The downstream targets of endosomal-derived ceramides and activates extracellular signal-regulated kinases (ERKs) and activates extracellular signal-regulated kinases (ERKs) mitogen-activated kinase (MEK-1), which eventually phosphorylates and activates Raf-1 kinase (4, 5). Activated Raf-1 activation of ceramide-activated protein kinase (CAPK) (3), lipid second messenger ceramide and phosphorylcholine. Ceramide produced by sphingomyelinases (SMases) 1 has been recognized as an important second messenger in membrane receptor signaling. Through binding to the p55 TNF receptor-associated proteins TRADD and FADD. Overexpression of TRADD and FADD in 293 cells did not change basal activity of A-SMase but enhanced TNF-induced stimulation of A-SMase. Other TNF R55-associated proteins like TRAF2 and RIP, which were reported to mediate TNF R55-mediated activation of nuclear factor-κB, did not affect activation of A-SMase. Caspase inhibitors markedly reduced A-SMase activity, suggesting the involvement of an IκB-like protease in TRADD/FADD-mediated activation of A-SMase. Overexpression of caspase-8/FLICE (MACH) or caspase-10/FLICE2 did not change A-SMase activity, suggesting that TRADD/FADD-mediated activation of A-SMase involves a yet to be defined caspase-like protease distinct from caspase-8/FLICE or -10/FADD.

Ceramide produced by sphingomyelinases (SMases) has been recognized as an important second messenger in membrane receptor signaling. Through binding to the p55 TNF receptor (TNF-R55), TNF rapidly activates two distinct forms of SMases, a membrane-associated neutral (N-) SMase and an acid (A-) SMase (1), which reside in caveolae (2) and the endosomal-lysosomal compartment. Each type of SMase hydrolyzes the phosphodiester bond of sphingomyelin to yield the neutral lipid second messenger ceramide and phosphorylcholine. Ceramide generated by N-SMase at the plasma membrane directs the activation of ceramide-activated protein kinase (CAPK) (3) that phosphorylates and activates Raf-1 kinase (4, 5). Activated Raf-1 in turn phosphorylates and stimulates a dual specificity mitogen-activated kinase (MEK-1), which eventually phosphorylates and activates extracellular signal-regulated kinases (ERKs). The downstream targets of endosomal-derived ceramide produced by A-SMase are not yet defined but may include protein kinase C (PKCζ), JNK, and caspases (6–8). N-SMase and A-SMase are activated independently by distinct cytoplasmic domains of TNF-R55 (1). N-SMase activation is mediated by neutral sphingomyelinase domain (NSD), spanning an 11-amino acid motif N-terminally adjacent to the death domain of TNF-R55 (9, 10). The NSD binds a WD-repeat protein, FAN, that mediates activation of N-SMase.

The domain of TNF-R55 activating the A-SMase pathway strikingly corresponds to the death domain signaling the cytotoxic effects of TNF (1, 11). The cytoplasmic protein TRADD was recently identified to associate with the death domain of TNF-R55 in a TNF-dependent process (12, 13). TRADD serves as an adapter protein that recruits other proteins to the cytoplasmic TNF receptor complex (14, 15). Overexpression of TRADD potently activates distinct signaling cascades leading to activation of NF-κB and induction of cell death (13). FADD, which directly binds to TRADD, has been shown to mediate activation of a pro-apoptotic protease, caspase-8/FLICE (MACH), eventually leading to apoptosis (16, 17).

Here we show that TNF-induced activation of A-SMase is mediated through TRADD and FADD. Neither caspase-8/FLICE nor a dominant negative mutant of caspase-8/FLICE alter the TNF-induced activation of A-SMase. However, several tetrapeptide caspase inhibitors as well as cytokine response modifier A (cIAP1) attenuated TNF-induced activation of A-SMase activity, suggesting that FADD mediates enhancement of A-SMase activity through activation of a protease distinct from FLICE.

**MATERIALS AND METHODS**

**Cell Culture and Biological Reagents**—The human embryonic kidney cell line HEK 293 was kindly provided by Dr. M. Schmidt, Essen, FRG. Hela and COS7 cells were obtained from ATCC. Cells were maintained in high glucose Dulbecco’s modified Eagle’s medium (ICN) supplemented with 10% fetal calf serum, 10 mm glutamine, and 50 μg/ml each of streptomycin and penicillin in a humidified incubator at 5% CO₂.

Highly purified recombinant human TNF (3500 U/mg) was kindly provided by Dr. G. Adolf, Boehringer Research Institute, Vienna, Austria. Tetrapeptide caspase inhibitors were obtained from Bachem, Heidelberg, FRG.

**Expression Vectors**—Mammalian cell expression vectors encoding TRADD, TRAF2, RIP, cIAP1, caspase-8/FLICE and dominant negative deletion mutants of this protein, as well as the control expression plasmids pRK5 and pCMV-β-gal were kindly provided by Drs. D. V. Goeddel and M. Rothe, Tularik Inc., S. San Francisco, and have been described previously (12, 13, 15). FADD and caspase-10b cDNA were kindly provided by Dr. V. Dixit, Genentech, San Francisco. The expression vector pRK-FADD was generated by inserting a SalI-HindIII fragment of FADD cDNA into the pRK5 vector. Deletion mutants of this protein, as well as the control expression plasmids pRK5 and pCMV-β-gal were kindly provided by Drs. D. V. Goeddel and M. Rothe, Tularik Inc., S. San Francisco, and have been described previously (12, 13, 15). FADD and caspase-10b cDNA were kindly provided by Dr. V. Dixit, Genentech, San Francisco. The expression vector pRK-FADD was generated by inserting a SalI-HindIII fragment of FADD cDNA into the pRK5 vector. Deletion mutants of FADD were generated by inserting corresponding sequences in-frame into the expression vector pRK5.

For transient expression experiments, 1.5 × 10⁶ HEK 293 cells or 1.0 × 10⁶ COS7 or Hela cells were seeded on 100-mm dishes (Falcon, 3080). Cells were transfected the following day by the calcium phosphate precipitation method (19). After 9 h of incubation, cells were harvested, and enzymatic SMase assays were performed.

**Assays for Neutral and Acidic SMases**—HEK 293 or COS7 cells were transfected with different TRADD expression constructs, or with iden-
tactical amounts of expression vectors, either alone or in combination with other expression constructs for TNF-R55-associated proteins. After 9 h, cells were harvested either for SMase assays or Western blot analysis. The micellar SMase assay using exogenous radiolabeled sphingomyelin was performed as described previously (1). Briefly, at 9 h post-transfection, cells were treated in triplicate in 0.5 ml of medium with 100 ng/ml human recombinant TNF for the indicated periods of time. To measure acid SMase, cells were homogenized in 0.2% Triton X-100 lysis buffer. Radioactive phosphorylcholine produced from \(^{14}C\)sphingomyelin (labeled in the choline moiety, 47 mCi/mmol, NEN Life Science Products) was determined in the aqueous phase by liquid scintillation counting.

**Western Blot Analysis**—Cells were washed once with phosphate-buffered saline (PBS) and lysed for 20 min at 4 °C in 0.4 ml of lysis buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 10% glycerol, 5 mM EDTA, and protease inhibitor mix Complete® (Boehringer Mannheim, FRG)). Cell lysates were fractionated by 10% SDS-PAGE and transferred on Porablot NCL filters (0.45 μm, Macherey-Nagel, Düren, FRG) Filters were blocked overnight in Tris-buffered saline containing 0.1% Tween-20, 5% milk powder, and 1% bovine serum albumin. After incubation at room temperature with either mAb FLAG M2 (Kodak Inc.), mAb myc (Life Technologies, Inc.), rabbit anti-TRADD, rabbit anti-TRAF2, (M. Rothe), or mAb FADD (Transduction Laboratories), filters were washed in Tris-buffered saline containing 0.1% Tween-20 and incubated with a 1:2400 dilution of goat anti-mouse/goat anti-rabbit horseshadish peroxidase conjugate (Coulter-Immunotech) in blocking buffer. Filters were washed and developed with ECL detection reagent (Amersham Corp.).

**Hypodiploidy Apoptosis Assay**—Cells were washed twice with cold PBS/EDTA, 5 mM, and 1 × 10^6 cells were resuspended in 0.5 ml of PBS/EDTA and fixed by adding 0.5 ml of ethanol. After 30 min of incubation at room temperature, cells were centrifuged and resuspended in 0.5 ml of PBS/EDTA. 20 μl of RNase A (1 μg/ml) was added, and after incubation for 30 min at room temperature, 0.5 ml staining solution (500 μg/ml propidium iodide in PBS/EDTA, 5 mM) was added. After 1 h of incubation at 4 °C, cell cycle analyses were performed by flow cytometry using a Becton Dickinson Calibur FACS Analyzer.

**Materials and Methods**—TNF-induced A-SMase activity was determined as described under “Materials and Methods.” TNF-induced A-SMase activities are expressed as percent of untreated control vector transfected cells.

**Results**

Recent studies have identified a C-terminal domain, corresponding to the death domain of TNF-R55, that is responsible for activation of A-SMase and generation of the lipid second messenger ceramide (1, 22). To examine whether one of the so far identified TNF-R55 death domain-associated proteins mediates the activation of A-SMase, 293 cells were first transfected with 1.5 μg of TRADD expression plasmid or control vector, and TNF-induced A-SMase activity was determined.
SMase. TRAF2 belongs to a family of signal transducers sharing a highly conserved C-terminal TRAF domain, which binds to a N-terminal region of TRADD distinct from the death domain (13, 23). TRAF2 was shown to mediate TNF-R55-induced activation of nuclear transcription factor κ B (NFκB) (13, 15). As shown in Fig. 2A, TRAF2 had no effect on A-SMase activity. Similarly, overexpression of a dominant negative mutant of TRAF2 (amino acids 87–501), lacking the N-terminal RING finger motif essential for NFκB activation, had no inhibitory effect on A-SMase activity (Fig. 2A). Finally, co-transfection with TRADD cDNA did not reveal TRAF2 action on A-SMase (Fig. 2D). The expression and biological function of TRAF2 cDNA were ensured by Western blot analysis and by NFκB-activation in 293 cells, respectively (Fig. 2, B and C). These findings strongly suggest that TRAF2 is not involved in TNF-induced activation of A-SMase.

The receptor interacting protein kinase RIP has been shown to directly interact with TRAF proteins and the death domain of TRADD (13) and to be capable of inducing both the activation of NFκB and apoptosis (24). As shown in Fig. 3A, neither overexpression of RIP nor a dominant negative deletion mutant RIP-DN, encoding only the death domain of RIP, altered TNF-induced activation of A-SMase. Notably, both expression constructs induced apoptosis when transfected into 293 cells (Fig. 3B). The lack of the ability of RIP-DN to signal for the activation of NFκB may contribute to the greater extent of hypodiploid nuclei in HeLa cells when compared with the full-length RIP expression construct. Co-transfection of TRADD and RIP did not result in enhanced activity of A-SMase (Fig. 3C). These results suggest that RIP, like TRAF2, is not involved in TNF-induced activation of A-SMase.

It has been recently reported that overexpression of FADD causes the activation of caspase-8/a (FLICE/MACH) and eventually apoptosis (16, 17). As shown in Fig. 4A, expression of FADD cDNA in 293 cells led to enhanced A-SMase activity in a dose- and TNF-dependent manner. Basal activity of A-SMase remained unchanged. A dominant negative mutant of FADD, lacking the N-terminal death effector domain (DED), FADD-DN, was found to decrease TNF-induced activation of A-SMase in a dose-dependent manner (Fig. 4B). Co-expression of FADD along with a constant amount of TRADD cDNA (Fig. 4C) enhanced the A-SMase activity slightly over the values obtained with FADD expression constructs alone. These findings indicate, that FADD, like TRADD, can enhance TNF-induced A-SMase activation.

To obtain a more detailed estimation for TRADD and FADD-mediated enhancement of A-SMase activity, we performed enzyme kinetic analysis from cells transfected with expression constructs for TRADD and FADD. Apparent $V_{\text{max}}$ and $K_m$ values were determined for the substrate [N-methyl-14C]sphingomyelin by fitting initial rates of SM hydrolysis at various substrate concentrations to the Michaelis-Menten equation. As shown in Fig. 5, A-SMase has a basal activity of $V_{\text{max}} = 21$ nmol/h/mg and a $K_m$ of 11 μM for sphingomyelin in 293 cells. The maximal velocity $V_{\text{max}}$ is enhanced upon TNF treatment to $V_{\text{max}} = 33$ nmol/h/mg, whereas $K_m$ remained unchanged. Overexpression of TRADD further enhanced the activity of A-SMase, yielding a maximal velocity $V_{\text{max}} = 45$ nmol/h/mg. The highest maximal velocity was observed with TNF-treated cells previously co-transfected with expression vectors for TRADD and FADD ($V_{\text{max}} = 83$ nmol/h/mg). In all instances, $K_m$ values remained unchanged.

The kinetic profile of A-SMase activation after TNF treatment is shown in Fig. 6. After stimulation of 293 cells with TNF, the activity of A-SMase is transiently increased, peaking at 3 min, and declined over a period of 10 min to reach basal levels again. Cells transfected with expression vectors for TRADD and FADD showed enhanced A-SMase activity at any time after TNF treatment, while basal levels of A-SMase activity remained unaffected.

Caspase-8/a (FLICE/MACH) is a downstream signaling molecule of FADD that transduces the TNF and FAS-induced death signal. When 293 cells were transfected with various doses of expression constructs for caspase-8/a the basal activity of A-SMase remained unchanged (Fig. 7A). Furthermore, overexpression of caspase-8/a (FLICE/MACH) did not alter the magnitude of TNF-induced A-SMase activation. However, caspase-8/a strongly induced apoptosis in these cells, within 15–24 h post-transfection, which was blocked by the coexpression of a 2-fold excess of crmA expression construct (Fig. 7B).
FIG. 4. FADD altered TNF-induced activation of A-SMase. A, 293 cells were transfected either with 10 μg of control vector pRK5 or 3 μg of expression construct for FADD or a dominant negative deletion mutant FADD-DN (559–671). Cells were stimulated with 100 ng/ml TNF for 3 min or were left untreated. [14C]Sphingomyelin hydrolysis was determined from cellular extracts, and A-SMase activity is expressed relative to untreated vector control cells. B, HeLa cells were transfected with expression constructs for FADD, a dominant negative deletion mutant of FADD, encoding the death domain of FADD and control vector. Hypodiploidy apoptotic assays were performed 24 h later. C, 293 cells were transfected with control vector pRK5 or were co-transfected with indicated amounts of expression vector for FADD along with a constant amount of TRADD cDNA, and A-SMase activity was determined. D, cells were analyzed by Western blotting using anti-FLAG antibody mAbM2. The positions of FLAG-TRADD and FLAG-FADD proteins are indicated by arrows.

FIG. 3. TNF-mediated activation of A-SMase is independent of RIP. A, 293 cells were transfected either with 10 μg of control vector pRK5 or 3 μg of expression construct for RIP or a dominant negative deletion mutant RIP-DN (559–671). Cells were stimulated with 100 ng/ml TNF for 3 min or were left untreated. [14C]Sphingomyelin hydrolysis was determined from cellular extracts, and A-SMase activity is expressed relative to untreated vector control cells. B, HeLa cells were transfected with expression constructs for RIP, a dominant negative deletion mutant of RIP, encoding the death domain of RIP and control vector. Hypodiploidy apoptotic assays were performed 24 h later. C, 293 cells were transfected with control vector pRK5 or were co-transfected with indicated amounts of expression vector for RIP along with a constant amount of TRADD cDNA, and A-SMase activity was determined. D, cells were analyzed by Western blotting using anti-FLAG antibody mAbM2. The positions of FLAG-TRADD and FLAG-RIP proteins are indicated by arrows.
These findings ensured the expression of a functional caspase-8/a zymogen. A dominant negative mutant of caspase-8/a lacking 43 amino acids of the C terminus was shown to prevent TNF-induced cell death (25). However, overexpression of FLICE-DN did not alter activity of A-SMase (data not shown). Similarly, overexpression of caspase-10/b (FLICE2) did not alter basal or TNF-induced A-SMase activation in 293 cells (data not shown). These findings suggest that caspase-8/a or caspase-10/b are not involved in TNF-induced A-SMase activation in 293 cells.

To investigate a possible involvement of caspases other than FLICE/MACH in A-SMase activation, we finally employed a set of potent caspase inhibitors. When cow pox virus crmA was overexpressed in 293 cells, TNF-induced activation of A-SMase was markedly reduced (Fig. 8A). As shown in Fig. 8D, crmA also inhibited enhancing effects of TRADD and FADD on TNF-induced A-SMase activity. Again, the basal A-SMase activity in these cells remained unaltered. Similar results were obtained with tetrapeptide inhibitors of caspases, z-VAD.fmk, Ac-DEVD.CHO, and Ac-YVAD.cmk. Treatment of cells with Ac-DEVD.CHO, which preferentially inhibits caspase-3-like death proteases, resulted in reduced TNF activation of A-SMase (Fig. 8A). A more pronounced inhibitory effect was obtained with Ac-YVAD.cmk, an inhibitor of caspase-1 (Fig. 8C). In contrast to Ac-DEVD.CHO, Ac-YVAD.cmk also slightly reduced basal activity of A-SMase. The broad range caspase inhibitor z-VAD.fmk also inhibited the enhanced activation of A-SMase in TRADD- and FADD-transfected 293 cells. These results suggest that FADD-mediated activation of A-SMase involves an ICE-like caspase activity distinct from caspase-8/a (FLICE/MACH) and caspase-10/b (FLICE2).

\[ V_{\text{max}} \text{ and } K_m \text{ values were determined for sphingomyelin by fitting initial rates of SM hydrolysis at substrate concentrations from 1 to 60 } \mu\text{M to the Michaelis-Menten equation.} \]

\[ B \text{ and } C, \text{ kinetic parameters } V_{\text{max}} \text{ and } K_m \text{ were ascertained by double-reciprocal Lineweaver-Burk plot.} \]

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**DISCUSSION**

Here we show that acid sphingomyelinase is activated through p55 TNF receptor-associated proteins TRADD and FADD. Overexpression of TRADD and FADD in 293 cells enhanced the TNF-induced activity of A-SMase. Activation of A-SMase could be blocked in cells expressing a dominant negative mutant of FADD. Basal velocity of A-SMase in cells transfected with TRADD/FADD expression plasmids was not altered, suggesting that cross-linking of the TNF-R55 zTRADDzFADD complex is essential for the activation of A-SMase. Enzyme kinetic analysis revealed that the maximal velocity \( V_{\text{max}} \) of A-SMase is enhanced in TNF-treated 293 cells overexpressing TRADD and FADD. The highest maximal velocity of A-SMase was obtained in 293 cells transfected with both TRADD and FADD expression plasmids. Notably, the \( K_m \) remained unchanged, indicating that the enzyme activity of A-SMase was enhanced rather than the accessibility of the substrate.

In contrast, overexpression of neither TRAF2 nor a dominant negative deletion mutant TRAF2-DN altered TNF-induced activity of A-SMase in 293 cells, suggesting that TRAF2 is not required for the activation of A-SMase by TNF. TRAF2 has been shown to mediate TNF-induced activation of SAP/JNK (26) and NF\( \kappa \)B (15), which involves a MAP3 kinase-related protein kinase, NIK, and the IkB-kinase IKK\( \alpha \) (27–29). In 293
cells, TRAF2 is apparently not involved in activation of a A-SMase, suggesting that the A-SMase activation pathway is distinct from that causing induction of NFκB and SAPK/JNK. These data confirm a recent report by Hannun et al. (30), who showed that ceramide is not a direct member of the NFκB activation pathway and rather may antagonize NFκB activation in some systems. The previous notion, that A-SMase and ceramide activate NFκB may be explained by tissue-specific activation and function of A-SMase or as a result of a nonspecific stress response caused by exogenous ceramide or SMase.

Overexpression of the receptor interacting protein RIP has been shown to be capable of inducing both activation of NFκB and apoptosis. (26, 31). Notably, RIP was found to be important for TNF-R55-mediated activation of NFκB but not for Fas/Apo1-initiated induction of apoptosis (32). The results of our study indicate that RIP does not alter TNF-induced activation of A-SMase, suggesting that RIP is not a component of TNF R55-triggered A-SMase activation pathway.

It is well established that FADD, through its death effector domain (DED), recruits caspase-8 (FLICE/MACH) to the DISC where it is activated (16, 17, 33, 34). However, overexpression of caspase-8/a or of its dominant negative, did not affect TNF-induced activation of A-SMase. Thus, the apoptotic signaling of caspase-8 pathway to segregate from the A-SMase activation pathway at the level of FADD. While caspase-8/a did not seem to be involved in activation of A-SMase, tetrapeptide caspase inhibitors markedly inhibited TNF-induced A-SMase activation. In addition, overexpression of crmA, a potent inhibitor of ICE-like proteases, was able to block the TRADD and/or FADD-mediated enhancement of TNF-induced activation of A-SMase. Together, these results suggest that ICE-like protease distinct from caspase-8/a are involved in A-SMase activation. Hannun and co-workers (36) reported that ceramide signaled apoptosis in vircinistine-treated ALL-697 leukemia cells via a caspase-3 (CPP32)-like protease and that this effect was inhabitable by Bcl-2 (35, 36). Cells overexpressing Bcl-2, like wild-type cells, still responded to vincristine treatment with ceramide generation but failed to show PARP cleavage and apoptosis in response to a C6-ceramide analog. Further support for the notion that ceramide may be downstream of caspases is derived from experiments which showed that REAPER-induced ceramide generation was blocked by caspase inhibitor z-VAD.fmk (37). Furthermore, crmA, a pox virus serpin that is an inhibitor of ICE-like proteases but does not block caspase-3 (CPP32), abolished ceramide generation in TNF-treated cells (38). These studies suggested that ceramide generation occurs downstream of caspases. However, the source of ceramide was not addressed in these studies. The data of this report indicate that overexpression of TRADD and FADD enhances TNF-induced activation of A-SMase, which favors A-SMase to be downstream of the death domain adapter complex rather than ceramide synthase and other pathways of ceramide generation.

The question arises whether A-SMase is able to confer apoptosis in some instance of a specific cell type/stimulus combination. This idea is consistent with previous reports demonstrating that deletions of the death domain region of the TNF-R55 and CD95 (1, 39–41), as well as overexpression of dominant negative FADD-blocked ligand-induced ceramide generation and apoptosis (42). Further, Dixit and co-workers (42) reported that treatment with ceramide analogs can bypass the anti-apoptotic effect of dominant negative FADD and restore apoptosis, indicating that apoptotic elements downstream of ceramide generation are intact in these cells.

The results of the present study clearly indicate that A-SMase activation can be dissociated from caspase-8-induced apoptosis. Overexpression of caspase-8 readily induced apoptosis of 293 cells in the absence of changes of A-SMase activity levels. Thus, A-SMase does not seem to be necessary for caspase-8-driven apoptosis. A-SMase seems neither sufficient for apoptosis of 293 cells because overexpression of A-SMase did not cause cell death. The data do not rule out, though, that A-SMase constitutes an accessory pathway that can enhance the apoptotic response of 293 cells. The role of A-SMase in apoptosis induced by various stimuli in diverse cell types remains an open issue (for review see Ref. 43), and its precise delineation will require further extensive investigation.

Acknowledgments—We thank Drs. S. Adam-Klages and D. Adam for assistance with fluorescence-activated cell sorter analysis and helpful discussions. We thank Drs. M. Rothe and D. V. Goeddel, Tularik Inc., 2 R. Schwandner, K. Wiegmann, K. Bernardo, D. Kreder, and M. Kronke, unpublished observation.
TRADD and FADD Signal Activation of A-SMase

FIG. 8. Inhibition of TNF-induced A-SMase activity by several caspase inhibitors. 293 cells were transfected with 10 μg of control vector pRK5 or with 3 μg of crmA expression construct (A) or cotransfected with 0.5 μg of TRADD, 4.5 μg of FADD, and/or 5 μg of crmA expression constructs and were left untreated or treated with 25 μM z-VAD (D). 293 cells were left untreated or treated with 25 μM Ac-DEVD-CHO (B) or YVAD.cmK (C). After 9 h, cells were stimulated for 3 min or the indicated times, and A-SMase activity was determined. TNF-induced A-SMase activities are expressed as percent of untreated control cells.

S. San Francisco, for providing reagents and fruitful discussions and Dr. V. Dixit for FADD and FLICE2 expression vectors.

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