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Apoptotic Signaling through CD95 (Fas/Apo-1) Activates an Acidic Sphingomyelinase

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Summary

Intracellular pathways leading from membrane receptor engagement to apoptotic cell death are still poorly characterized. We investigated the intracellular signaling generated after cross-linking of CD95 (Fas/Apo-1 antigen), a broadly expressed cell surface receptor whose engagement results in triggering of cellular apoptotic programs. DX2, a new functional anti-CD95 monoclonal antibody was produced by immunizing mice with human CD95-transfected L cells. Crosslinking of CD95 with DX2 resulted in the activation of a sphingomyelinase (SMase) in promyelocytic U937 cells, as well as in other human tumor cell lines and in CD95-transfected murine cells, as demonstrated by induction of in vivo sphingomyelin (SM) hydrolysis and generation of ceramide. Direct in vitro measurement of enzymatic activity within CD95-stimulated U937 cell extracts, using labeled SM vesicles as substrates, showed strong SMase activity, which required pH 5.0 for optimal substrate hydrolysis. Finally, all CD95-sensitive cell lines tested could be induced to undergo apoptosis after exposure to cell-permeant C_2-ceramide. These data indicate that CD95 cross-linking induces SM breakdown and ceramide production through an acidic SMase, thus providing the first information regarding early signal generation from CD95, and may be relevant in defining the biochemical nature of intracellular messengers leading to apoptotic cell death.

The CD95 (Fas/Apo-1 antigen) is an ~45-kD single transmembrane receptor expressed on a variety of normal and neoplastic cells (1, 2). It has been suggested that CD95 may play a fundamental role in regulation of tissue development and homeostasis, since its molecular cross-linking results in apoptotic cell death (3, 4). In lymphoid cells, CD95 is preferentially induced on the cell surface after cellular activation (5) and its expression might be crucial to allow clonal selection by cell deletion in the thymus, as well as suicide of autoreactive clones in the periphery. This is strongly suggested by the evidence that the autoimmune disease prone *lpr* mouse carries a mutation in the murine CD95 gene (6). Primary sequence analysis of the extracellular portion of CD95 has revealed strong homologies with the extracellular domain of receptors belonging to the TNF receptor family, which includes TNF receptor types 1 and 2 (TNFR1/2), the low affinity nerve growth factor receptor, and lymphocyte receptors such as CD27, CD30, CD40, and OX40 (1, 2). An integral membrane protein, with strong homology to TNF-α and -β, has been recently identified as a Fas ligand (7). Moreover, a moderate degree of homology (26% identity in a stretch

of 65 amino acids [aa]) between the intracellular portion of the human CD95 and the 55-kD TNFR1, has been observed. Mutational analysis of this domain has revealed its involvement in the generation of the apoptotic signal from both CD95 and TNFR1 (8, 9). These data suggested that common effectors may transduce the apoptotic signal from both receptors.

Whereas no information is available about the biochemical nature of the signals generated via CD95, signaling through TNFR1 has been extensively characterized. Its 221-aa intracellular portion has been shown to be functionally coupled with different phospholipases, including phospholipase A2 (PLA2)[1] (10), phosphatidylcholine-specific phospholipase C (PC-PLC) (11), and sphingomyelinase (SMase) (12, 14). A specific role for the SMase pathway, and in particular for ceramide, produced from sphingomyelin (SM) hydrolysis, in

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[1] *Abbreviations used in this paper:* PC-PLC, phosphatidylcholine-specific phospholipase C; PLA2, phospholipase A2; SM, sphingomyelin; SMase, sphingomyelinase.

the generation of the apoptotic signal has been recently suggested by the demonstration that synthetic cell–permeant ceramides can directly promote apoptosis (15), by inducing double-stranded DNA fragmentation (16). We therefore investigated whether SM hydrolysis and ceramide production could be induced by CD95. Our data indicate that cross-linking of the CD95 receptor triggers SM breakdown in U937 promyelocytic cells, as well as in other tumor cell lines, through an acidic SMase.

Materials and Methods

Generation of Anti-CD95 mAbs. Human Fas cDNA was generated by RT-PCR (17) from the Jurkat cell line and was subsequently subcloned into pBJ. Primers used to generate a full-length Fas cDNA were: sense, GGG CTC GAG ACA ACC ATG CTG GGC ATC TGG (including an XhoI cloning site); anti-sense, GGG GAT ATC TTC ACT CTA GAC CAA GCT TTG (containing an EcoRV cloning site). Murine L cells were cotransfected with 15 μg human Fas-pBJ plasmid using 100 μg lipofectin (GIBCO BRL, Gaithersburg, MD) and G418-resistant cells were selected, as described previously (18). Anti-CD95 hybridomas DX2 (IgG1) was generated by immunizing C3H/He mice with CD95 transfected L cells and fusing immune splenocytes with Sp2/0 myeloma cells.

Cell Lines. The human T cell lymphoma HUT78, human T cell leukemia Jurkat, and human promyelocytic leukemia U937 cell lines were grown in RPMI supplemented with 10% FCS, 1 mM glutamine, and antibiotics (complete medium). The murine lymphocytic leukemia L1210 cell line was transfected with human CD95 (Fas-pBJ plasmid) and G418 selected, as described previously (18). The resulting L1210-Fas cell line, stably expressing human CD95, was grown in complete medium.

DNA Labeling and Flow Cytometry Analysis. Cells at 5 × 10⁵/ml in complete medium were incubated in 24-well cell culture plates (Costar Corp., Cambridge, MA) coated with saturating amounts of DX2 antibody. In different experiments, cells were treated with 50 μM C₂-ceramide (N-acetyl-D-sphingosine; Sigma Chemical Co., St. Louis, MO) or C₂-dihydroceramide (N-acetyl-D-dihydrosphingosine; Biomol, Plymouth Meeting, PA). After different times of incubation, cells were recovered and washed in PBS and processed for apoptotic cell detection (19). Briefly, the cell pellet was gently resuspended in 1 ml hypotonic fluorochrome solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma Chemical Co.) in 12 × 75 polystyrene tubes and kept overnight at 4°C in the dark until flow cytometry analysis. The propidium iodide fluorescence emission of individual nuclei was filtered through a 585/42-nm band pass filter and measured on a logarithmic scale by a FACScan® cytometer (Becton Dickinson & Co., Mountain View, CA). Cell debris were excluded from analysis by appropriately gating on physical parameters. The number of apoptotic cells was determined by evaluating the percentage of hypodiploid nuclei (20).

SMase assay. Cells were labeled for 48 h with [N-methyl-¹⁴C]choline (1 μCi/ml, sp act 56.4 mCi/mmol) and then serum starved for 4 h in medium supplemented with 2% BSA (14). Aliquots of 10⁷ cells were suspended in 1 ml PBS and treated for the indicated times with control mAb or with DX2 mAb (1 μg/ml), cross-linked by goat anti–mouse Ig (1 μg/ml), or with recombinant TNF-α (Genzyme Corp., Cambridge, MA) (100 ng/ml). Stimulation was stopped by immersion of samples in methanol/dry ice (−70°C) for 10 s followed by centrifugation at 4°C in a microfuge. Cell pellets were resuspended in ice-cold CH₃OH/CHCl₃/H₂O

(2.5:1.25:1). Phospholipids were extracted, dried under nitrogen, resuspended in 200 μl chloroform, and applied to a Silica Gel TLC plate (Merck, Darmstadt, Germany), with an automatic applicator (Linomat IV; Camag, Muttenz, Switzerland). Samples containing equal amounts of radioactivity were loaded. The amount of labeled PC, which remained constant when labeled at equilibrium, was used as internal control to normalize for equal amounts of loaded material. Phospholipids were separated by TLC using a solvent system containing CHCl₃/CH₃OH/CH₃COOH/H₂O (100:60: 20:5). LysoPC, PC, and bovine brain SM (Sigma Chemical Co.) were used as standards and visualized in iodine vapor. The radioactive spots were visualized by autoradiography, scraped from the plate, and counted by liquid scintillation. SMase activity was expressed as pmoles of SM hydrolyzed/10⁶ cells.

For in vitro SMase assay, the cells were treated with DX2 mAb (1 μg/ml) cross-linked by goat anti–mouse Ig (1 μg/ml) or with TNF-α (100 ng/ml) at 37°C for the indicated times, washed, and then resuspended in Tris buffer, pH 7.4, or sodium acetate buffer, pH 5.0, containing 10 mM PMSF, 100 mM bacitracin, 1 mM benzamidine, 1 mM aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin, and 5 mg/ml soybean trypsin inhibitor (Sigma Chemical Co.). Cells were lysed by sonication with a cell sonifier (Vibracell, Sonic & Materials Inc., Danbury, CT). Protein concentrations were determined using a protein assay (Bio-Rad Laboratories, Richmond, CA). 100 μg of the whole cell lysate was added to 250 μl reaction buffer containing the substrate [N-methyl-¹⁴C]SM (0.2 μCi/ml, sp act, 56.6 mCi/mmol), and 50 mM Tris or 50 mM sodium acetate (pH 5.0), 150 mM NaCl, and 10 mM Ca²⁺ (pH 7.4), with or without 6 mM Mg²⁺. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 250 μl CHCl₃/CH₃OH/CH₃ COOH (4:2:1). Phospholipids were extracted, TLC was performed as described above and [¹⁴C]SM hydrolysis was quantitated by autoradiography and liquid scintillation. SMase activation was expressed as picomoles of SM hydrolyzed/10⁶ cells.

Ceramide Mass Measurement (Diacylglycerol Kinase Assay). After stimulation, lipids were extracted and then incubated with Escherichia coli diacylglycerol kinase (21). Ceramide phosphate was then isolated by TLC using CHCl₃/CH₃OH/CH₃COOH (65/15/5, vol/vol/vol) as solvent. Authentic ceramide-1-phosphate was identified by autoradiography at Rf 0.25. Quantitative results for ceramide production are expressed as pmoles of ceramide-1-phosphate/ 10⁶ cells.

Results

DX2 Is a New Functional Anti-CD95 mAb. A series of mAbs recognizing CD95 was produced by immunizing C3H/He mice with murine L cells transfected with the human Fas/Apo-1 gene (see Materials and Methods). DX2 mAb (IgG1) specifically reacts with murine L cells, murine L1210 leukemia cells, and murine P815 mastocytoma cells transfected with human Fas cDNA, but does not react with the untransfected parental cells, by FACS® analysis (data not shown). The ability of the DX2 mAb to deliver an apoptotic signal was investigated on L1210 cells stably transfected with human CD95 (L1210-Fas). Apoptosis induction was evaluated as decrease of cellular DNA content by propidium staining and FACS® analysis (19). Fig. 1 C shows that >70% of L1210-Fas cells were undergoing apoptosis within 12 h from the stimulation with DX2 mAb. Apoptotic cells within untransfected L1210 treated with DX2 mAb or within L1210-Fas cells treated
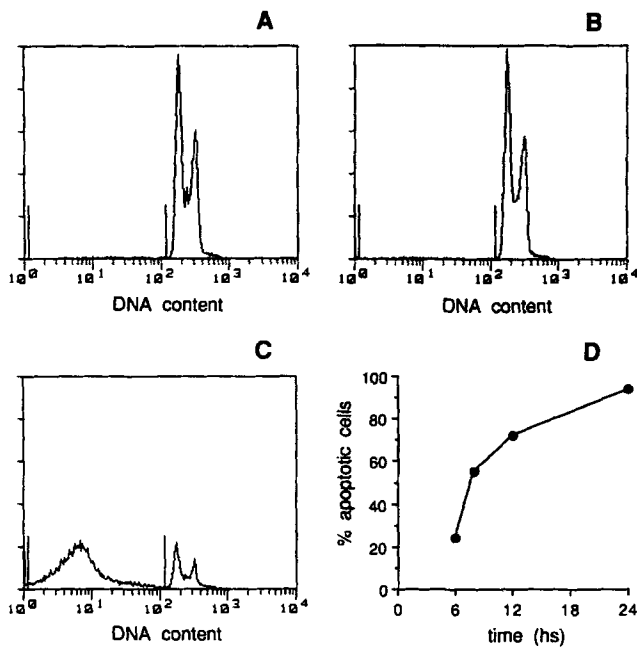
Figure 1. Apoptosis induction by DX2 mAb. L1210 cells incubated for 12 h on DX2-coated plates (A), L1210-Fas cells incubated for 12 h on Leu3a (anti-CD4)-coated (B), or DX2-coated (C) plates, were processed for DNA content analysis by propidium iodide staining. Nuclei were analyzed with a FAScan® cytofluorimeter and data plotted on log histograms as red fluorescence intensity (x axis) vs. relative cell number (y axis). Hypodiploid nuclei (between markers), are 1, 2, and 73% in A, B, and C, respectively. (D) Kinetic analysis of apoptosis induction in L1210-Fas cells after CD95 cross-linking by DX2 mAb. Cells treated as above described were collected at different time points and processed for DNA content analysis by propidium iodide staining. Data shown are from one representative out of several experiments performed.

different time points and analyzed by TLC. Fig. 2 A shows that cross-linking of CD95 in U937 cells resulted in significant hydrolysis of SM, which reached maximal levels by 5 min, and was completed within 30 min. Comparable peak levels of SM hydrolysis were observed by treating U937 cells for 5 min with 100 ng/ml TNF-α, used as positive control (Fig. 2 B). Significant levels of SM hydrolysis were also observed 5 min after CD95 cross-linking in HUT78 and Jurkat cell lines (Fig. 2 B), indicating that CD95-induced SM turnover was not restricted to U937 cells. Finally, remarkable SM hydrolysis was induced by anti-CD95 mAb DX2 in L1210-Fas cells (Fig. 2 B), further indicating that expression of CD95 is sufficient to enable functional coupling with a SMase.

SM hydrolysis was paralleled by generation of ceramide, as detected by TLC analysis of phospholipids extracted from DX2-stimulated U937 cells and subjected to diacylglycerol kinase assay (Fig. 3 A). Ceramide accumulation peaked at 10 min after CD95 cross-linking (Fig. 3 B). Sphingosine for-
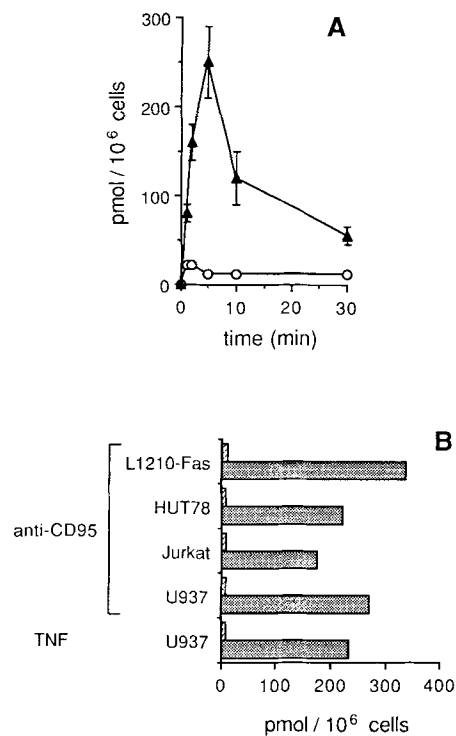
with control mAb for 12 h were <2% (Fig. 1, A and B). Kinetic analysis showed that a significant fraction of cells was undergoing apoptosis within 6 h from CD95 stimulation, and that almost all nuclei were hypodiploid by 24 h (Fig. 1 D). Very similar results were obtained with P815 cells transfected with human CD95 (data not shown). These data indicated that the DX2 mAb recognized a functional epitope of the CD95 antigen.

SM Breakdown is Induced by CD95 Cross-linking. Cross-linking of TNFR-1 by TNF-α in promyelocytic U937 cells results in SM hydrolysis and ceramide production (14). The activation of SM turnover has been suggested to play an important role in initiating the biochemical pathway leading to active cell death, since synthetic ceramide analog C_2-ceramide has been shown to be directly responsible for apoptosis induction in U937 cells (15). As U937 cells express CD95 and are susceptible to CD95-mediated apoptosis induction (3), we investigated whether cross-linking of CD95 could induce SM breakdown in U937 cells. U937 cells were labeled with [N-methyl-14C] choline for 48 h and stimulated with 1 μg/ml anti-CD95 mAb DX2, together with 1 μg/ml GaM to maximize cross-linking, since molecular cross-linking has been shown to be critical for CD95-mediated apoptosis induction (22). Cellular phospholipids were then extracted at

Figure 2. SM hydrolysis by anti-CD95 mAb. (A) U937 cells were 14C-choline labeled and stimulated for different times with control anti-CD45 mAb (open circles) or anti-CD95 mAb (closed triangles). Phospholipids were then extracted, separated by TLC, and visualized by autoradiography. Relevant spots were scraped from the plate and counted by liquid scintillation. Data are expressed as pmoles of SM hydrolyzed in stimulated samples (closed triangles) and unstimulated ones (open circles). Mean values and standard deviations at each time point, from four different experiments are shown. (B) Different cell lines were 14C-choline labeled and stimulated for 5 min with anti-CD95 mAb or TNF-α. Phospholipids were then extracted, separated by TLC, and visualized by autoradiography. Relevant spots were scraped from the plate and counted by liquid scintillation. Data are expressed as pmoles of SM hydrolyzed in stimulated samples (shaded bars) and unstimulated ones (hatched bars).
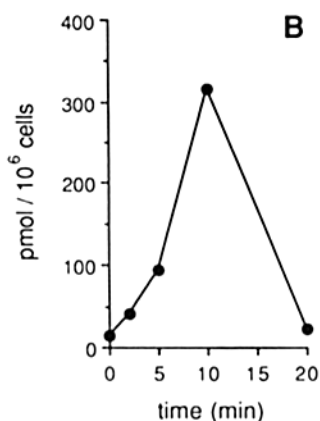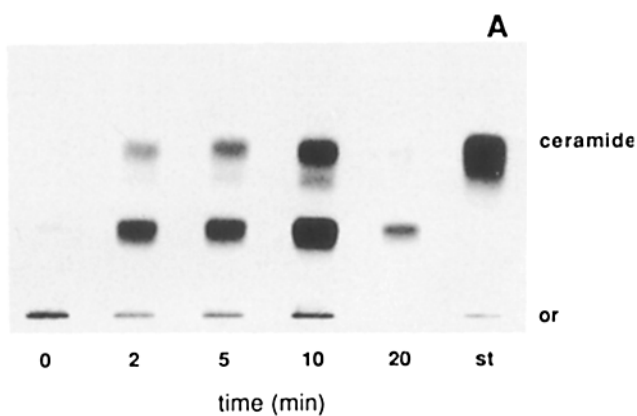
A

ceramide

or
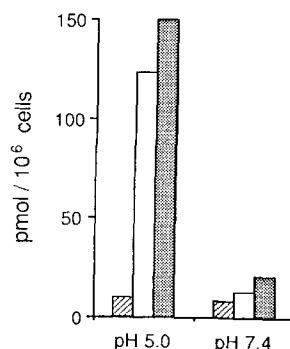
0   2   5   10   20   st

time (min)

B

Figure 4. pH dependency of CD95-dependent SMase. U937 cells were stimulated with anti-CD95 mAb or TNF-α, and cell lysates were then adjusted to the indicated pH. After reaction of cell lysates with labeled SM vesicles in the proper buffer, phospholipids were extracted, separated by TLC, and visualized by autoradiography. Relevant spots were scraped from the plate and counted by liquid scintillation. Data are expressed as pmoles of SM hydrolyzed in samples from U937 cells stimulated with TNF (*open columns*), anti-CD95 mAbs (*shaded columns*), or left untreated (*hatched columns*).

Figure 3. Ceramide accumulation after anti-CD95 stimulation. (*A*) U937 cells were stimulated with anti-CD95 mAb for different times, and lipids were then extracted and subjected to diacylglycerol kinase assay. Lipids were separated by TLC and radioactive spots visualized by autoradiography. (*st*) Ceramide authentic standard, (*or*) chromatographic origin. (*B*) Quantitative results for ceramide-1-phosphate accumulation, expressed as picomoles/10^6 cells. Two different experiments gave similar results.

mation was observed, (band migrating at lower Rf in TLC), possibly due to rapid in vitro ceramide catabolism by ceramidases. The above results suggested that cross-linking of CD95 could transiently activate a cellular SMase to hydrolyse SM and generate ceramide.

CD95 Cross-linking Activates an Acidic SMase. SMases represent a family of widely distributed type-C phospholipases. Although all SMases hydrolyse SM to generate ceramide and phosphorylcholine, they are heterogeneous in activation requirements and cellular localization (23). Neutral SMases operate preferentially outside or at the plasma membrane, with a pH optimum of 7.4, and require Mg^{2+}, whereas acidic SMase are localized intracellularly and in lysosomes, have a pH optimum of 5.0, are Mg^{2+}-independent and require 1,2-diacylglycerol for activation (24, 25). It was of interest to characterize the SMase species involved in CD95 signaling. CD95-activated SMase activity was therefore evaluated in vitro on purified substrate, under different pH conditions. U937 cells were stimulated for 5 min with 1 μg/ml anti-CD95 mAb DX2 and 1 μg/ml GaM, and cell extracts were then incubated
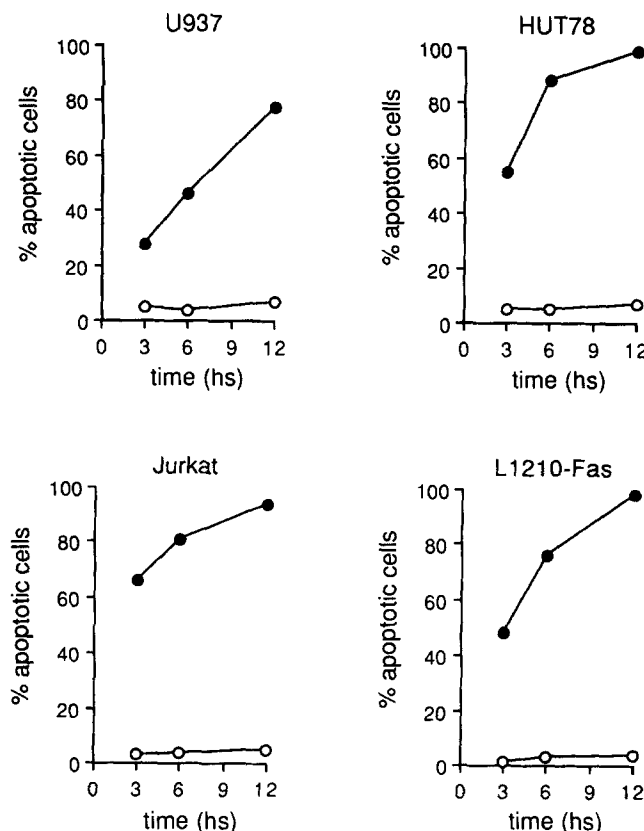

Figure 5. Sensitivity to ceramide. U937, HUT78, Jurkat, and L1210-Fas cell lines were treated with 50 μM C2-ceramide (*closed circles*) or 50 μM C2-dihydroceramide (*open circles*). At different time points, cells were analyzed for apoptosis induction by propidium iodide staining. Nuclei were analyzed with a FACScan® cytofluorimeter. Percent hypodiploid nuclei in each cell line is plotted versus time elapsed.

with labeled SM vesicles using pH 5.0 or 7.4 reaction buffers. As shown in Fig. 4, optimal SMase activity was detected at pH 5.0, whereas at pH 7.4 enzymatic activity was minimal. Addition of 6 mM Mg^{2+} to the reaction buffers did not result in any change in CD95-triggered SMase activity (data not shown). Cell extracts from TNF-α–stimulated U937 cells, used as positive control, also contained acidic SMase activity, as reported (14). These data indicated that CD95 cross-linking was activating an acidic SMase.

Ceramide Mediates Apoptosis in CD95-sensitive Cell Lines. To further investigate the role of ceramide in CD95-induced cell death, we tested whether cell lines which were shown to generate ceramide upon CD95 cross-linking, were in fact induced to undergo apoptosis by exogenous ceramide. U937 cells, as already described (15), but also Jurkat, HUT78, and CD95-transfected L1210 cells, rapidly underwent massive apoptosis upon exposure to cell-permeant synthetic C_2-ceramide. By contrast, cell-permeant structural analog C_2-dihydroceramide was totally ineffective (Fig. 5). These data strongly suggest that CD95-mediated ceramide generation is responsible for apoptosis induction in CD95-sensitive cells.

Concluding Remarks. Different cytokine receptors, including those for TNF-α, IFN-γ, and IL-1β, have been shown to trigger SM turnover, as part of their signaling capabilities, upon ligand binding (12, 13, 26). SM hydrolysis with ceramide production is emerging as a major receptor-operated cell activation pathway (27), highly conserved along evolution (28) and possibly implicated in multiple gene regula-

tory events, leading to as diverse outcomes as growth inhibition and cell differentiation (29, 30) or cellular proliferation (31). Ceramides, in fact, can activate at least two distinct Ser/Thr kinases (32), one of which was identified as the 42-kD mitogen-activated protein kinase (33), and a cytosolic Ser/Thr protein phosphatase 2A (termed ceramide-activated protein phosphatase or CAPP) (34). The effects of C_2-ceramide on apoptosis induction of U937 cells (15), and the identification of a "death domain" common to CD95 and TNFR-1 (8, 9), suggest the possibility that the SM pathway could mediate apoptotic signaling through CD95.

CD95-generated early signaling has remained elusive so far, as no early enzymatic activity or intracellular $[Ca^{2+}]_i$ elevations, after CD95 cross-linking, have been reported. The data presented here provide the first attempt to characterize the signaling pathway originated at the CD95 receptor. They demonstrate that cross-linking of CD95 activates an acidic SMase in U937 cells and suggested that released ceramide could be involved in mediating CD95-dependent apoptosis. Although ceramides are likely candidates as mediators of CD95-dependent apoptosis, and CD95 triggers SM breakdown in all CD95$^+$ cell lines tested, a marked heterogeneity in susceptibility to CD95-mediated apoptosis induction among the different cell lines or among freshly isolated cells has been observed (5 and our unpublished data). This suggests a complex and possibly cell-specific regulation of the CD95-dependent SMase pathway, which will require further investigation.

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