Requirement of FADD for Tumor Necrosis Factor-induced Activation of Acid Sphingomyelinase*

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The generation of mice strains deficient for select members of the signaling complex of the 55-kDa tumor necrosis factor receptor (TNF-R55) has allowed the assignment of specific cellular responses to distinct TNF-R55-associated proteins. In particular, the TNF-R55-associated protein FADD seems to be responsible for recruitment and subsequent activation of caspase 8. In this report we demonstrate the requirement of FADD for TNF-induced activation of endosomal acid sphingomyelinase (A-SMase). In primary embryonic fibroblasts from FADD-deficient mice the activation of A-SMase by TNF-R55 ligation was almost completely impaired. This effect is specific in that other TNF responses like activation of NF-κB or neutral (N-)SMase remained unaffected. In addition, interleukin-1-induced activation of A-SMase in FADD-deficient cells was unaltered. In FADD‡ embryonic fibroblasts reconstituted by transfection with a FADD cDNA expression construct, the TNF responsiveness of A-SMase was restored. The results of this study suggest that FADD, in addition to its role in triggering a proapoptotic caspase cascade, is required for TNF-induced activation of A-SMase.

Sphingomyelinase (SMase)§ activation and ceramide generation have emerged as a lipid signaling pathway transducing diverse biological effects of cytokine receptors like p55 tumor necrosis factor (TNF) receptor or CD95 (Fas, Apo-1) (1, 2). Through binding to the 55-kDa TNF receptor (TNF-R55), TNF rapidly activates two distinct forms of SMase, a membrane-associated neutral (N-)SMase and an acid (A-)SMase (3) residing in caveolae (4) and in the endosomal-lysosomal compartment. Each type of SMase hydrolyzes the phosphodiester bond of sphingomyelin to yield the neutral lipid second messenger ceramide and phosphorylcholine. Studies on TNF receptor signaling suggested a model that clarifies how distinct SMases might function through ceramide in diverse pathways (3, 5, 6). Using mutants of the cytoplasmic domain of TNF-R55, we have shown previously that specific receptor domains link to different sphingomyelinases. The activation of N-SMase is signaled by a cytoplasmic portion of TNF-R55 containing a small motif of 9 amino acid residues at position 310–318 that is both necessary and sufficient for activation of N-SMase (5, 6). This region was termed NSD for neutral sphingomyelinase activation domain.

The domain of TNF-R55 activating the A-SMase pathway strikingly corresponds to the death domain signaling the cytotoxic effects of TNF (1, 7). The molecular mode of action of the death domain has been extensively investigated. The death domain of TNF-R55 binds an adaptor protein, TRADD, that in turn recruits at least three further proteins, TRAF2, FADD, and RIP (for review, see Ref. 8). The emerging picture based on studies of many investigators indicates that TRAF2 mediates the activation of the c-Jun N-terminal kinase (JNK) (9), RIP is essentially involved in the NF-κB activation pathway (10), and FADD signals apoptosis through activation of caspase 8 (FLICE/MACH) (11–14). We have obtained evidence recently that neither TRAF2 nor RIP affected A-SMase activation (15). In contrast, overexpression of TRADD and FADD in 293 cells enhanced TNF-induced A-SMase activation without changing the basal level of A-SMase enzymatic activity. Strikingly, overexpression of the FADD-associated caspase 8 does not lead to enhanced A-SMase activation after TNF treatment, indicating that the apoptotic cascades initiated by caspase 8 segregate from the A-SMase activation pathway. Enzyme kinetic analysis revealed that the observed enhancement of A-SMase activation by TRADD and FADD is due to an increased maximal velocity of substrate hydrolysis rather than a higher affinity for the substrate sphingomyelin.

Here we further delineate the role of FADD for TNF-induced activation of A-SMase. Using embryonic fibroblasts from FADD-deficient mice, we provide evidence for a stringent requirement of FADD for TNF-induced activation of A-SMase.

MATERIALS AND METHODS

Cell Culture and Expression Vectors—Primary embryonic fibroblasts (EF) were derived from embryos at day 9.5 of gestation as described previously (14). EF cells were cultured 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 (3 × 10⁹ units/mg) was kindly provided by Dr. G. Adolf, Bender, Vienna, Austria.

Mammalian cell expression vectors encoding FADD 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. For transient expression experiments, 1.5 × 10⁵ EF cells were seeded on 100-mm dishes (Falcon, 3803). Cells were transfected the following day by the calcium phosphate precipitation method (16). After 9 h of incubation, cells were harvested, and enzymatic SMase assays were performed.

Assays for Neutral and Acid SMases—The micellar SMase assay using exogenous radiolabeled sphingomyelin was performed as described previously (5). Briefly, 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 A-SMase, cells were homogenized in 0.2% Triton X-100 lysis buffer. Radioactive phosphorylcholine produced from [N-methyl-³¹C]sphingomyelin (labeled in the choline moiety, 47 mCi/
FADD-dependent Activation of A-SMase

**RESULTS AND DISCUSSION**

**Unresponsiveness of A-SMase in FADD<sup>−/−</sup> EF Cells**—Primary EF cells obtained from FADD<sup>−/−</sup>, FADD<sup>+/−</sup>, or FADD<sup>+/+</sup> mice were stimulated with TNF and assayed for A-SMase activity. As shown in Fig. 1, TNF did not enhance A-SMase activity in FADD<sup>−/−</sup> EF cells within the time frame investigated. These results suggest that FADD is indispensable for TNF-induced A-SMase activation. In contrast, the A-SMase activation profile of FADD<sup>−/−</sup> EF cells was unaltered when compared with wild-type EF cells. Because the amplitude and kinetics of A-SMase activation were not diminished in FADD<sup>−/−</sup> cells, we conclude that FADD is not a rate-limiting factor for A-SMase activation in wild-type fibroblasts.

TNF treatment of FADD-deficient EF cells resulted in activation of N-SMase (Fig. 2A) and NF-κB (Fig. 2B), which was indistinguishable from TNF-stimulated wild-type EF cells. These findings indicate that FADD-independent TNF signaling pathways are intact. Furthermore, like TNF, IL-1 readily induced NF-κB in FADD<sup>−/−</sup> EF cells, which provides further evidence that the status of FADD deficiency does not establish a general unresponsiveness of EF cells.

**Specificity of FADD Action**—Unlike TNF, IL-1-induced activation of A-SMase in FADD<sup>−/−</sup> EF cells was similar in terms of amplitude and kinetics to that observed with heterozygous FADD<sup>+/−</sup> or wild-type EF cells (Fig. 3). Thus, IL-1 stimulates activation of A-SMase in a FADD-independent manner. Information about the molecular mechanisms of IL-1-induced activation of A-SMase is sparse. Recently, we reported that an accessory chain of the IL-1 receptor, IL-1RαCp, is required for both IL-1 internalization and activation of A-SMase (18). The functional domain of IL-1RαCp responsible for A-SMase activation has not yet been mapped. Neither has a putative IL-1RαCp-associated protein been identified that mediated A-SMase activation. At any rate, FADD, in general, does not seem to play a role in IL-1 receptor signaling. This is consistent with the unaltered A-SMase activation observed in IL-1-treated FADD-deficient EF cells.

**Restoration of A-SMase Responsiveness in FADD-deficient Cells**—In order to provide further pertinent evidence for the essentiality of FADD for the TNF-induced A-SMase activation pathway, FADD<sup>−/−</sup> EF cells were transiently transfected with a FADD cDNA expression vector. As shown, Fig. 4A, FADD-transfected cells responded to TNF with approximately half-maximal A-SMase activation when compared with FADD-deficient cells stimulated with IL-1 for control. This partial A-SMase response corresponded well with the transfection efficiency, which ranged from 35 to 45% as judged from parallel transfection with a GFP expression plasmid (not shown). In TNF-stimulated FADD-deficient EF cells, transfected with an empty vector for control, the changes of A-SMase activity were not significant as inferred from the respective S.E. In addition, results obtained from different independent experiments did not reveal a significant increment of A-SMase activity (Fig. 4B and data not shown). When different amounts of FADD expression vector were transfected into FADD<sup>−/−</sup> EF cells, a dose-dependent increase of A-SMase activation was observed (Fig. 4B). Thus, the A-SMase responsiveness to TNF in FADD-deficient EF cells can be reconstituted by transfection with FADD expression plasmids. Together, the results of this study indicate that FADD is an essential member of the TNF-induced A-SMase pathway.
The question then arises how FADD signals A-SMase activation. More specifically, how does the FADD-derived signal cross the membrane bilayer to target the endosomal A-SMase. Caspase 8 does not appear to mediate FADD activation of A-SMase (15), and A-SMase is not a target for caspase 8 proteolytic cleavage. Rather, recent evidence suggests that internalization of TNF-R55 is required for A-SMase activation (19). While the formation of TNF-R55-containing endosomes brings together TNF-R55 and A-SMase at the same subcellular compartment, TRADD and FADD will stay at the cytoplasmic face of the endosomal membrane, which does not allow any direct physical interaction between A-SMase and TRADD or FADD. Thus, TRADD and FADD may either activate a cytosolic enzyme that generates a lipid messenger able to cross the endosomal membrane. Alternatively, TRADD and FADD may simply serve to stabilize a TNF-R55 multimer to allow activation of A-SMase through the extracellular domains of TNF-R55 located in the endosomal lumen. Further studies will be necessary to address in detail the molecular mechanisms of FADD-mediated A-SMase activation.

It is well established that FADD can interact through a C-terminal death domain with DD-containing proteins like CD95, TRADD, and death receptor 3 (DR3). This interaction unmasks a N-terminal death effector domain of FADD resulting in the recruitment and activation of caspase 8 triggering an apoptotic caspase cascade (20). Using FADD-deficient EF cells, Yeh et al. (14) demonstrated recently that FADD is essential for signaling TNF-R55-, CD95-, or DR3-induced apoptosis. Remarkably, FADD was also found to be essential for mouse embryo development in particular for the ventricular myocardium. The lethality of FADD-deficient mice contrasts with the phenotypically normal embryonic development and viability of CD95- and TNF-R55-deficient mice (21, 22). This led to the conclusion that FADD, besides its signaling function for death receptors, may also be used by other receptors regulating embryonic development. In addition, the early lethality of FADD-deficient embryos points to physiologic functions of FADD other than caspase 8 activation. In the present study we identify A-SMase as a further FADD-dependent enzyme, indicating that FADD, indeed, may couple to signaling systems distinct from caspase 8. Although A-SMase has been implicated in numerous cellular responses including apoptosis (for review, see Ref. 1), A-SMase can be viewed an “orphan” enzyme, whose precise role in signaling transduction has become elusive. Notably, A-SMase-deficient mice do not show early embryonic lethality (23, 24). Thus the effects of FADD deficiency on embryogenesis cannot be explained by a lack of A-SMase activation. Further investigations are needed to clarify the role of FADD and A-SMase in nonapoptotic signaling.

**Fig. 3.** IL-1-induced activation of A-SMase in FADD–/– EF cells. EF cells were stimulated with 150 pg/ml IL-1. At indicated times, cellular lysates were prepared and assayed for A-SMase activity.

**Fig. 4.** Overexpression of FADD restores TNF responsiveness of A-SMase in FADD–/– EF cells. A, FADD–/– EF cells were left untreated or transfected with either control vector or 3 μg of expression construct for FADD. Cells were either left untreated or stimulated with 100 ng/ml TNF or 150 pg/ml IL-1, respectively, for 3 min (solid bars) or 4 min (hatched bars). Cellular lysates were prepared and assayed for A-SMase activity. B, FADD–/– EF cells were left untreated or transfected with the indicated amounts of FADD expression construct. EF cells were stimulated with 100 ng/ml TNF for 3 min and analyzed for A-SMase activity.
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