular amino-terminal region of the receptor. It was further suggested that Fas ligand may orchestrate the multimerization of trimerized receptors, generating aggregates of activated receptors, thus producing high local concentration of Fas death domain complexes (4). Consistent with this paradigm, FLAG-tagged soluble Fas ligand (sFasL) that retained the capacity to interact with Fas failed to induce apoptosis unless it was cross-linked with anti-FLAG antibody, apparently forming a super-aggregated state required for Fas signaling (5).

Aggregation and clustering of cell surface receptors upon binding to their specific ligands has been reported for a variety of receptors, including the insulin (6), epidermal growth factor (7), L-selectin (8), immunoglobulin (9), and T-cell receptor (10, 11). In several of these systems, patches of aggregated receptors were found to migrate toward one pole of the cell and coalesce to form a cap via an energy-dependent process involving cytoskeleton reorganization (7, 9). Such ligand-induced capping appears to facilitate transduction of extracellular signals by local assembly of the various signaling elements. For example, Huang et al. (12) show activation of the T-cell receptor-induced co-capping of numerous proteins required for efficient signal transmission (CD4, Rac-1, F-actin, LFA-1 CD28, PKCζ), whereas T-cell receptor antagonists generated caps deficient in CD28 and PKCθ. These and other studies support the notion that the assembly of molecules in caps is specific for ligand/receptor combinations and appears critical for delivery of their signals. There is emerging evidence for a role of capping in Fas signaling of apoptosis. Belka et al. (13) report that Fas ligand induced Fas capping and apoptosis in IL-2-treated and irradiated peripheral blood lymphocytes, whereas von Reynier et al. (14) report that Fas ligand...
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(14) demonstrate defective Fas capping and apoptosis in colon carcinoma cell lines.

Indirect evidence suggests that membrane structures known as glycosphingolipid-enriched microdomains (GEMs), or membrane rafts, may be associated with cap assembly. Rafts are organized membrane formations composed of glycosphingolipids, sphingomyelin, cholesterol, and specific membrane proteins (15, 16). When caveolin-1 is integrated into rafts, they form plasma membrane invaginations known as caveolae that are implicated in a variety of cellular processes, including membrane sorting in polarized cells (17, 18), endocytosis (19, 20), cholesterol trafficking, and signal transduction from cell surface receptors (21–27). It has been suggested that these specialized lipid microdomains provide a milieu for spatial segregation of specific sets of proteins and enhance the efficacy and specificity of interactions between enzymes involved in signal transduction. Disruption of GEMs resulting from cholesterol depletion abrogated signaling through this compartment in a variety of distinct cell types (28). Cholesterol depletion also abrogated capping of immunoglobulins and the Fc receptor on the surface of lymphocytes (29, 30). One study reports that cholesterol depletion of U937 cells abrogated TNF-β-stimulated apoptosis (31). Other Fas-stimulated cell systems have, however, not been explored as yet.

Another unresolved issue that challenges the classical model of Fas signaling relates to the role of the sphingolipid ceramide in the apoptotic response. There are numerous reports of ceramide elevation upon Fas activation (32–36), generated by either acid (ASMase) or neutral sphingomyelinase (33, 37). Recent investigations provided evidence that Fas-induced ceramide generation is dependent on Caspase-8 and occurs before commitment to the effector phase of the apoptotic process (38). In these studies, the amount of FADD or pro-caspase 8 transected into HeLa or 293T cells was titrated down to the point where they no longer induced apoptosis. Under these conditions, FADD and pro-caspase 8 still induced maximal ceramide generation that was blocked by the initiator caspase inhibitor crmA.

Whether ceramide generated in response to Fas ligation is involved in the apoptotic process or whether it represents an epi-phenomenon has been a matter of ongoing debate. Testi and co-workers (39) claim that Epstein-Barr virus-transformed lymphoblasts from Niemann-Pick disease patients, which have an inherited deficiency of ASMase activity, displayed deficits in Fas-induced ceramide generation and apoptosis, whereas Borst and co-workers find no differences (40). Recently, we provided definitive evidence for the role of ceramide in Fas-mediated apoptosis (35, 41). Hepatocytes from asmase−/− mice failed to manifest rapid ceramide elevation in response to Jo2 anti-Fas antibody and were 1 log unit more resistant to Jo2-mediated apoptotic death than paired asmase+/+ hepatocytes. The addition of low nanomolar quantities of natural C16-ceramide to asmase−/− hepatocytes had no direct effect on apoptosis, yet completely restored Fas sensitivity. Other sphingolipids could not substitute for natural ceramide and restore Fas sensitivity. The ability of ceramide to reverse the Fas resistance phenotype of asmase−/− cells without affecting the genotype provides evidence that ceramide is obligate for at least one form of Fas-induced apoptosis. The mechanism of ceramide regulation of Fas-induced apoptosis remains, however, unknown.

In the present studies, we show that ceramide, generated in response to Fas engagement, mediates apoptosis via facilitating Fas clustering and cap formation. Jurkat T lymphocytes and primary cultures of hepatocytes responded to CH-11 or Jo2 anti-Fas antibody, respectively, with ceramide elevation, capping, and apoptosis. asmase−/− hepatocytes were defective in these responses, but nanomolar concentrations of natural C16-ceramide restored capping and apoptosis. sFasL failed to induce capping or apoptosis in Jurkat cells, but cross-linking of sFasL with M2 anti-FLAG antibody restored both responses. Pretreatment with C16-ceramide bypassed the necessity for forced M2 antibody cross-linking and enabled sFasL to cap and induce apoptosis. Integrity of sphingolipid-enriched membrane domains may be necessary for the Fas response, since their disruption with cholesterol-depleting agents abrogated capping and prevented apoptosis. These data provide a mechanistic basis for the involvement of ceramide in one form of Fas-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bis-benzimide trihydrochloride (Hoechst 33258), γ-cyclohexanecarboxylic acid methyl ester (γ-CMC), methyl-β-cyclodextrin, filipin, nystatin, mouse monoclonal M2 anti-FLAG antibody, FITC-conjugated anti-mouse IgG and Alexa Fluor 594-conjugated anti-mouse IgG, anti-human Fas IgM (clone CH-11) from Biozol (Plymouth Meeting, PA). C16-dihydroceramide was from Toronto Research Chemical Inc. (North York, Ontario). The stimulatory mouse anti-human Fas IgM (clone CH-11) was from Upstate Biotechnology Inc. (Lake Placid, NY). Jo2 hamster anti-human Fas IgG was from Pharmingen (San Diego, CA). Jo2 mouse anti-human Fas IgG was from Toronto Research Chemical Inc. FITC-conjugated anti-hamster IgG was from Transduction Labs (West Grove, PA).

**Cell Culture**—Jurkat T lymphocytes were grown 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 nonessential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were grown in a 5% CO2 incubator at 37 °C.

**Animals**—ASMase knockout mice, maintained in an s129 × C3H/BL6 background, were propagated using heterozygous breeding pairs and genotyped as described (35). Experimental mice were 8–12 weeks old and were sacrificed by carbon dioxide asphyxiation.

**Ex Vivo Hepatocyte Culture**—Primary cultures of hepatocytes were prepared by mechanical disruption as described (35). Briefly, harvested livers were washed three times with phosphate-buffered saline at 37 °C, cut into small pieces, and separated into small clumps with a spatula. Individual hepatocytes were mechanically dispersed by passage through an 18-gauge needle, filtered through a 100-μm cell strainer, washed once with RPMI 1640 Complete medium, and resuspended into the same medium containing 10% fetal bovine serum. Hepatocytes were rested in a 37 °C incubator with 5% CO2 for 1 h before treatment. After that, 5 × 106 cells were placed in 24-well plates precoated with 10 mg of bovine serum albumin fraction V (Sigma/ml) and treated with anti-CD95 Jo2 antibody or sFasL for the indicated times while under constant agitation using a cell rotator.

**Sphingolipid and Antibody Treatment**—FasL was preincubated with 1 μg of anti-FLAG antibody/ml for 10 min before the addition to the cells to allow cross-linking. Cells were pretreated with 1 μg/ml of Jo2 anti-Fas antibody or sFasL and then permeabilized with a solution of 0.1% Triton X-100 and 0.1% sodium citrate at 4 °C for 5 min. Apoptosis was assessed by TUNEL according to the manufacturer's instructions (Roche Molecular Biochemicals). At least 200 cells were counted for each point. For Jurkat cell studies, the transincapation step was omitted.

**Bis-Benzimide Staining**—Bis-benzimide bis-Benzimide Staining—Bis-benzimide staining was used to visualize the nucleus as described (36). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34).

**Apoptosis Assay**—Apoptosis was assessed by two different techniques.

**TUNEL Assay**—TUNEL assay was performed. Cells were trypsinized after treatment with anti-Fas antibody or sFasL and then permeabilized with a solution of 0.1% Triton X-100 and 0.1% sodium citrate at 4 °C for 5 min. Apoptosis was assessed by TUNEL according to the manufacturer's instructions (Roche Molecular Biochemicals). At least 200 cells were counted for each point. For Jurkat cell studies, the transincapation step was omitted. bis-Benzimide Staining—Morphological changes in the nuclear chromatin of cells undergoing apoptosis were detected by staining with the DNA binding fluorochrome bis-benzimide trihydrochloride as previously described (34). 300 cells were examined for the incidence of apoptosis using an Axiolux S-100 Zeiss fluorescence microscope. Apoptotic cells exhibited chromatin condensation and segmentation, its compaction along the periphery of the nucleus as well as the appearance of apoptotic bodies.

**Capping of Fas**—To visually examine surface Fas after administration of cross-linking CH-11 antibody, 0.5–0.8 × 106 cells were treated with 1 μg CH-11/ml at 4 °C for 60 min in Hepes-buffered saline (H/S) (132 mM NaCl, 20 mM Hepes (pH 7.4), 5 mM KCl, 1 mM CaCl2, 0.7 mM MgCl2, 0.8 mM MgSO4) containing 2% fetal bovine serum. Unbound antibody was removed by washing twice with H/S. Cells were incubated...
FIG. 1. Treatment with CH-11 antibody rapidly induces capping of Fas on the surface of Jurkat cells. A, 0.5–0.8 × 10^6 Jurkat cells, treated sequentially at 4 °C for 60 min each with 1 μg CH-11/ml and Texas Red-conjugated secondary antibody, were warmed to 37 °C, harvested
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Comparison of ED<sub>50</sub> values for capping, ceramide elevation, and apoptosis

|                     | Hepatitis | Jurkat         |
|---------------------|-----------|----------------|
| ED<sub>50</sub>     | (ng of Jo2/ml) | (ng of CH-11/ml) |
| Ceramide elevation* | 454 ± 73    | 43 ± 18        |
| Capping*            | 397 ± 13    | 27 ± 2         |
| Apoptosis*          | 441 ± 8     | 55 ± 25        |

* Ceramide levels were measured using the diacylglycerol kinase assay as described under "Experimental Procedures." Capping was determined by immunofluorescence as described under "Experimental Procedures." Apoptosis was determined by bis-benzimide and/or TUNEL staining as described elsewhere. The data (mean ± S.E.) are compiled from three experiments. 200 cells were analyzed per point.

RESULTS

Jurkat T Cells Cap in Response to Fas Engagement by CH-11 Antibody—Because recent studies suggest that Fas oligomerization may be critical for optimal signaling, we examined the pattern of Fas migration in the plasma membrane of Jurkat cells upon engagement with 1 µg of anti-Fas IgM CH-11 antibody/ml. Fas-CH-11 complexes were detected with Texas Red-conjugated anti-mouse secondary antibody. In unstimulated cells, Fas was diffusely distributed across the cell surface (Fig. 1A, 0 min). Cross-linking Fas with CH-11 induced within 30 s amassing of Fas into patches along the cellular perimeter in 60% of the cells accompanied by central clearing (Fig. 1A, 30 s). Fas patching was rapidly followed by capping, which peaked at 2 min of stimulation and was sustained at a lower level for as long as 30 min (Fig. 1B). Not all of the cells capped simultaneously. At 2 min, 50 ± 4% of the cells capped, whereas 25 ± 3% still exhibited patching. The ED<sub>50</sub> for capping at 2 min of stimulation was 27 ± 2 ng CH-11/ml (Table I). As previously reported and validated here (Fig. 5), CH-11 induced a rapid ceramide elevation to 148 ± 6% of control within 15 s of stimulation (p < 0.001) and a maximal increase after 1 min. The ED<sub>50</sub> for this event of 43 ± 18 ng CH-11/ml was similar to that for capping and apoptosis (Table I). These data indicate that cross-linking Fas results in rapid ceramide elevation and capping in Jurkat cells destined to undergo apoptosis.

Natural Ceramide Reverses the Capping Defect of asmasen<sup>−/−</sup> Hepatocytes—Our prior investigations showed that ceramide generation is a critical element of the apoptotic pathway induced by Fas in hepatocytes in vitro (35) and in vivo (41). While hepatocytes obtained from asmasen<sup>−/−</sup> mice failed to generate ceramide and underwent apoptosis only in response to very high doses of Jo2 anti-Fas antibody, exogenous C<sub>16</sub>-ceramide reversed the resistance phenotype and completely restored Fas sensitivity. To investigate whether ceramide is also involved in capping of Fas, hepatocytes isolated from asmasen<sup>−/−</sup> and asmasen<sup>+/−</sup> mice were treated with Jo2 antibody. As previously reported (35), ceramide levels increased after 30 s in asmasen<sup>+/−</sup> hepatocytes (p < 0.01 versus control), peaked at 3-fold of control at 5 min of stimulation, and remained elevated for at least 20 min (not shown). As little as 50 ng Jo2/ml was effective, and a maximal effect was achieved with 1000 ng/ml Jo2 (not shown); the ED<sub>50</sub> for this event was 454 ± 74 ng/ml Jo2 (Table

| Jo2 (ng/ml) | Percent Capping |
|------------|-----------------|
| 0          | 60%             |
| 50         | 60%             |
| 100        | 60%             |
| 200        | 60%             |
| 400        | 60%             |
| 600        | 60%             |
| 800        | 60%             |
| 1000       | 60%             |

at the indicated times, and fixed with 2% paraformaldehyde. Fas-CH-11 complexes were localized using an Axiovert S-100 Zeiss fluorescence microscope equipped with a SPOT digital camera. Unstimulated control cells were exposed to CH-11 and secondary antibody at 4 °C only. B, quantitation of Fas capping as described in A. The data (mean ± S.E.) are compiled from three separate experiments. 150–250 cells were analyzed per time point.
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Recent investigations demonstrate that sFasL, a natural proteolytic product of Fas ligand (5), binds trimerized Fas but fails to signal apoptosis unless it is super-aggregated by cross-linking. To determine whether cross-linking resulted in capping, we used a recombinant sFasL engineered to carry a FLAG tag (5, 44). For these studies, Jurkat cells were stimulated with sFasL alone, or with sFasL precross-linked with 1 μg of M2 anti-FLAG antibody/ml, and sFasL-Fas complexes were detected with a FITC-conjugated secondary antibody. Cells stimulated with sFasL alone failed to cap (Fig. 3, left panel). As observed with ligation of Fas in wild-type hepatocytes or Jurkat cells, precross-linked sFasL induced rapid Fas capping on the surface of Jurkat cells (Fig. 3, middle panel). Since ceramide appeared to confer capping upon Jo2 in asmase−/− hepatocytes, we preincubated cells with 25 nm C16- ceramide for 10 min followed by stimulation with sFasL for 2 min. Although ceramide alone had no effect on Fas capping of Jurkat cells (not shown), sFasL induced capping as efficiently when cells were pretreated with C16-ceramide (Fig. 3, right panel) as when it was directly precross-linked by M2 anti-FLAG antibody. Similar results were obtained in primary cultures of hepatocytes (not shown).

Ceramide Elevation Precedes Capping—Although our prior investigations demonstrate a requirement for ceramide generation for efficient hepatocyte apoptosis, these studies also suggest that the necessity for ceramide can be bypassed under some circumstances. In particular, precross-linked Jo2 induced apoptosis of asmase−/− hepatocytes as efficiently as asmase+/+ hepatocytes (41). Similarly, high Jo2 doses (>1000 ng/ml), which spontaneously aggregate, induced apoptosis of asmase−/− hepatocytes (35). To explore whether forcing coupled of sFasL bypasses ceramide generation entirely, we measured ceramide levels in Jurkat cells treated with CH-11 anti-Fas antibody, sFasL alone, or sFasL cross-

Figure 3. Cross-linking of sFasL with anti-FLAG antibody or addition of C16-ceramide induces capping in Jurkat cells. Jurkat cells were stimulated for 2 min with 50 ng of sFasL/ml alone or with sFasL precross-linked with 1 μg of M2 anti-FLAG antibody/ml. Alternatively, cells were preincubated with 25 nm C16-ceramide for 10 min before the addition of sFasL. After stimulation, cells were washed and then incubated with additional sFasL plus anti-FLAG antibody (Ab) at 4 °C. Capping was detected as described in Fig. 1A.

I). As in CH-11-treated Jurkat cells, asmase−/− hepatocytes responded to Jo2 anti-Fas antibody with rapid capping that was time- and dose-dependent (Fig. 2). As little as 100 ng of Jo2/ml was effective and 70 ± 6% of the population displayed capping after 2 min of treatment with 1000 ng of Jo2/ml. An E50 value of 397 ± 13 ng of Jo2/ml for capping (Table I) was similar to the ED50 for ceramide generation and apoptosis. asmase−/− hepatocytes, which fail to generate ceramide and are resistant to apoptosis up to 1000 ng of Jo2/ml (35), also manifested marked resistance to capping. Only at Jo2 doses of 1000 ng/ml or more, at which immunoglobulins spontaneously aggregate and likely artificially cross-link the receptor (42, 43), was capping detected (up to 70% capping was achieved with doses up to 8000 ng of Jo2/ml (not shown). These data correlate closely with our prior data showing Jo2 doses greater than or equal to 2000 ng/ml were required to induce apoptosis of asmase−/− hepatocytes (35).

To determine whether the lack of ASMase or of ceramide yielded the resistance to capping, natural C16-ceramide was added to asmase−/− hepatocytes immediately before Jo2. In previous studies, we showed that the addition of C16-ceramide, but not other sphingolipids, restored apoptosis sensitivity to anti-Fas in asmase−/− hepatocytes (35). Whereas pretreatment of cells with 25 nm C16-ceramide for 10 min before stimulation had no direct effect on Fas capping in the absence of Jo2 antibody, it completely restored Fas capping in response to Jo2 (Fig. 2). In contrast, the addition of C16-dihydroceramide, which fails to restore Fas-induced death in asmase−/− hepatocytes, was without effect (% capping at 10, 100, and 1000 ng/ml Jo2 in the presence of 25 nm C16-dihydroceramide was 5 ± 2, 4 ± 3, and 16 ± 4%, respectively; n = 3). These studies demonstrate that the defect in anti-Fas-induced capping of asmase−/− hepatocytes, like the defect in apoptosis, results from lack of ceramide generation, and is reversible upon provision of natural ceramide.

Natural Ceramide and Anti-FLAG Antibody Enable sFasL to Induce Fas Capping in Jurkat Cells—Recent investigations demonstrated that sFasL, a natural proteolytic product of Fas ligand (5), binds trimerized Fas but fails to signal apoptosis unless it is super-aggregated by cross-linking. To determine whether cross-linking resulted in capping, we used a recombinant sFasL engineered to carry a FLAG tag (5, 44). For these studies, Jurkat cells were stimulated with sFasL alone, or with sFasL precross-linked with 1 μg of M2 anti-FLAG antibody/ml, and sFasL-Fas complexes were detected with a FITC-conjugated secondary antibody. Cells stimulated with sFasL alone failed to cap (Fig. 3, left panel). As observed with ligation of Fas in wild-type hepatocytes or Jurkat cells, precross-linked sFasL induced rapid Fas capping on the surface of Jurkat cells (Fig. 3, middle panel). Since ceramide appeared to confer capping upon Jo2 in asmase−/− hepatocytes, we preincubated cells with 25 nm C16-ceramide for 10 min followed by stimulation with sFasL for 2 min. Although ceramide alone had no effect on Fas capping of Jurkat cells (not shown), sFasL induced capping as efficiently when cells were pretreated with C16-ceramide (Fig. 3, right panel) as when it was directly precross-linked by M2 anti-FLAG antibody. Similar results were obtained in primary cultures of hepatocytes (not shown).

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linked with anti FLAG antibody. Stimulation with CH-11 antibody induced elevations in endogenous ceramide levels detected as early as 15 s after the antibody addition (Fig. 5). Ceramide levels peaked after 1 min of stimulation, reaching 170 ± 63% of control (p < 0.001), and remained elevated for at least 4 min. Elevations in ceramide levels were dose-dependent, with the highest increase observed with 100 ng/ml CH-11 (not shown). sFasL alone failed to induce changes in ceramide levels, whereas cross-linked sFasL, at concentrations that induce capping and apoptosis, resulted in elevations similar to those induced by CH-11 (p < 0.001 versus sFasL).

Disruption of Membrane Microdomains Abrogates Capping and Inhibits Apoptosis—Although developing literature suggests that GEMs may be involved in cap formation of some stimulated receptors (29–31), their involvement in Fas capping requires definition. To address this issue, we pretreated Jurkat cells with the cholesterol-depleting agents methyl-β-cyclodextrin, filipin, and nystatin, which disrupt these domains before sFasL treatment. α-Cyclodextrin, the inactive isomer of β-cyclodextrin, was used as a control. Jurkat cells were treated for 60 min at 37 °C with 2 mM α- and methyl-β-cyclodextrin, 1 μg of filipin/ml, or 10 μg of nystatin/ml before stimulation with CH-11 antibody for 2 min. Methyl-β-cyclodextrin, filipin, and nystatin all effectively reduced capping of Jurkat cells, whereas α-cyclodextrin had little or no effect (Fig. 6A). These data suggest that the presence of intact rafts may be necessary for Fas capping. Treatment with cholesterol-depleting agents also inhibited apoptosis. Cells were pretreated with the above-mentioned concentrations of drugs for 60 min followed by the addition of 50 ng of CH-11 antibody/ml in the presence of the drugs. Samples were taken at various time points and analyzed for apoptosis using bis-benzimide staining. Data for filipin, which prevented Fas-induced apoptosis for up to 9 h, are shown in Fig. 6B. Nystatin and methyl-β-cyclodextrin were also effective, whereas α-cyclodextrin did not affect apoptosis induction (not shown). Consistent with a role for ceramide in Fas-induced death, these data suggest that the presence of intact sphingolipid microdomains may be essential for full activation of Fas-mediated apoptosis.

**Fig. 4.** Natural ceramide confers apoptotic capacity upon sFasL in Jurkat cells and mouse hepatocytes. *A*, mouse hepatocytes were prepared as described under “Experimental Procedures” and treated with increasing concentrations of precross-linked or uncross-linked sFasL or sFasL with 25 nM C16-ceramide for 8 h at 37 °C. *B*, Jurkat cells were treated as described in *A*. The data (mean ± S.E.) are compiled from 3 experiments each, examining 200 cells per point. Ab, antibody.

**Fig. 5.** Time course of ceramide generation in Jurkat cells in response to Fas activation. Jurkat cells were treated with 100 ng CH-11/ml or precross-linked or uncross-linked sFasL for the indicated times as described under “Experimental Procedures.” Ceramide content was determined by the diacylglycerol kinase assay. The data (mean ± S.E.) are compiled from three experiments performed in triplicate. Ab, antibody.
membrane signaling in response to interleukin-1β (45, 46), heat (47), nerve growth factor (48), and ionizing radiation (49, 50) via an ASMase-mediated mechanism (45, 46, 48, 51, 52). Preliminary experiments similarly show ASMase translocation into GEMs and ceramide elevation within GEMs upon Fas stimulation of hepatocytes.

Our investigations also show that ceramide-dependent capping can be bypassed by forced clustering of Fas. Either cross-linking Jo2 by binding to plastic (41) or the use of Jo2 doses known to effect spontaneous aggregation (42, 43) permit Fas-mediated clustering and apoptosis in the absence of ASMase. Whether forced clustering ever represents a physiologic process is uncertain. For some in vivo events, however, ASMase appears indispensable for Fas-FasL-mediated apoptosis. In particular, for PHA-induced hepatitis (41), anti-CD4-induced deletion of CD3+ and CD4+ populations (41), and Pseudomonas aeruginosa-induced sloughing of pulmonary epithelium, events which model pathophysiology, increasing the inductive signal does not restore Fas responsiveness in asmase−/− mice. Furthermore, it is unknown whether cells that signal Fas-mediated apoptosis independent of ASMase-generated ceramide, such as thymocytes or the B cell clone responsible for the lpr phenotype, still cap Fas. In this regard, there are other mechanisms for ceramide generation such as stimulation of neutral sphingomyelinase or de novo synthesis which might be available for capping.

Recent biophysical studies of ceramide in model membranes provide insight into the properties that may enable ceramide to confer capping. In resting cells, the outer leaflet of the plasma membrane, which contains most cellular sphingomyelin, displays low fluidity. This is an intrinsic property of sphingomyelin and may be enhanced by sphingomyelin-mediated trapping and packing of cholesterol within the bilayer (54). Thus, sphingomyelin hydrolysis, which releases cholesterol from the membrane (55, 56), would serve to increase membrane fluidity, increasing lateral mobility of membrane constituents. Perhaps more critical to the capacity to elicit capping is the generation of ceramide. A number of groups have now identified specific properties of ceramide in model membrane systems devoid of protein (57). In particular, ceramide has the propensity to spontaneously self-assemble into microdomains, as detected by a variety of physical techniques including 2H NMR (58), the combination of differential scanning calorimetry and infrared spectroscopy (59), and atomic force spectroscopy (60). If these properties are also operative in biologic membranes, locally generated ceramide might re-organize into lipid patches capable of fusing into larger platforms (29). The interaction of Fas with these structures might generate a high local concentration of Fas and its intracellular effectors. Consistent with this paradigm, a recently developed anti-ceramide antibody detected high ceramide levels within Fas caps by confocal microscopy. An unanswered question is, How does this system initiate? Previous studies demonstrated that Fas-mediated ASMase activation is initiator caspase-dependent (38), although ASMase does not appear to be a direct caspase target. Whether trimerized Fas or minimally oligomerized Fas initiates ASMase activation and ceramide generation is presently unknown. However, the studies by Grullich et al. (38) suggest that weak Fas signals, which may not be sufficient to induce apoptosis, may nonetheless be sufficient to initiate ceramide generation. Although unliganded receptor does not cap upon C16-ceramide addition, capping occurred with a combination of the above properties.

**DISCUSSION**

The present studies provide mechanistic information regarding the role of ceramide in Fas signaling. Ceramide is generated via ASMase within seconds of Fas ligation, preceding Fas patching and capping in Jurkat cells and primary cultures of hepatocytes. The ED₅₀ values for ceramide generation, capping, and apoptosis correlated closely within each cell population. Genetic evidence supports a role for ceramide in the clustering process, as asmase−/− hepatocytes manifest a log defect in capping. That ceramide is obligatory for capping in asmase−/− hepatocytes is evidenced by the restoration of Fas capping by nanomolar amounts of natural ceramide. Further evidence that ceramide-mediated capping may be necessary for optimal Fas-induced apoptosis is derived from studies using cholesterol-depleting agents, which reduce cholesterol availability, disrupt sphingolipid microdomains (28), and in the present studies, prevent capping and apoptosis. These domains represent known sites for ceramide production during trans-

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Footnotes:

2 A. Cremesti and R. Kolesnick, unpublished observation.
3 E. Gulbins and R. Kolesnick, unpublished observation.
4 E. Gulbins, submitted for publication.
of exogenous ceramide and sFasL, indicating that receptor ligation confers interaction with GEMs but not death-inducing signaling complex formation. Similar results have been obtained with ceramide and the inactive DX2 anti-Fas antibody in Jurkat cells.4 Perhaps the conformational change, our recent studies show that cysteine 149 within the tor concentrates within a cap, it is fully capable of activating enriched rafts at the outer membrane. Once liganded receptor concentrates within a cap, it is fully capable of activating the death-inducing signaling complex formation. Similar results have been obtained with ceramide and the unactive DX2 anti-Fas antibody in Jurkat cells.4 Regarding the mechanism of Fas cap-obtained with ceramide and the unactive DX2 anti-Fas antibody in Jurkat cells.4 We thank Desiree Ehleiter, Sylvia Menendez, and Dr. Thomas McNabb for assistance with these experiments.

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REFERENCES

1. Krammer, P. H. (1998) Toxicol. Lett. 102-103, 131–137
2. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomasselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. (1998) EMBO J. 17, 1675–1687
3. Siegel, R. M., Frederiksen, J. K., Chan, F. K., Johnson, M., Lynch, D., Ten, E. Y., and Lenardo, M. (2000) Science 288, 2354–2357
4. Schneider, P., Holler, N., Bodmer, J. L., Hahne, M., Frei, K., Fontana, A., and Gulbins, E. (1998) J. Cell Biol. 139, 333–340
5. E. Gulbins, unpublished observation.

E. Gulbins, unpublished observation.