Sphingosine Kinase Interacts with TRAF2 and Dissects Tumor Necrosis Factor-α Signaling*

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Tumor necrosis factor-α (TNF) receptor-associated factor 2 (TRAF2) is one of the major mediators of TNF receptor superfamily transducing TNF signaling to various functional targets, including activation of NF-κB, JNK, and antiapoptosis. We investigated how TRAF2 mediates differentially the distinct downstream signals. We now report a novel mechanism of TRAF2-mediated signal transduction revealed by an association of TRAF2 with sphingosine kinase (SphK), a lipid kinase that is responsible for the production of sphingosine 1-phosphate. We identified a TRAF2-binding motif of SphK that mediated the interaction between TRAF2 and SphK resulting in the activation of the enzyme, which in turn is required for TRAF2-mediated activation of NF-κB but not JNK. In addition, by using a kinase inactive dominant-negative SphK and a mutant SphK that lacks TRAF2-binding motif we show that the interaction of TRAF2 with SphK is required for the subsequent activation of SphK. These findings show a role for SphK in the signal transduction by TRAF2 specifically leading to activation of NF-κB and antiapoptosis.

Tumor necrosis factor-α (TNF)1 is a pleiotropic cytokine that elicits a wide spectrum of physiologic and pathogenic events such as cell activation, proliferation, cell death, and inflammation. The different cellular responses to TNF are signaled through cell surface receptors (p55, TNFR1 and p75, TNFR2), and their adaptor proteins, initiating different signaling pathways. These distinct signals can lead to opposing cellular effects as best exemplified by TNF’s proapoptotic and antiapoptotic role (1). TNF-induced apoptosis primarily depends on the recruitment of a complex of adaptor proteins, including TRADD and FADD/MORT1 leading to the further recruitment and activation of various caspases and, subsequently, to programmed cell death (2, 3). On the other hand, the cell activation, inflammatory reaction, and antiapoptotic function of the TNF receptor superfamily are predominantly mediated by another class of adaptor proteins, TNF receptor-associated factors (TRAF) (1, 4, 5). To date, six members of TRAF proteins have been identified in mammals from TRAF1 to TRAF6. TRAF2 is the prototypical member of TRAF family. It can interact directly or indirectly with various members of TNF receptor superfamily to mediate the signal transduction of these receptors. TRAF2 can also interact with numerous intracellular proteins, such as I-TRAF/TANK, RIP, MAPK kinase kinase, NIK, and the caspase inhibitors cIAPs, and thereby transduces signals required for the activation of the transcription factor NF-κB, the stress-activated protein kinase (SAPK or JNK) and antiapoptosis (6–9). While structural studies have revealed the complexity and flexibility of TRAF2 (10) as a signal junction transducing various signal pathways, it is still not clear how TRAF2 can differentially activate its distinct downstream signals such as NF-κB and JNK, leading to different biological functions.

Sphingolipids have recently emerged as signaling molecules that mediate various activities of TNF (11, 12). TNF signaling via sphingolipids is exemplified by two distinct pathways: the formation of ceramide resulting from the activation of sphingomyelinase or de novo synthesis and the production of sphingosine 1-phosphate (S1P) upon sphingosine kinase (SphK) activation. While ceramide has been variably implicated as a mediator of TNF-induced apoptosis (13), S1P has been emerged as an antiapoptotic and mitogenic factor (14–16). We have recently reported that TNF activated SphK independently of its activation of sphingomyelinase activity and that the resulting production of S1P is a potent antagonist of TNF-induced apoptosis (17). Thus we investigated whether SphK could mediate a subset of TRAF2 signaling in response to TNF stimulation. We further demonstrated a physical and functional interaction between TRAF2 and SphK that specifically transduces TNF signal to activation of NF-κB and antiapoptosis.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, Mutagenesis, and Transfections—HEK 293T were obtained from the American Type Culture Collection and maintained in Dulbecco’s modified Eagle’s medium (Invitrogen), supplemented with 10% fetal calf serum. Human umbilical vein cells (HUVEC) were isolated and maintained as described previously (18). Human SphK1 (SphK) cDNA (GenBank accession number AF200328) was FLAG epitope-tagged at the 3′ end and subcloned into pcDNA3 vector (Invitrogen) as described previously (19). For generation of SphK mutants, the FLAG-tagged SphK was cloned into pALTER (Promega) site-directed
interaction of sphingosine kinase and TRAF2

mutagenic vector. Single-stranded DNA was prepared and used as a template for oligonucleotide-directed mutagenesis as detailed in the manufacturer’s protocol. The mutagenic oligonucleotides (5′-TGCACTGCGGGCCAGTGGC-3′ and 5′-CACCACCAAGGGCCGCCCT-TAGA-3′) were designed to generate the TB1-SphK and TB2-SphK mutants, respectively, and in combination for TB1/2-SphK. The mutants were sequenced to verify incorporation of the desired modifications and then subcloned into pcDNA3 vector. Generation of SphK<sup>cat210</sup> was described previously (20). Expression plasmids of pRK5-TRAF2-FLAG and pRK5-TRAF2<sup>ΔA-T501</sup>-FLAG were gifts from Dr. V. Dixit (Genentech Inc., South San Francisco). LipoFectAMINE 2000 (Invitrogen) was used for transient transfections according to the manufacturer’s protocols.

**Immunoprecipitations and Immunoblot Assays—**Transiently transfected 293T cells from each 10-cm dish were lysed in 1 ml of lysis buffer (50 mM HEPES, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 5 mM EDTA, 0.1% Nonidet P-40, 100 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and aprotonin). The lysates were analyzed with the same amount of proteins were immunoprecipitated with anti-FLAG, anti-HA, or control mouse IgG1 monoclonal antibodies (Sigma) for 2 h at 4 °C, respectively. The immune complexes were precipitated by incubation with protein A/G PLUS-agarose beads (Santa Cruz) for another 1 h. The agarose beads were washed twice with 1 ml of lysis buffer, twice with 1 ml of high salt (1 M NaCl) lysis buffer, and twice more with lysis buffer. The immunoprecipitates were separated by 10% SDS-PAGE and transferred to Hybond-P (Amersham Biosciences, Inc.). Subsequent immunoblotting analyses were performed as described elsewhere (17). Antibodies against FLAG-epitope (M2, Eastman Kodak Co.), HA-epitope (Sigma), TRAF2, and Ibα (Santa Cruz) were used at a 1:5,000, 1:2,500 and 1:1,000 dilution, respectively, for immunoblotting assays.

**GST Fusion Protein Binding Assay—** The human SphK cDNA was subcloned in-frame into the GST fusion protein expression vector, pGEX-1 (Amerham Biosciences, Inc.). Expression and purification of the derived GST-SphK fusion proteins were performed as described previously (21). Cell lysates from each T75 flask of HUVEC or 293T cells overexpressed with TRAF2 or GST alone fusion proteins for 24 h were analyzed by an immunoblot assay with anti-TRAF2 antibodies.

**Cell Viability Assay—** The transfected 293T cells were seeded on a 48-well plate at a density of 2 × 10<sup>4</sup> cell/well and stimulated with TNF (10 ng/ml) in the presence or absence of cycloheximide (1 μg/ml) for 18 h. Cell viability was assessed by an MTT dye reduction assay and expressed as a proportion of cells maintained in normal culture medium as described previously (17).

**Kinase Activity Assays—** SphK activity was measured by incubating the cytosolic fraction with 5 μM sphingosine dissolved in 0.1% Triton X-100 and [γ-<sup>32</sup>P]ATP (1 mm, 0.5 mCi/ml) for 15 min at 37 °C as described previously (18). SphK kinase activity was expressed as picomoles of [γ-<sup>32</sup>P]ATP incorporated per min per mg of protein. JNK activity was measured by the immune complex kinase assay in anti-HA immunoprecipitates from the cells coexpressed with HA-tagged JNK. The activity of immunoprecipitated complexes was determined by incubation with GST-c-Jun(1–79) fusion protein as substrate as described previously (22).

**Electrophoretic Mobility Shift Assay—** 293T cells were cotransfected with the desired expression vectors or empty vector. Nuclear extracts were prepared 24 h after transfection followed by TNF stimulation. The double-stranded oligonucleotides used as a probe in these experiments included 5′-GGACTGGCATTGGGGATTTCTCTTTACTGAGAT-3′, which contains a consensus NF-κB binding site in E-selectin promoter that is underlined. Gel mobility shift of a consensus NF-κB oligonucleotide was performed by incubating a [α-<sup>32</sup>P]-labeled NF-κB oligonucleotide with nuclear extracts of 293T cells as described previously (22). The specific DNA-protein complexes were completely abolished by addition of a 50-fold molar excess of unlabeled NF-κB oligonucleotides.

**Reporter Assay—** Stable transfected 293 cell lines overexpressing SphK, SphK<sup>cat210</sup>, or empty vector were cotransfected with pRK5-TRAF2-FLAG or pRK5 vector together with IgB-luciferase reporter gene plasmid (pTK81-IgB, 200 ng per transfection) and Renilla luciferase control vector (pRL, 20 ng per transfection). Total amounts of transfected DNA were kept constant by supplementing empty vector as needed. Cell extracts were prepared 24 h after transfection, and reporter gene activity was determined by the dual-luciferase assay system (Promega) and normalized relative to Renilla luciferase activity.

**Interaction of Sphingosine Kinase and TRAF2**

As TRAF2 does not contain intrinsic catalytic activity, protein-protein interactions are essential for TRAF2-mediated activation of downstream signals (5). We therefore tested the possibility of a physical interaction between TRAF2 and SphK. We initially performed overexpression-based communoprecipitation assays in HEK 293T cell line coexpressed HA-epitope-tagged SphK with FLAG-epitope-tagged TRAF2 or ΔTRAF2. The cell lysates were immunoprecipitated with anti-FLAG monoclonal antibodies, and the coprecipitated HA-tagged SphK was detected by immunoblot assay with anti-HA antibodies. SphK...
was found to be associated with TRAF2 in the immunoprecipitation complexes from the transfected cells (Fig. 2a). Conversely, by using anti-HA-epitope antibodies to perform the immunoprecipitation assays, we also found that FLAG-tagged TRAF2 or ΔTRAF2 was coprecipitated with HA-tagged SphK (data not shown). In addition, we examined whether endogenous TRAF2 could also interact with SphK by using GST-SphK fusion protein to pull-down the associated cellular proteins. As shown in Fig. 2b, GST-SphK fusion protein was capable of interacting with not only the overexpressed TRAF2 in 293T cells, but also the endogenous TRAF2 in HUVEC, confirming a physical interaction of TRAF2 with SphK. The dominant-negative TRAF2 (ΔTRAF2) was also shown to be associated with SphK (Fig. 2), indicating that the N-terminal RING finger of TRAF2 is not required for the interaction.

A TRAF2-binding Motif, PPEE, Is Responsible for the Interaction of TRAF2 with SphK—A structure-based sequence alignment of TRAF2 binding sequences in various members of TNF receptor superfamily demonstrated a major consensus motif of (P/S/T/A)XQ/E/I and a minor motif of PXXQXYD (23–24). Analysis of the SphK sequence (human SphK-1) revealed two possible TRAF2-binding motifs in positions 240–243 (PLEE) and 379–382 (PPEE), respectively, providing a potential structural basis for the interaction of SphK and TRAF2. To test whether these two TRAF2-binding motifs are responsible for the binding of SphK to TRAF2, we generated three mutants of SphK, TB1-SphK, TB2-SphK, and TB1/2-SphK, in which the first, second, or both TRAF2-binding motifs were mutated with alanines, i.e. PLEE → PLAA and PPEE → PPAE, respectively (Fig. 3a). We found that expression of either TB2-SphK or TB1/2-SphK (data not shown), but not TB1-SphK, deleted the ability of SphK to coimmunoprecipitated with TRAF2 (Fig. 3b), indicating that the second TRAF2-binding motif is essential for the interaction of these two molecules. The cells enforced expressing TB1-SphK, TB2-SphK, or TB1/2-SphK raised an unstimulated SphK activity to similar levels found with wild-type SphK-transfected cells (Fig. 4a), revealing an undiminished intrinsic enzyme catalytic activity in these SphK mutants. Strikingly, the activity of TB2-SphK, but not TB1-SphK, failed to respond to TNF stimulation (Fig. 4, a and b), suggesting an important role for C-terminal TB2 site of SphK not only in its capacity of interaction with TRAF2, but also in mediating TNF-induced activation of SphK. By contrast, the response of TB-2 SphK to phorbol ester (phorbol 12-myristate 13-acetate), an activator of SphK through protein kinase C activation (15–17), was undiminished (Fig. 4a), suggesting a TNF-specific defect of TB2-SphK. Taken together, these data indicate that SphK interacts with TRAF2 through the binding motif of PPEE379–382 and that this interaction is responsible for mediating TNF-induced SphK activation.

Interaction of TRAF2 with SphK Is Required for TNF-induced NF-κB Activation—Given the fact that TRAF2 interacted with and subsequently activated SphK and that SphK has been implicated in signaling to regulate cell survival and activation (15, 16), we sought to determine the role of SphK in the TRAF2-transduced signals. In agreement with previous report (7), overexpression of TRAF2 was capable of activating NF-κB as determined here by degradation of IκBα (Fig. 5a) and gel shift assay of NF-κB DNA binding complex (Fig. 5b). Coexpression of TB2-SphK markedly inhibited IκBα degradation (Fig. 5a) and decreased NF-κB DNA binding activity (Fig. 5, b and c) induced by either TNF stimulation or overexpression of TRAF2. By contrast, overexpression of wild-type SphK increased NF-κB activity (Fig. 5b), suggesting a potential effect of SphK on NF-κB activation. To further establish the role of the interaction of SphK with TRAF2 in mediating TNF-induced NF-κB activation, we used a point mutant of SphK (SphKG82D) that reserves intact TRAF2-binding motif but lacks the enzyme catalytic activity (20). As anticipated, SphKG82D had diminished binding ability to TRAF2 as determined by immunoprecipitation (data not shown) and completely abolished the SphK activity in response to TNF stimulation (Fig. 5a). Expression of SphKΔG82D dramatically blocked the degradation of IκBα (Fig. 5a) and inhibited the NF-κB DNA binding activity in a dose-dependent manner (Fig. 5, b and c). We further performed NF-κB reporter gene assays that confirmed the result from the assays of IκBα degradation and NF-κB DNA binding, showing that overexpression of TRAF2 or SphK increased NF-κB-dependent gene activity, whereas the effect of TNF or TRAF2 on NF-κB activation was blocked by coexpression of SphKG82D (Fig. 5d). Thus, the TRAF2-mediated SphK activation is apparently necessary for TNF-induced NF-κB activation.

Interaction of TRAF2 and SphK Does Not Signal JNK Activation—Since JNK is another well documented major signal pathway mediated by TRAF2 during TNF stimulation (25, 26), we tested whether the interaction of TRAF2 with SphK could also regulate the TRAF2-dependent JNK activation. Strikingly, neither TNF stimulation nor overexpression of TRAF2-induced JNK activity was affected by expression of TB2-SphK or SphKΔG82D (Fig. 6). In addition, overexpression of wild-type SphK had no significant effect on JNK activation. Hence, in contrast with the effect of SphK on NF-κB, the activation of JNK induced by TNF or TRAF2 is independent of SphK.
SphK Activation Is Involved in TRAF2 Antiapoptotic Signaling—An essential role of TRAF2 in antiapoptosis has been definitively identified based on the studies with the dominant-negative TRAF2 and deletion of TRAF2 gene in vivo (7, 9, 26, 27). We further investigated whether the interaction of TRAF2 with SphK is involved in TRAF2-mediated antiapoptotic signaling pathways. Consistent with previous reports (7, 26), expression of ΔTRAF2 increased cell sensitivity to killing by TNF (Fig. 7), indicating the role of TRAF2 in antiapoptosis. The effect of ΔTRAF2 was completely prevented by overexpression of SphK, even in the presence of an inhibitor of protein synthesis, cycloheximide, suggesting an independent of de novo protein synthesis antiapoptotic pathway promoted by SphK activation (Fig. 7). While overexpression of TRAF2 had a partially protective effect against TNF-induced apoptosis in the presence of cycloheximide, it was substantially enhanced by coexpression with SphK (Fig. 7, right panel). By contrast, the protective effect of TRAF2 against apoptosis was abolished by coexpression of SphK^{G82D} (Fig. 7). Taken together, our findings suggest that SphK activity is essential to determine the antiapoptotic capacity of TRAF2 during TNF stimulation.

DISCUSSION

In this report, we describe an association of TRAF2 with SphK, the first lipid kinase to interact with this signal transducer, which provides a novel mechanism for the specific signaling pathway leading from TRAF2 to the activation of NF-κB and antiapoptosis (Fig. 8). We demonstrate the association between TRAF2 and SphK by coimmunoprecipitation assays from the transfected cells and in vitro binding assays, which

**Fig. 3.** Site-directed mutagenesis of TRAF2-binding motif in SphK ablates the interaction of SphK with TRAF2. *a,* diagrams of the putative TRAF2-binding motifs (TB1 and TB2) in wild-type human SphK-1 (wt-SphK) and the mutants of SphK, TB1-SphK, TB2-SphK, and TB1/2-SphK. *b,* 293T cells were cotransfected with the indicated expression vectors. 48 h after transfection, cells were lysed, and the lysates were immunoprecipitated (IP) with anti-TRAF2 antibodies and coimmunoprecipitated SphK or its mutants were detected by immunoblotting assay (IB) with anti-FLAG antibodies (top panel). The expression of proteins in whole-cell lysates was shown in bottom panel.

**Fig. 4.** TB2-SphK and SphK^{G82D} block TNF-induced SphK activation. *a,* 293T cells were transfected with the indicated expression vectors, and SphK activity was determined after stimulation with TNF (1 ng/ml), phorbol 12-myristate 13-acetate (100 nM), or nil for 10-min post-transfection at 48 h. Data are the mean (± S.D.) of relative activity of three individual experiments. The mean of unstimulated (Nil) levels of SphK activity in the cells transfected with SphK, TB1-SphK, TB2-SphK, and SphK^{G82D} were 42,600, 43,100, 41,800, and 34 pmol/min/mg of protein, respectively. *b,* SphK activity was assayed in the SphK- or TB2-SphK-transfected 293T cells at the indicated time points of TNF (1 ng/ml) stimulation. Data shown are mean of activity of one representative experiment done in duplicate.
show that SphK associated with not only the transfected proteins but also endogenous TRAF2. In addition to the physical association, we provide four lines of evidence for a functional role of SphK in TRAF2 mediated TNF signaling: (i) either TNF or overexpression of TRAF2 was capable of activating SphK; (ii) TNF-induced SphK activation was blocked by the dominant-negative TRAF2, \( \Delta \text{TFR2} \); (iii) overexpression of SphK potentiated the ability of TRAF2 in activation of NF-kB and anti-

**FIG. 5. Effect of SphK on NF-κB activation.** 293T cells were cotransfected with the indicated expression vectors. 48 h after the transfection, cells were stimulated with or without TNF (1 ng/ml) for 30 min. a, Western blot assay with anti-IκBα antibodies showing IκBα degradation. b, NF-κB activation was determined by gel shift assay of NF-κB DNA binding complex as described under “Experimental Procedures.” *, individual reactions were supplemented with a 50-fold excess of unlabeled competitor oligonucleotide, indicating a specificity of the binding of NF-κB. c, NF-κB binding complex determined in the cells transfected with an increasing amount (1–4 μg) of SphK\(^{\text{G82D}}\) or TB2-SphK followed by TNF stimulation. d, stable transfected 293 cells overexpressing SphK, SphK\(^{\text{G82D}}\), or empty vector were cotransfected with TRAF2 or pRk5 vector together with IgκB-luc reporter plasmid and Renilla luciferase control vector. 24 h after transfection, cells were stimulated with TNF (1 ng/ml) for 4 h, and then the reporter gene activity was determined and normalized relative to Renilla luciferase activity. Data shown are mean of relative luciferase activity of one representative experiment done in quadruplicate. Similar results were obtained in four independent experiments.
 apoptosis and restored the effect of ΔTRA2; and (iv) SphK mutants lacking either TRA2-binding motif or enzyme catalytic activity abrogated the effect of TRA2. Thus, the interaction of TRA2 with and subsequent activation of SphK appears critically involved in the process of TRA2 mediated signal transduction.

TRA2 is a signal-transducing adapter protein that contains a conserved C-terminal TRA2 domain and an N-terminal region consisting of a RING finger domain and an additional array of zinc finger-like structures (28). The TRA2 domain is involved in receptor association and homo/hetero-oligomerization of TRA2 proteins and serves as a docking site for a number of other signaling proteins (4, 5). A structure-based sequence alignment has revealed a consensus motif of (P/S/T/A)X(Q/E)E existing among the TRA2-binding receptors including TNFR2, CD40, CD30, OX40, 4-1BB, CD27, LTβ-R, and ATAR (23, 24). Several biochemical studies with mutagenesis have also supported the definition of the TRA2-binding motifs (29–32). Interestingly, the presence of two TRA2-binding motifs in positions 240–243 (PLEE) and 379–382 (PPEE), respectively, were found in SphK, a lipid kinase that has been implicated in signaling of cell survival, activation, and proliferation (14–16, 18). Alanine mutagenesis delineated the PPEE motif being responsible for the binding of SphK to TRA2. TB2-SphK containing a mutated TRA2-binding motif (PPAA379–382) not only abolished the ability of SphK to associate with TRA2 (Fig. 3b), but also specifically blocked the TNF-induced activation of SphK (Fig. 3c). These data reveal a critical role of the TRA2-binding motif for the physical and functional interaction between SphK and TRA2.

The most prominent signaling pathways mediated by TRA2 are activation of NF-κB and JNK (4, 5). Previous reports that overexpression of TRA2 activated NF-κB and ΔTRA2 blocked TNF-induced NF-κB suggested a central role of TRA2 in NF-κB activation (33). Although TRA2-deficient cells are only partially deficient in NF-κB activation (26, 27), TRA2/TRA5 double knockout cells exhibit a complete defect in TNF-induced NF-κB activation (34), revealing the importance of TRA2 proteins for NF-κB activation. Nevertheless, the mechanisms of NF-κB activation mediated by TRA2 are far from being understood. Recent reports indicated that the receptor-interacting kinase RIP is required for TRA2-mediated NF-κB activation (35). TRA2 recruits IκB kinase (IKK, essential for NF-κB activation) to the TNF receptor complex, while RIP promoted IKK activation (36). However, as the kinase activity of RIP seems not to be involved in IKK activation (36), the precise role of RIP in TRA2-mediated NF-κB is still unclear (5). The NIK that associates with TRA2 was reported as a component of IKK complexes to be implicated in TNF-induced NF-κB activation (37, 38). The role of NIK in NF-κB activation has also been questioned based on knockout and other genetic experiments (39, 40). In the present report, we describe a novel TRA2-binding protein SphK that appears playing an important role in mediating TNF- and/or TRA2-induced NF-κB activation. Our previous studies (18, 22) and others (41) have shown that S1P, the product of SphK, activated NF-κB and a specific inhibitor of SphK (N,N-dimethylsphingosine) blocked NF-κB activation. Consistent with these observations, we found that overexpression of SphK was capable of activating NF-κB and enhancing TRA2-induced NF-κB activity. Furthermore, the dominant-negative SphK completely abrogated TNF- and/or TRA2-induced NF-κB activation (Fig. 5, a–d). The effect of SphK on NF-κB was demonstrated in the present study by the degradation of IκBα, NF-κB DNA binding, and the NF-κB-dependent reporter gene activity, pointing toward SphK being involved in a critical step of regulation of NF-κB, probably in IKK complexes. The precise role of SphK in NF-κB activation needs to be further elucidated.

TRA2 is also known to activate JNK, and indirect evidence based on the interaction between TRA2 and NIK suggested that TNF-induced activation of NF-κB and JNK was likely

![Fig. 6. Effect of SphK on JNK activation.](http://www.jbc.org/)

293T cells were cotransfected with the indicated amounts of expression vectors. After 48 h cells were stimulated with or without TNF (1 ng/ml) for 30 min. JNK activity was assayed as described under "Experimental Procedures." An HA immuno blot is shown in the bottom panel, indicating equivalent levels of HA-JNK.

![Fig. 7. Effect of SphK on TRA2-mediated antiapoptosis.](http://www.jbc.org/)

293T cells were cotransfected with SphK, SphK<sup>Δ</sup>, or an empty vector together with TRA2 or ΔTRA2 as indicated. 48 h after the transfection, cells were stimulated with TNF (10 ng/ml) in the absence (left panel) or presence (right panel) of cycloheximide (CHX, 1 μg/ml) for a further 18 h. Cell viability was then assessed by an MTT assay and expressed as a proportion of cells maintained in normal culture medium containing 10% of fetal calf serum. Data shown are mean (± S.D.) of one representative experiment done in triplicate. *p < 0.001, compared with cells cotransfected with an empty vector.
bifurcated at the site of TRAF2 (6). In accordance with this idea, we found that while SphK mediated TRAF2-promoted NF-κB activation (Fig. 5), JNK activation was SphK-independent (Fig. 6). Neither wild-type SphK nor the dominant-negative SphK influenced JNK activity induced by TNF and/or TRAF2 (Fig. 6). These results demonstrate that the interaction of TRAF2 with SphK appears responsible for one of the arms of TRAF2-mediated signaling, namely the activation of NF-κB. However, the other arm of TRAF2 signals, activation of JNK, is independent of SphK. Hence, these two distinct signaling cascades initiated by TRAF2 appear to be disengaged by the interaction of TRAF2 with SphK.

An obvious question raised from this finding is how TRAF2 activates SphK. Although the details are currently unknown, our experiments provide several indications. That interaction of TRAF2 and SphK occurs even in the absence of TNF stimulation seemingly suggests a signal-independent association between these two molecules. However, this observation does not exclude a signal-dependent association that is modulated by TNF, since overexpressed TRAF2 is already in an active state presumably due to its oligomerization (42). Indeed, like most of the downstream signaling events such as NF-κB activation (25, 33), overexpression of TRAF2 is sufficient to activate SphK without TNF stimulation. The data that ΔTRAF2 retains the ability to associate with SphK, but fails to activate SphK, indicates the requirement of N-terminal RING and zinc-finger motifs of TRAF2 for the activation of SphK.

Following the activation of distinct signal transduction pathways, the pivotal biological function of TNF is determining the choice between cell survival and death and control of cell proliferation and inflammation (1, 43). The understanding that the signaling mechanisms underlying TNF regulate these choices will provide insights into the physiological and pathophysiological role of TNF and thereby provide new avenues for therapeutic intervention. The choice can be made initially by the access of TNF to different cell surface receptors (44), i.e. TNFR1 and TNFR2 (with or without a death domain). This choice is, however, not definitive as the actions of TNF mediated by two types of receptor are somewhat overlapping (1, 43). The choice between TNF’s functions can be additionally determined by the recruitment of distinct intracellular signal mediators to the receptor complexes as exemplified by the interaction of TRADD with FADD or TRAF2 (9, 45). Indeed, the most widely accepted TNF signaling pathways are that the interaction of TRADD, FADD, and caspases mediates TNF-induced apoptosis and that the interaction of TRADD, TRAF, and MAPK kinase kinase transduces signaling to cell survival, proliferation, and inflammation (1, 43). Even though TRAF2 is established as a central transducer to mediate TNF antiapoptotic signaling, the biological function of TRAF2-mediated activation of JNK is very different from that of NF-κB (1). JNK activation was also suggested to mediate TNF-induced cell death (46). The present findings provide evidence showing that the choice of TNF actions could be further defined on the level of TRAF2 through the interaction with SphK. As discussed above, that TRAF2 interacts with and subsequently activates SphK could bifurcate the TRAF2-integrated signals specifically leading to activation of NF-κB but not JNK. This interconnection signaling model was further strengthened by the effectiveness of SphK in protecting against cell death. We found that overexpression of SphK potently block the proapoptotic effect of TNF and the ΔTRAF2-potentiated cell death (Fig. 7), which is consistent with the previous finding that SphK and/or S1P is a potent protector against a wide range of factor-induced apoptosis (15, 17, 47–49). In addition, the dominant-negative SphK, SphK<sup>ΔC2D</sup>, was capable of triggering TNF-induced apoptosis and also deleted the antiapoptotic potential of TRAF2. The data that SphK<sup>ΔC2D</sup> abrogated SphK activity (Fig. 3c) and inhibited NF-κB activation (Fig. 4) pointed to the antiapoptotic effect of SphK being related to NF-κB activation. However, SphK protected against TNF-induced or ΔTRAF2-potentiated cell death even in the presence of cycloheximide (Fig. 7) that suppressed any <i>de novo</i> synthesis of NF-κB-dependent antiapoptotic proteins, suggesting the antiapoptotic effect of SphK is, at least partly, independent on NF-κB activation.

Although NF-κB activation has been implicated in suppression of apoptosis via the production of antiapoptotic proteins, including the inhibitor-of-apoptosis proteins (8), it does not appear essential for TRAF2-mediated antiapoptosis (26, 27), revealing an NF-κB-independent antiapoptotic pathway(s) promoted by TRAF2 during TNF-induced apoptosis. Thus, the interaction of TRAF2 with SphK and subsequent activation of SphK indicate a novel mechanism for TRAF2-mediated antiapoptosis independent of NF-κB, providing an insight into the understanding of signal machinery of cell death and survival.

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Interaction of Sphingosine Kinase and TRAF2

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Sphingosine Kinase Interacts with TRAF2 and Dissects Tumor Necrosis Factor-α Signaling
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Vadim Budagian, Elena Bulanova, Luba Brovko, Zane Orinska, Raja Fayad, Ralf Paus, and Silvia Bulfone-Paus

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Natural soluble interleukin-15Rα is generated by cleavage that involves the tumor necrosis factor-α-converting enzyme (TACE/ADAM17).

Vadim Budagian, Elena Bulanova, Zane Orinska, Andreas Ludwig, Stefan Rose-John, Paul Saftig, Ernest C. Borden, and Silvia Bulfone-Paus

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PAGE 8000:
It has come to our attention that there appears to be two duplications of lanes in Fig. 5b. Upon investigation of this, we conclude that an error has taken place in assembling Fig. 5b (containing 11 lanes) from the original gel retardation assay of 15 samples. The corrected figure is shown below.
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Figs. 1b and 5b in the article and in the published correction (Volume 286 (2011), page 9894) were inadvertently non-uniformly resized from the original images. The corrected figures are shown below.

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Human T-cell leukemia virus type I Tax down-regulates the expression of phosphatidylinositol 3,4,5-trisphosphate inositol phosphatases via the NF-κB pathway.

Ryu-ich Fukuda, Kiyohito Tsuchiya, Koji Suzuki, Katsuhiko Itoh, Jun Fujita, Atae Utsunomiya, and Takashi Tsuji

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