CD40 Signals Apoptosis through FAN-regulated Activation of the Sphingomyelin-Ceramide Pathway*

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Bruno Séguin§§, Nathalie Andrieu-Abadie§§, Sabine Adam-Klages§, Olivier Meilhac**, Dirk Kreder†, Virginie Garcia‡, Alain P. Bruno**, Jean-Pierre Jaffrézou**, Robert Salvayre‡, Martin Krönke‡‡, and Thierry Levade$$$§§

From the §INSERM U466, Laboratoire de Biochimie, Institut Louis Buguinard, Bâtiment L3, Centre Hospitalier Universitaire Rangueil, 1 Avenue Jean Pouhéès, F-31403 Toulouse Cedex 4, France, the **Institut für Immunologie, Christian-Albrechts Universität zu Kiel, 24105 Kiel, Germany, the ‡INSERM U9910, Institut Claudius Regaud, 31052 Toulouse, France, and the §§Institut für Medizinische Mikrobiologie und Hygiene Universität zu Köln, 50935 Cologne, Germany

The possibility that the sphingomyelin (SM)-ceramide pathway is activated by CD40, a transmembrane glycoprotein belonging to the tumor necrosis factor receptor superfamily and that plays a critical role in the regulation of immune responses has been investigated. We demonstrate that incubation of Epstein-Barr virus-transformed lymphoid cells with an anti-CD40 antibody acting as an agonist results in the stimulation of a neutral sphingomyelinase, hydrolysis of cellular SM, and concomitant ceramide generation. In addition, SM degradation was observed in acid sphingomyelinase-deficient cells, as well as after ligation by soluble CD40 ligand. The anti-CD40 antibody, as well as the soluble CD40 ligand induced a decrease in thymidine incorporation and morphological features of apoptosis, which were mimicked by cell-permeant or bacterial sphingomyelinase-produced ceramides. Stable expression of a dominant-negative form of the FAN protein (factor associated with neutral sphingomyelinase activation), which has been reported to mediate tumor necrosis factor-induced activation of neutral sphingomyelinase, significantly inhibited CD40 ligand-induced sphingomyelinase stimulation and apoptosis of transformed human fibroblasts. Transformed fibroblasts from FAN knockout mice were also protected from CD40-mediated cell death. Finally, anti-CD40 antibodies were able to co-immunoprecipitate FAN in control fibroblasts but not in cells expressing the dominant-negative form of FAN, indicating interaction between CD40 and FAN. Altogether, these results strongly suggest that CD40 ligation can activate via FAN a neutral sphingomyelinase-mediated ceramide pathway that is involved in the cell growth inhibitory effects of CD40.

CD40, a transmembrane glycoprotein mainly expressed on the surface of B cells and other cell types (reviewed in Refs. 1–3), is a member of the tumor necrosis factor (TNF) receptor superfamily, which includes the low affinity nerve growth factor (NGF) receptor and CD95/Fas (4, 5). Following interaction with its natural ligand, CD40L, a protein belonging to the TNF gene family (1–3), CD40 mediates a number of major immunoregulatory functions, including B cell homotypic adhesion, proliferation, immunoglobulin isotype switch, and secretion (1–3). Activation of CD40 has also been shown to inhibit the growth of certain B cell lymphomas (6–9) and to induce the death of transformed cells of mesenchymal or epithelial origin (10–12).

The signal transduction pathways triggered through CD40 have not yet been fully delineated. Early biochemical events include activation of Rac (13), of Lyn and Syk protein tyrosine kinases (14–16), phosphorylation and activation of phospholipase Cγ2 and phosphoinositide-3-kinase (15), and induction of mitogen-activated protein kinases (17–19). Activation processes seem to be initiated by association of the CD40 receptor with signaling molecules of the TRAF family (see Ref. 1).

During the last years, a crucial function of sphingomyelin (SM) ceramide phosphocholine), a major structural constituent of mammalian cell membranes, has been documented in the signal transduction of cytokines, neurotransphins, antibodies, and various stress agents (see Refs. 20–25 for reviews). In this pathway of cell regulation, SM is hydrolyzed by a sphingomyelinase to ceramide, which in turn serves as a second messenger mediating certain effects of the above extracellular agonists and in particular the cell growth inhibitory effects. The intracellular targets of ceramide include a proline-directed protein kinase, a protein phosphatase, the mitogen-activated protein kinase cascade, and transcription factors (20–25). Both acidic and neutral sphingomyelinas have been described to be responsible for the generation of bioactive ceramide. For instance, TNFα has been reported to activate an acid sphingomyelinase through proteins that bind the 55-kDa receptor death domain and a neutral sphingomyelinase through the adapter protein FAN (factor associated with neutral sphingomyelinase activation) that specifically binds to a distinct cytoplasmic domain of the TNF receptor called the neutral sphingomyelinase activation domain (24, 26, 27). Although activation of the former enzyme by TNFα has been associated with apoptotic signaling, stimulation of the latter via FAN has been linked to noncytotoxic effects of TNF (24).

Interestingly, the SM-ceramide signal transduction pathway has been shown to be activated by triggering of three members of the TNF receptor superfamily, i.e. the TNF receptor 1 (28–29), TNFR, TNF receptor; TRAF, TNF receptor-associated factor; mAb, monoclonal antibody.

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31), the low affinity NGF receptor (32), and CD95/Fas (33–35). Because CD40 belongs to this family, sharing some homology both in the extracellular and cytoplasmic regions with the above-mentioned receptors, because of the recruitment of common TRAF molecules, and because of the activation of intracellular targets also described to be stimulated by ceramide, we examined whether CD40 is able to activate the SM-ceramide pathway.

The present study was undertaken to investigate the effects of CD40 engagement on SM and ceramide levels in EBV-transformed lymphoid B cells or in transformed fibroblasts. Evidence is presented for the first time that in these cells triggering of CD40 results in early neutral sphingomyelinase activation, SM hydrolysis, and ceramide generation and that these events, which precede apoptosis, are regulated, at least partially, by interaction with the FAN adapter protein.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Monoclonal mouse anti-human CD40 (IgG1, clone M3) and anti-human CD40L were purchased from Genzyme (Cergy, France), rabbit anti-human CD40 (clone C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA), and murine anti-CD40 antibodies (Cergy, France), rabbit anti-human CD40 (clone C-20) was from Santa Cruz, and anti-human α-actin was from Sigma. Soluble trimeric human CD40L/leucine zipper fusion protein was a gift of ImmuneX (Seattle, WA). [methyl-3H]Choline chloride (81 Ci/mmol), [methyl-3H]thymidine (6.7 Ci/mmol), and [choline-methyl-3H]SM (54 Ci/mmol) were obtained from NEN Life Science Products, and [γ-32P]ATP (3500 Ci/mmol) from ICN (Orsay, France). Sphingomyelinase (Bacillus cereus), bovine brain type III ceramide, N-acetylphosphosine (C2-ceramide), N-hexanoylphosphosine (C8-ceramide), and ExtrAvidin-peroxidase were supplied from Sigma; phospholipase C (B. cereus, grade I), bovin-16-DTUP and terminal transferase were from Roche Molecular Biochemicals, 1,4-Acetyl-1-Phe-nyl-2-decanoylamin-5-morpholin-1-propanol was from Alexis-Cogex (Paris, France). All solvents and other reagents obtained from Merck or SDS (Pepin, France) were of analytical grade.

Cell Lines and Transfections—Long-term lymphoid cell lines were established by EBV transformation of human peripheral blood B lymphocytes as reported (36). Line TRE was derived from a patient affected with Niemann-Pick disease Type B. More than 90% of the lymphoid cells were positive for CD40 as determined by flow cytometry. Cells were routinely grown in RPMI 1640 medium supplemented with Glutamax (2 mmol/liter), penicillin (100 units/ml), and heat-inactivated fetal calf serum (10%). Cells were grown in a humidified 5% CO2 atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium containing 10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, and 1.9 mM CaCl2. These were then incubated at 4 °C for 15 min in the presence of 1 μg/ml of annexin V, 1 μg/ml of propidium iodide, and 2 μg/ml of Syto-13. The percentage of apoptotic cells (having a condensed and fragmented nucleus), including postapoptotic cells (characterized by nuclear fragmentation, cell condensation, and propidium iodide staining), was evaluated by counting cells (100 cells in at least three different fields for each well) under a Leica fluorescence-equipped inverted microscope.

Fibroblasts viability was estimated directly on six-well culture plates (3/4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or by incorporation with propidium iodd (4 μM) and SYTO-13 (1 μM) (Molecular Probes, Leiden, The Netherlands). The percentage of apoptotic cells (having a condensed and fragmented nucleus), including postapoptotic cells (characterized by nuclear fragmentation, cell condensation, and propidium iodide staining), was evaluated by counting cells (100 cells in at least three different fields for each well) under a Leica fluorescence-equipped inverted microscope.

To measure annexin V binding, cells (5 × 10^6/ml) were sedimented, washed with phosphate-buffered saline, and resuspended in a buffer containing 10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM KCl, and 1 mM MgCl2. These were then incubated at 4 °C for 15 min in the presence of 1 μg/ml of annexin V, 1 μg/ml of propidium iodide, and 2 μg/ml of SYTO-13.

Western Blotting and Immunoprecipitation Analyses—Cells were lysed in a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl2, and 1 mM CaCl2. The cellular lysates were assayed as described previously (41, 42) using [choline-methyl-3H]SM (100,000 dpm/assay) as substrate.

Cell Proliferation, Toxicity, and Morphological Studies—Lymphoid cell lines suspended in RPMI medium containing 10% fetal calf serum and distributed in 24-well flat-bottomed plates (250,000 cells/well) were tested for viability directly on six-well culture plates (3/4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay or by incorporation with propidium iodd (4 μM) and SYTO-13 (1 μM) (Molecular Probes, Leiden, The Netherlands). The percentage of apoptotic cells (having a condensed and fragmented nucleus), including postapoptotic cells (characterized by nuclear fragmentation, cell condensation, and propidium iodide staining), was evaluated by counting cells (100 cells in at least three different fields for each well) under a Leica fluorescence-equipped inverted microscope.

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**FIG. 1. Activation of the SM-ceramide pathway by CD40 ligation in EBV-transformed lymphoid cells.** A and B, SM hydrolysis. Lymphoid cells (LCL) were labeled for 48 h with [3H]choline. After a 2-h chase, cells (5 × 10^6/ml) were incubated at 37 °C in the presence of 5 μg/ml of anti-CD40 mAb (αCD40) or control murine IgG (IgG1 m) (A), or 3 μg/ml of soluble CD40L (sCD40L) (B). At the indicated time points, incubations were stopped, and SM levels were determined as described under “Experimental Procedures.” The SM levels are expressed as percentage of the value observed before treatment. The values correspond to the mean ± S.E. of five independent experiments. In untreated cells, cell-associated SM radioactivity averaged 70,000 dpm/mg of protein. C, stimulation of neutral sphingomyelinase activity. Lymphoid cells (10 × 10^6/ml) were incubated at 37 °C in the presence or absence of 5 μg/ml of anti-CD40 mAb (αCD40). At the indicated time points, incubations were stopped, and acid (A SMase) and neutral (N SMase) sphingomyelinase activities were determined. The values correspond to the mean ± S.E. of at least three independent experiments. D, ceramide generation. Lymphoid cells (LCL) (10 × 10^6/ml) were incubated at 37 °C in the presence of 5 μg/ml of anti-CD40 (αCD40) or control murine IgG (IgG1 m). At the indicated time points, incubations were stopped, and ceramide levels were determined using the diacylglycerol kinase assay. The values correspond to the mean ± S.E. of four independent experiments.

**RESULTS**

Activation of SM Hydrolysis by CD40 Ligation in Lymphoid Cell Lines—Engagement of CD40 on EBV-transformed lymphoid cells was studied by using either a monoclonal anti-human CD40 antibody (acting as an agonist) or a soluble recombinant CD40L. As shown in Fig. 1, A and B, CD40 ligation on [3H]choline-labeled lymphoid cells (but not an irrelevant IgG) induced a time-dependent SM hydrolysis, which was detectable after 5 min and represented about 20% of total cellular SM. The SM levels then returned to baseline values by 90–120 min. Similar results were obtained when the SM levels were monitored by a mass quantitative assay (see Fig. 4) or after metabolic labeling with 32P-orthophosphate (data not shown).

The degradation of SM in the SM signaling pathway has been shown to be mediated by the stimulation of a sphingomyelinase, which hydrolyzes SM to ceramide and phosphocholine (20–25). However, different sphingomyelinases have been reported to be activated in this signal transduction pathway, we characterized the sphingomyelinase involved in CD40 signaling. Fig. 1C shows that CD40 stimulated the activity of a neutral sphingomyelinase. This activation was detectable as early as 2 min and peaked at about 30 min. There was no detectable activation of an acidic sphingomyelinase (Fig. 1C).

Activation of SM breakdown in the SM signaling pathway should be accompanied by an increase in cellular ceramide levels. As illustrated in Fig. 1D, CD40 triggering by anti-CD40 induced a significant elevation of ceramide levels. This increase was apparent by 5 min and maximal at 30–60 min, i.e. concomitantly to the peak of SM hydrolysis (see Fig. 1A).

CD40 Ligation and Ceramide Induce Inhibition of EBV-transformed Lymphoid Cell Growth—Previous studies have demonstrated that CD40 stimulation inhibits either in vitro or in vivo the growth of human or murine B-cell lymphoma (6–9) or myeloma (12). It was of interest to examine the effect of CD40 ligation on the EBV-transformed lymphoid cells we used. As shown in Fig. 2, addition of the anti-CD40 antibody to lymphoid cells (but not an irrelevant IgG) resulted in growth inhibition (20% growth inhibitory effect after 72 h incubation), as determined either by [3H]thymidine incorporation (Fig. 2A and D) or cell counting (Fig. 2C). Very similar results were obtained using 1–5 μg/ml soluble CD40L (Fig. 2A).

Treatment of cells with cell-permeant analogs of ceramide or indirect manipulation of endogenous ceramide levels have been shown to mimic the biological effects of inducers of the SM pathway (20). To determine whether the SM-ceramide pathway could mediate the observed growth inhibitory effect of CD40,
we tested the effects of exogenous or endogenous ceramide. Treatment of lymphoid cells with exogenous sphingomyelinase (which hydrolyzed at least 20% of SM; Fig. 2, B and E), with low concentrations of cell-permeant C₂-ceramide (data not shown) or C₆-ceramide (Fig. 2, C and F), or even with bovine brain ceramide (Fig. 2B) resulted in about 20–40% decrease in [³H]thymidine incorporation, with kinetics close to that observed with anti-CD40. A similar effect was also obtained with D,L-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (Fig. 2B), which inhibits the utilization of ceramide for glucosylceramide biosynthesis (45). Of note, incubation of the cells with bacterial phospholipase C, which degraded 20–30% cellular phosphatidylcholine to diacylglycerol, and which, as sphingomyelinase, liberates phosphocholine, was devoid of any growth inhibitory effect (Fig. 2B).

**CD40 Ligation and Ceramide Induce Apoptotic Features in EBV-transformed Lymphoid Cells**—CD40 has been reported to induce apoptotic cell death in transformed or myeloma cells (11, 12, 46). As previously observed on carcinoma cells (10), flow cytometric analysis of DNA content in EBV-transformed lymphoid cells showed that anti-CD40- or ceramide-induced growth inhibition did not involve growth arrest at specific points in the cell cycle, nor was any increase in the number of Trypan blue-stained cells after treatment with anti-CD40. However, morphological studies revealed that lymphoid cells exhibited chromatin condensation and membrane blebbing (Fig. 3, B and H) after exposure to anti-CD40 as well as cell-permeant ceramide (Fig. 3, C and I). These condensed cells proved to be labeled by the in situ terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining procedure (Fig. 3, E and F), strongly suggesting DNA fragmentation. In addition, annexin V binding studies indicated that anti-CD40 induced phosphatidylserine externalization (Fig. 2G), a hallmark of apoptosis. Quantification of these phenomena showed that CD40 ligation was accompanied by about 40% annexin V-positive cells; a similar proportion was found by counting apoptotic cells after May-Grünwald-Giemsa staining (Fig. 2H).

**CD40 Induces Activation of the SM Pathway and Apoptosis in Niemann-Pick Cells**—To further characterize the sphingomyelinase activated in response to CD40 ligation, we examined the response of cells derived from patients affected with Niemann-Pick disease Type A, i.e. cells genetically deficient in acid sphingomyelinase activity. Both EBV-transformed lymphoid cells and SV40-transformed skin fibroblasts from Niemann-Pick patients were tested. The latter cell type was also employed because it responds to CD40 ligation by undergoing apoptotic cell death (11). As shown in Fig. 4A, CD40 ligation induced SM hydrolysis in acid sphingomyelinase-deficient lymphoid cells. Indeed, both in control and mutant cells (which
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**Fig. 4. Activation of SM hydrolysis and growth inhibition by CD40 ligation in Niemann-Pick cells.** Lymphoid cells (5 × 10^6/ml) from a control subject or a patient affected with Niemann-Pick disease (NPD) were incubated in the absence or presence of 5 μg/ml of anti-CD40 mAb (aCD40) or isotype-matched murine (IgG1 m) antibody (A and B). A, intracellular SM levels were determined after 30 min incubation by mass assay (mean ± S.E. of three independent experiments). B, [3H]thymidine incorporation after 72 h of incubation. Results are expressed as percentage of the values in untreated cells; the data correspond to the mean ± S.E. of at least three independent experiments.

**Expression of a Mutant FAN Affects CD40-induced Apoptosis—**Because CD40 ligation seemed to activate a neutral sphingomyelinase, and because TNF-induced stimulation of neutral sphingomyelinase has been reported to be mediated by FAN (27), we investigated whether FAN could signal the apoptotic effects of CD40. To test this hypothesis, SV40-transformed fibroblasts were transfected with an empty vector or with a vector encoding the C-terminal part of FAN to stably express a truncated FAN protein which exhibits a dominant-negative effect on neutral sphingomyelinase (27). Expression of the truncated FAN (Fig. 5A) resulted in the abrogation of CD40L-triggered neutral sphingomyelinase activation (Fig. 5B) and subsequent SM hydrolysis (Fig. 5C). Importantly, stable expression of the mutated FAN led to considerable inhibition of the pro-apoptotic effect of CD40 ligation (Fig. 6). Similar results were observed when transformed fibroblasts were treated with soluble CD40L in either the presence of a protein synthesis inhibitor (Fig. 6, A and B) or in serum-free medium (Fig. 6, C–E). Of note, both mock-transfected and deleted FAN-transfected cells were sensitive to the cytotoxic action of exogenous cell-permeant ceramide (Fig. 6, A and D), suggesting that the signaling events downstream ceramide were unaffected in the truncated FAN-transfectants.

To further document the role of FAN on CD40-triggered cytotoxic signals, we tested the response of transformed fibroblasts derived from mice lacking a functional FAN protein that have been generated by targeted gene disruption (38). As shown in Fig. 7, FAN-deficient murine fibroblasts exhibited little sensitivity to an agonistic anti-murine CD40 antibody (47), whereas a dose-dependent cytotoxic effect was observed in wild-type cells.

**Interaction of CD40 with FAN—**Because the above observations suggested the implication of the FAN protein in CD40 cytotoxic signaling, we investigated whether CD40 receptor, similarly to TNFR1 (27), could physically interact with FAN in intact cells. In lysates from human lymphoid cells that were immunoprecipitated with an anti-CD40, Western blotting using an anti-FAN antibody detected a protein of about 120 kDa that was present in total extracts from these cells (Fig. 8A). The same protein was found in lysates from human fibroblasts and was overexpressed in cells stably transfected with the full-length FAN (Fig. 8A). Most notably, this protein was detected in immunoprecipitates with two different anti-CD40 antibodies (Fig. 8, C and D) and an anti-TNFR1 antibody (Fig. 8C) only in empty vector-transfected cells but not in cells expressing the dominant-negative form of FAN (Fig. 8C) or in immunoprecipitates with a control antibody (Fig. 8D).

**DISCUSSION**

Sphingolipid second messengers are emerging as key signaling components in a variety of cellular systems, including the regulation of the immune response (20-25). Different members of the TNF receptor superfamily, including TNFR1 (p55), low-affinity NGF receptor, and CD95 (Fas), have been described to trigger the SM-ceramide pathway (20-25). This event is believed, through the generation of ceramide or its metabolites, to mediate a number of biological responses of these receptors.

CD40L-CD40 is a ligand-receptor pair belonging to the family that includes TNF and its receptor, FasL-Fas, NGF-NGF receptor, and others (4). Although there is no strong homology (but still some (11)) between the cytoplasmic domains of CD40 and other members of the same family, CD40 shares with TNF and Fas receptors identical TRAF signaling molecules (1). This prompted us to investigate whether or not CD40 could signal through activation of SM hydrolysis. The present study indeed establishes for the first time that CD40 ligation triggers the SM-ceramide pathway.

Using two different means for CD40 ligation and two different transformed cell types, we demonstrate that CD40 activates SM turnover, with characteristics very close to those seen

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2 Human fibroblasts stably transfected with a plasmid carrying the full-length FAN and control fibroblasts showed a comparable sensitivity to CD40L (data not shown).
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with TNFα (28, 29, 31), NGF (32), CD95/Fas (33), or other agonists (48). In good agreement with our data, a transient increase in intracellular ceramide levels has also been reported recently in CD40L-stimulated human dendritic cells (49).

Different sphingomyelinases have been reported to be activated in response to extracellular stimuli (20–25). These enzymes include an acid, endosomal/lysosomal sphingomyelinase (29, 33), a neutral, Mg2+-dependent, membrane bound sphingomyelinase (27, 33, 41), and a neutral, Mg2+-independent, cytosolic enzyme (50). It appears that, conversely to the TNF (41) and Fas (33) systems, in which both neutral and acid sphingomyelinases can be activated, CD40 triggered SM turnover through activation of a neutral sphingomyelinase. In addition, our observation that CD40 is able to promote SM hydrolysis and growth inhibition and cell death in Niemann-Pick diseased cells, makes unlikely the hypothesis of the involvement of an acid enzyme.

These results suggest that CD40 activates a neutral sphingomyelinase that might be identical to that stimulated by TNF in fibroblasts (31) or by vitamin D3 in HL-60 cells (50). The products of SM hydrolysis are phosphocholine and ceramide. Whereas the latter has been described to mediate a number of cellular responses (20–25), the former has recently...
been shown to be involved in mitogenesis through an extracellular site (51, 52) but not apoptosis. In addition, the participation of phosphocholine to CD40-mediated growth inhibition, at least in our experimental cell system, is unlikely because the generation of phosphocholine by bacterial phospholipase C had no effect. Numerous studies have emphasized the role of the SM-ceramide pathway in growth regulation (20–25). Ceramide appears as a likely candidate for mediating the growth inhibitory effects of CD40 on EBV-transformed cells, and perhaps on other cell types. Indeed, not only intracellular ceramide was found to accumulate early after CD40 ligation but also manipulation of the intracellular ceramide levels by addition of exogenous ceramides, bacterial sphingomyelinase, or inhibitors of ceramide utilization could recapitulate the morphological alterations, DNA fragmentation, and decrease of [3H]thymidine incorporation induced by CD40.

Further evidence that the SM-ceramide pathway plays an important role in CD40 apoptotic signals is provided by the observation that stable expression of a truncated version of the FAN protein (amino acids 703–917) led to inhibition of both neutral sphingomyelinase activation and induction of cell death. This N-terminal deleted FAN has been described by Kronke and co-workers (27) to behave as a dominant-negative mutant when transiently expressed in COS cells. Indeed, this mutant abrogated the stimulation of neutral sphingomyelinase upon treatment with TNFα. Moreover, cells derived from the recently generated FAN-deficient mice (38) proved to be resistant to the cytotoxic effect of CD40 ligation. Thus, our findings indicate that the protein FAN plays a pivotal role, not only in TNF signaling but also in CD40-triggered pathways, at least those that lead to growth arrest or apoptosis.6 It is therefore tempting to speculate that, very much like the p55 TNF receptor, CD40 interacts with FAN. Consistent with this is the observation that in intact human cells anti-CD40 antibodies were able to comnunoprecipitate FAN. Binding of FAN to the TNF receptor requires the presence of a so-called neutral sphingomyelinase activation domain, having the amino acid sequence QKWEASLK (26, 27). Intriguingly, the cytoplasmic domain of CD40 contains a short sequence, i.e. QETLH, sharing some homology with the EDSAH of the neutral sphingomyelinase activation domain of p55 TNF receptor. Whether this sequence is involved in binding of FAN remains to be established.

In summary, this study provides evidence for (i) the activation by CD40 of the so-called SM signal transduction pathway, (ii) the potential role of ceramide (or its metabolites) in the regulation of B cell and transformed cell growth, and (iii) the modulation by FAN of CD40-induced production of ceramide and cell death. Hence, this work not only extends the functions of FAN to another member of the TNF receptor superfamily but is also the first description of a biological effect mediated by FAN, i.e. the regulation of apoptosis. These findings open new avenues of research as to the nature of the signaling molecules (such as TRAF proteins) involved in CD40 activation of sphingomyelinase. Moreover, pharmacological inhibition of the SM-ceramide pathway initiated by CD40-CD40L interactions might help elucidating the role of this receptor-ligand pair in the regulation of immune and inflammatory processes.

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The possibility that CD40 ligation triggers SM turnover, ceramide generation, and subsequent apoptosis through the secretion of TNFα was examined. Several lines of evidence argue against this hypothesis: (i) no TNFα was detected in the culture medium of CD40L-treated cells; (ii) TNFα and CD40L activated SM breakdown with similar kinetics; (iii) coinubcation of cells with CD40L and a blocking anti-p55 TNF receptor antibody (1 µg/ml; clone H388) did not affect the apoptotic response; and (iv) fibroblasts expressing deleted FAN were not completely resistant to TNF-induced cell death. (B. Ségui and T. Levade, unpublished data.) The possibility of a CD40-induced synthesis of TNFα (46) was also excluded because cell death was observed even in the presence of cycloheximide.

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