Tumor necrosis factor receptor 1 (TNF-R1) signaling elicits a wide range of biological responses, including inflammation, proliferation, differentiation, and apoptosis. TNF-R1 activates both caspase-mediated apoptosis and NF-κB transcription of anti-apoptotic factors. We now report a link between the TNF-R1 and inositol phosphate signaling pathways. We observed that overexpression of inositol 1,3,4-trisphosphate 5/6-kinase (5/6-kinase) inhibited apoptosis induced by TNFα. The anti-apoptotic effect by 5/6-kinase is not attributable to NF-κB activation, as no changes were detected in the levels of NF-κB DNA binding, IκB degradation, or anti-apoptotic factors, such as x-linked inhibitor of apoptosis protein. Decreased expression of 5/6-kinase by RNA interference rendered HeLa cells more susceptible to TNFα-induced apoptosis. Overexpression of 5/6-kinase in human embryonic kidney 293 cells inhibited TNFα-induced activation of caspases-8, -9, and -3, BID, and poly(ADP-ribose) polymerase. However, 5/6-kinase did not protect against Fas-, etoposide-, or cycloheximide-induced apoptosis. Further, 5/6-kinase protected against apoptosis induced by the overexpression of TNF-R1-associated death domain but not Fas-associated death domain. Therefore, we suggest that 5/6-kinase modifies TNFα-induced apoptosis by interfering with the activation of TNF-R1-associated death domain.

Inositol phosphate signaling is involved in many different cellular processes, ranging from intracellular calcium regulation to mRNA export from the nucleus (for review see Refs. 1–3). In mammalian cells, activation of phospholipase C generates inositol 1,4,5-trisphosphate, which then becomes phosphorylated and dephosphorylated by a series of inositol phosphate kinases and phosphatases to generate inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate (3–6). Inositol 1,3,4-trisphosphate 5/6-kinase (5/6-kinase) functions at the branch point in this pathway (7, 8). Initially purified from calf brain, 5/6-kinase was identified by its inositol phosphate kinase activity. Using inositol 1,3,4-trisphosphate as a substrate, 5/6-kinase generates two different products, inositol 1,3,4,5,6-pentakisphosphate and inositol 1,3,4,6-tetakisphosphate. The latter product is then further phosphorylated to produce inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate (4–6). In addition, 5/6-kinase has been reported to phosphorylate inositol 3,4,5,6-tetakisphosphate to inositol 1,3,4,5,6-pentakisphosphate (8). Recently, we have reported that 5/6-kinase also exhibits protein kinase activity and associates with the COP9 signalosome complex (9, 10). The COP9 signalosome has been implicated in regulation of the cell cycle, proteolysis in the proteasome, and plant development (11–13). We have shown that 5/6-kinase associates with the CSN1 subunit of the COP9 signalosome and phosphorylates IκBα and c-Jun (9, 10). IκBα phosphorylation is an important regulatory mechanism for TNFα-induced NF-κB activation. To elucidate the physiological consequence of 5/6-kinase-mediated IκBα phosphorylation, we examined TNFα signaling in vivo.

TNFα, a member of a large class of cytokines, elicits a wide spectrum of cellular responses including differentiation, proliferation, inflammation, and cell death. TNFα signaling simultaneously triggers apoptotic and anti-apoptotic pathways (for review see Ref. 14). Although caspase activation results in apoptosis, NF-κB mediates transcription of anti-apoptotic factors that can block the caspase cascade; integration of these events determines the cellular response to TNFα stimulation. Upon TNFα binding to TNF-R1, interaction of the receptor with the TNF receptor-associated death domain (TRADD), a central adaptor protein for TNF-R1, leads to the formation of the death-inducing signaling complex (DISC) (15). TNFα stimulates both the mitochondrial-dependent (intrinsic) and -independent (extrinsic) apoptotic pathways. In response to extracellular stimuli, the extrinsic pathway of TNFα signaling induces the TRADD-dependent recruitment of Fas-associated death domain (FADD), which recruits and activates procaspase-8 leading to activation of the caspase cascade (16). Caspase-8 also stimulates the intrinsic pathway through the cleavage and activation of BID, resulting in cytochrome c release from mitochondria (17, 18).

Binding of TRADD to TNF-R1 is also crucial for anti-apoptotic signaling events. Receptor-interacting protein and TNF receptor-associated factor-2 are recruited to the receptor, and this complex activates the IκB kinase (IKK) signalosome, which phosphorylates IκBα (for review see Refs. 19 and 20). In resting cells, IκBα inhibits NF-κB by preventing translocation of NF-κB subunits into the nucleus. However, upon TNFα stimulation, phosphorylation of IκBα by the IKK signalosome, and subsequent ubiquitination, causes its degradation by the 26 S proteasome. As a result, NF-κB translocates to the nucleus.  

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The abbreviations used are: TNFα, tumor necrosis factor α; DISC, death inducing signaling complex; FADD, Fas-associated death domain; TNF-R1, TNF receptor 1; TRADD, TNF-R1-associated death domain; PARP, poly(ADP-ribose) polymerase; NF-κB, nuclear factor-κB; IKK, IκB kinase; XIAP, x-linked inhibitor of apoptosis protein; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; WB, Western blot; HEP, human embryonic kidney; MOPS, 4-morpholinepropanesulfonic acid; RNAi, RNA interference; SODD, silencer of death domain.
and activates the transcription of many anti-apoptotic factors, such as inhibitor of apoptosis protein 1, x-linked inhibitor of apoptosis protein (XIAP), and Bcl family members, which can inhibit the TNFα-induced apoptotic pathway (21–23).

We now report a link between the inositol phosphate and TNFα signaling pathways. We show that 5/6-kinase inhibits TNFα-induced apoptosis. In 5/6-kinase-overexpressing HEK 293 cells, activation of PARP, BID, caspases-8, -3, and -9 are inhibited in a time- and dose-dependent manner. Surprisingly, the 5/6-kinase effect of 5/6-kinase was not because of enhanced NF-κB activation. In addition, 5/6-kinase does not protect against Fas-, etoposide-, or cycloheximide-induced cell death. Therefore, we conclude that 5/6-kinase inhibits apoptosis by blocking the TNFα-induced activation of the caspase cascade.

MATERIALS AND METHODS

Reagents—All chemicals and reagents were obtained from Sigma, unless otherwise specified. Antibodies against PARP, BID, XIAP, caspase-3, and caspase-8 (1C12) were obtained from Cell Signaling. According to the manufacturer, purified anti-PARP antibody preferentially recognizes the cleaved form of PARP. Antibodies against IκBα, p50 (NF-κB), receptor-interacting protein, and α-tubulin were purchased from Santa Cruz Biotechnology, Inc. Anti-caspase-9 antibodies and irreversible caspase inhibitor Z-Glu-Val-Asp-fluoromethyl ketone (20 μM) were obtained from Oncogene, and anti-TRADD antibodies were from BD Biosciences. Antibodies against human 5/6-kinase were described previously (9). TNFα (Calbiochem) and anti-Fas antibody (BD Biosciences) were used to induce apoptosis. Protein G was purchased from Upstate Biotechnology. [3H]Insitol 1,3,4-trisphosphate was prepared from [3H]inositol 1,3,4,5-tetrakisphosphate as described previously (9).

DNA Constructs—An expressed sequence tag clone encoding human FADD (GenBank™ accession number BE794984 (ATCC) with primers 5’ by PCR using a template from the human expressed sequence tag clone CMV-14 expression vector (Sigma). Full-length TRADD was obtained with 2 mM glutamine and 10% fetal bovine serum (Invitrogen) and placed in puromycin-containing selection medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum). These were transfected with a plasmid containing either pSuper RNAi (Clontech) or p3XFLAG-CMV-14 expression vector (Sigma) to generate TRADD- or Fas- or FasL- or FasR- or Fas-interacting protein, and receptor-interacting protein- or TRADD-overexpressing 5/6-kinase or vector control cells were plated in triplicate in 24-well plates, and protein expression was measured with tetra-cycline for 48 h. After treatment with either TNFα/CHX (1 ng/ml and 0.5 μg/ml, respectively) or CHX (0.5 μg/ml alone), cells were trypsinized and counted with a hemocytometer. An average of three independent counts for each sample point was performed.

Apoptosis Assays—Trypan blue staining and APOPercentage Assay (Cell Lines—293T-Cells (Clontech) stably expressing 5/6-kinase or pcDNA4.0 vector-transfected cells were described previously (9). Tetra-cycline (0.1 μM) in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and 10% fetal bovine serum (Invitrogen) was added to cells 48 h prior to treatments. Apoptosis Assays—Trypan blue staining and APOPercentage Assay (manufacturer’s instructions). For trypan blue staining, HEK 293 cells overexpressing 5/6-kinase or vector control cells were plated in triplicate in 24-well plates, and protein expression was measured with tetra-cycline for 48 h. After treatment with either TNFα/CHX (1 ng/ml and 0.5 μg/ml, respectively) or CHX (0.5 μg/ml alone), cells were trypsinized and counted with a hemocytometer. An average of three independent counts for each sample point was performed.
5/6-Kinase Inhibits TNF-induced Apoptosis

5/6-Kinase Overexpression Protects against TNFα-induced Apoptosis—Previously, we reported that 5/6-kinase phosphorylated IκBα in vitro (10). IκBα is a key regulator of the NF-κB response to TNFα stimulation (for review see Ref. 19); therefore we examined the effect of 5/6-kinase overexpression on TNFα signaling. TNFα can induce activation of NF-κB and transcription of target genes that feed back to protect against TNFα-induced cell death. However, if the NF-κB pathway is blocked, cells become susceptible to TNFα-induced apoptosis. Overexpression of 5/6-kinase protected cells against TNFα-induced cell death, as shown by APOPercentage staining (Fig. 1A). Treatment with TNFα alone (without CHX) did not increase cell death in either 5/6-kinase-overexpressing or vector control cells (data not shown). The anti-apoptotic effect of 5/6-kinase was further analyzed using trypan blue staining of cells treated with either TNFα/CHX or CHX alone. Although 80% of vector-transfected cells died within 20 h of TNFα/CHX stimulation, cells expressing 5/6-kinase exhibited 50% cell death. Treatment by CHX alone caused only 20% cell death in either vector or 5/6-kinase-expressing cells (Fig. 1B). Similar results were observed in HeLa cells transiently transfected with an expression plasmid containing 5/6-kinase (data not shown). These results indicate that overexpression of 5/6-kinase inhibits TNFα-induced apoptosis.

5/6-kinase Does Not Enhance NF-κB Activation as a Mechanism for Protection—Signaling triggered by TNF-R1 can also cause activation of NF-κB signaling that leads to the expression of anti-apoptotic genes. To determine whether 5/6-kinase mediates its anti-apoptotic effect(s) by inhibiting the TNFα-induced apoptotic pathway and/or by promoting the TNFα-induced anti-apoptotic pathway, the stability of IκBα was measured after TNFα stimulation. Both vector control cells and 5/6-kinase-expressing cells showed IκBα degradation within 10 min (Fig. 2A). Although IκBα stability in response to TNFα signaling is not increased with the overexpression of 5/6-kinase, this does not directly show whether NF-κB activity is modulated in response to 5/6-kinase and TNFα. To determine whether 5/6-kinase directly affects NF-κB activity, the DNA binding activity of NF-κB was measured by EMSA as described under “Materials and Methods.” Treatment with TNFα for 30 min resulted in similar DNA binding activity of NF-κB in both vector control cells and 5/6-kinase-expressing cells (Fig. 2B). Furthermore, no significant differences were detected upon supershifting with an anti-p50 (NF-κB) antibody in 5/6-kinase or vector-expressing cells. Therefore, the ability of NF-κB to bind DNA does not seem to be increased by 5/6-kinase in response to TNFα.

To confirm that TNFα-induced NF-κB activity is not affected by 5/6-kinase, the expression of NF-κB anti-apoptotic target genes were measured in mRNA extracts from 5/6-kinase- or vector-transfected cells treated with TNFα for 6 h. As shown in Fig. 2C, in the absence of TNFα, mRNA levels of IκBα and NF-κB2 are low, and RelA is undetectable. After TNFα treatment, IκBα, RelA, and NF-κB2 mRNA levels increase significantly and to similar levels in both vector control cells and 5/6-kinase-overexpressing cells. The mRNA levels of β-actin and 5/6-kinase are shown as controls. Activation of NF-κB increases the synthesis of many anti-apoptotic proteins, such as inhibitor of apoptosis protein 1 and XIAP (21). However, the levels of XIAP are unchanged in cells expressing 5/6-kinase treated with TNFα/CHX (Fig. 2D). The mRNA levels of other NF-κB targets including TNF receptor-associated factor-2 and TNF receptor-associated factor-6 and Bcl family members, such as Bax, Bcl-2, and Bim were also not changed by the overexpression of 5/6-kinase (data not shown). Consistent with the lack of NF-κB up-regulation in response to 5/6-kinase overexpression, we have observed that 5/6-kinase continues to exert a protective effect against TNFα-induced apoptosis in the presence of inhibitors of NF-κB pathway, such as MG132, BAF (28), and BAY 11–7082 (data not shown). Therefore, these results argue that 5/6-kinase protects against TNFα-induced apoptosis through inhibition of the protease pathway and not through enhanced NF-κB activation and expression of anti-apoptotic genes.

5/6-Kinase RNA Interference Renders HeLa Cells More Susceptible to TNFα-induced Cell Death—To further confirm the protective effect(s) of 5/6-kinase against TNFα-induced apoptosis, endogenous levels of 5/6-kinase were reduced using RNA interference (RNAi). HeLa cells stably transfected with an
RNA interference plasmid for 5/6-kinase (5/6-kinase RNAi) or a control vector (pSuper) were treated with TNFα/CHX. Two stable cell lines (KD1 and KD2) were isolated that exhibit marked decreases in 5/6-kinase expression as shown by Western blot analysis (Fig. 3A). The activity of 5/6-kinase was reduced by 90–95% in these cell lines (Fig. 3B). To determine the effect of decreased 5/6-kinase expression on TNFα-induced apoptosis, 5/6-kinase RNAi cells (KD1) and control cells were treated with TNFα for 6 h, and total mRNA extracts were prepared. Northern blot analysis was performed using [32P]-labeled probes for IκBα, NF-κB2, RelA, 5/6-kinase, and actin. D, HEK 293 cells overexpressing 5/6-kinase or vector control cells were stimulated with TNFα/CHX (1 ng/ml and 0.5 μg/ml, respectively) for the indicated times. Total lysate (20 μg) was applied to SDS-PAGE, and Western blot analysis was performed using antibodies against XIAP. The results are representative of three independent experiments.

**Fig. 2. Effects of 5/6-kinase overexpression on NF-κB activation.** A, HEK 293 cells stably transfected with expression plasmids of vector or 5/6-kinase were treated with TNFα (1 ng/ml) for the indicated times. Western blot analysis was performed using anti-IκBα antibody. B, HEK 293 cells overexpressing 5/6-kinase or vector control cells were stimulated with TNFα (1 ng/ml) for 30 min. Nuclear extracts were prepared, and EMSA was performed as described under “Materials and Methods.” C, HEK 293 cells overexpressing 5/6-kinase or vector control cells were treated with TNFα for 6 h, and total mRNA extracts were prepared. Northern blot analysis was performed using [32P]-labeled probes for IκBα, NF-κB2, RelA, 5/6-kinase, and actin. D, HEK 293 cells overexpressing 5/6-kinase or vector control cells were stimulated with TNFα/CHX (1 ng/ml and 0.5 μg/ml, respectively) for the indicated times. Total lysate (20 μg) was applied to SDS-PAGE, and Western blot analysis was performed using antibodies against XIAP. The results are representative of three independent experiments.

5/6-Kinase Inhibits TNF-induced Apoptosis

Fas, another member of the TNF-R superfamily, shares a similar mechanism of caspase activation. Both TNF-R1 and Fas-mediated signaling involve FADD recruitment to the DISC for the activation of caspase-8 (25, 26). To determine whether 5/6-kinase overexpression could inhibit the mitochondrial pathway of apoptosis (24). To determine whether 5/6-kinase overexpression inhibited the mitochondrial pathway, cells expressing either 5/6-kinase or vector control cells were treated with etoposide for 20 h, and caspase levels were determined by Western blot analysis. As shown in Fig. 4A, etoposide-induced cleavage of caspase-9 was not inhibited by 5/6-kinase overexpression. These results suggest that 5/6-kinase overexpression does not inhibit the intrinsic apoptotic pathways.
To characterize the mechanism(s) by which 5/6-kinase mediates protective effect(s) against TNFα/H9251-induced apoptosis, we sought to determine whether 5/6-kinase directly affects caspase activation. One substrate of caspases is PARP, a DNA repair enzyme that is cleaved by caspase-3 (27). Cells overexpressing 5/6-kinase or vector control cells were treated with TNFα/H9251/CHX for 6 h, and full-length and cleaved PARP levels were measured. In vector control cells treated with CHX, PARP degradation is observed with increasing doses of TNFα/H9251. In contrast, cells expressing 5/6-kinase exhibited lower levels of degraded PARP in both a time- and dose-dependent manner (Fig. 5, A and B). The antibody against PARP preferentially recognizes the cleaved form of PARP, which explains the increase in the intensity of cleaved PARP as compared with that of full-length PARP in Fig. 5A. At this early time point (6-hr), the protein levels of major signaling intermediates in this pathway, including the p50 subunit of NF-κB (Fig. 5A),

**Fig. 3. Effects of 5/6-kinase RNA interference on TNFα-induced apoptosis.** A. HeLa RNAi cells from two clones of either vector (pSuper) or 5/6-kinase were analyzed by Western blotting analysis for 5/6-kinase expression with β-tubulin as a loading control. B. extracts from these clones were also assayed for 5/6-kinase activity. C. 5/6-kinase RNAi (KD1) cells were treated with TNFα/CHX (1 ng/ml and 0.5 µg/ml, respectively) for the indicated times. Western blot analysis was performed using antibodies against PARP, BID, and β-tubulin. The results are representative of three independent experiments using both KD clones. D. HeLa 5/6-kinase RNAi cells and HeLa vector RNAi cells (1 × 10⁶ cells) were treated with TNFα (1 ng/ml) and CHX (0.5 µg/ml) for 3 h and stained with APOPercentage dye. E. HeLa 5/6-kinase RNAi cells and HeLa vector RNAi cells were treated with TNFα (1 ng/ml) and CHX (0.5 µg/ml) for 6 h, and trypan blue was used to measure the percentage of live cells. Each time point was performed in triplicate, and the results are representative of three independent experiments. Comparison of lane 2 versus lane 4 shows increased cell death in RNAi of 5/6-kinase cells (56 versus 74%), p = 0.007 by Student’s t test.

5/6-kinase expression with β-tubulin as a loading control. B. extracts from these clones were also assayed for 5/6-kinase activity. C. 5/6-kinase RNAi (KD1) cells were treated with TNFα/CHX (1 ng/ml and 0.5 µg/ml, respectively) for the indicated times. Western blot analysis was performed using antibodies against PARP, BID, and α-tubulin. The results are representative of three independent experiments using both KD clones. D. HeLa 5/6-kinase RNAi cells and HeLa vector RNAi cells (1 × 10⁶ cells) were treated with TNFα (1 ng/ml) and CHX (0.5 µg/ml) for 3 h and stained with APOPercentage dye. E. HeLa 5/6-kinase RNAi cells and HeLa vector RNAi cells were treated with TNFα (1 ng/ml) and CHX (0.5 µg/ml) for 6 h, and trypan blue was used to measure the percentage of live cells. Each time point was performed in triplicate, and the results are representative of three independent experiments. Comparison of lane 2 versus lane 4 shows increased cell death in RNAi of 5/6-kinase cells (56 versus 74%), p = 0.007 by Student’s t test.
FIG. 5. Effects of 5/6-kinase overexpression on TNFα-induced cleavage of PARP, BID, caspase-3, and caspase-8. A, CHX (0.5 µg/ml) and TNFα at the indicated concentrations were added to either HEK 293 cells overexpressing 5/6-kinase or vector control cells (2 × 10⁶ cells on 6-well plates) for 6 h. Total lysate (20 µg) was applied to SDS-PAGE, and Western blot analysis was performed using antibodies against PARP, p50 (NF-κB), and 5/6-kinase. The graph shows the average of four experiments using 0.01, 0.1, and 1 ng TNFα/ml where 100% is the maximum PARP cleavage obtained. The mean ± S.D. is shown, p < 0.001. B, time course of PARP cleavage as above using 1 ng TNFα/ml. The graph shows the average of four experiments at 0, 4, and 8 h where 100% is the maximum PARP cleavage obtained. The mean ± S.D. is shown, p < 0.001. C–E, HEK 293 cells overexpressing 5/6-kinase or vector control cells were treated with TNFα/CHX (1 ng/ml and 0.5 µg/ml, respectively) for the indicated times. Total lysate (20 µg) was applied to SDS-PAGE, and Western blot analysis was performed using antibodies against caspase-3 (C), caspase-8 (D), and full-length BID (E). The graph shows the average of three experiments at 0, 4, and 8 h, where 100% is the maximum parcleaved caspase-3 obtained. The mean ± S.D. is shown, p < 0.001. D, caspase-8. The graph shows the average of four experiments at 0, 4, and 8 h where 100% is the maximum cleaved caspase-8 obtained. The mean ± S.D. is shown, p < 0.001. E, full-length BID and 5/6-kinase. The graph shows the average of three experiments at 0, 4, and 8 h, where 100% is the maximum full-length BID remaining. The mean ± S.D. is shown, p < 0.001.
TRADD, FADD, receptor-interacting protein, IKKα, and IKKβ, are not significantly changed (data not shown). These results demonstrate that 5/6-kinase overexpression inhibits PARP cleavage and suggest that 5/6-kinase inhibits the caspase cascade upstream of PARP.

PARP cleavage is mediated by caspases-3 and -7 (27, 28). To determine whether 5/6-kinase overexpression modulates caspase-3 activation, vector control cells or 5/6-kinase-overexpressing cells were examined for the levels of activated caspase-3. Upon TNFα/CHX treatment, cleaved caspase-3 is observed in vector control cells within 4 h, and full-length caspase-3 disappeared by 8 h (Fig. 5C). However, 5/6-kinase-expressing cells showed markedly reduced caspase-3 activation. Only low levels of activated caspase-3 were detected by 8 h. These results suggest that 5/6-kinase inhibits the activation of caspase-3.

Caspase-8 is a critical initiator of the apoptotic pathways triggered by TNF-R1 and is responsible for processing caspase-3 (29). To determine whether caspase-8 activation is affected by 5/6-kinase, caspase-8 levels were measured in TNFα-treated vector control and 5/6-kinase cells. As shown in Fig. 5D, vector control cells exhibit caspase-8 activation within 4 h, with the majority of caspase-8 cleaved by 8 h. However, 5/6-kinase-expressing cells show retarded activation of caspase-8, with only low levels of cleaved caspase-8 appearing within 8 h (20% of that seen in vector cells as shown in the graph.) Activation of caspase-8 induces BID truncation, which activates the mitochondrial pathway of apoptosis (30). Therefore, inhibition of BID cleavage is expected in response to 5/6-kinase overexpression. Consistently, 5/6-kinase-expressing but not vector control cells maintained full-length BID levels (4-fold greater than in vector cells as shown in the graph) (Fig. 5E). This result concurs with the observation that the levels of BID in 5/6-kinase RNAi cells were lower than that of control cells (Fig. 3). Consistent with the role of truncated BID in intrinsic apoptotic pathways, caspase-9 activation was reduced in 5/6-kinase-expressing cells (data not shown). These results demonstrate that the protective effect of 5/6-kinase on TNFα-induced apoptosis is because of inhibition of the TNF-R1-induced apoptosis upstream of caspase-8 activation.

**DISCUSSION**

We have shown that 5/6-kinase inhibits TNFα-induced cell death by specifically blocking apoptotic-signaling pathways. This phenotype is specific for TNFα, as 5/6-kinase did not protect cells against death induced by cycloheximide, etoposide,
5/6-Kinase Inhibits TNF-induced Apoptosis

Fig. 6. Effects of 5/6-kinase overexpression on TRADD- and FADD-induced caspase-8 cleavage. A–C, HEK 293 cells overexpressing 5/6-kinase or vector control cells are transiently transfected with empty vector in the presence of Z-Glu-Val-Asp-fluoromethyl ketone (20 μM) for 48 h. After TNFα/CHX (1 ng/ml and 0.5 μg/ml, respectively) treatment for the indicated times, Western blot analysis was performed using antibodies against TRADD, FADD, caspase-8, and 5/6-kinase. A, caspase-8. The graph shows the average of three experiments at 0, 2, 4, and 6 h where 100% is the maximum cleaved caspase-8 obtained. The mean ± S.D. is shown, p < 0.001. B, transfected with TRADD-FLAG. The graph shows the average of three experiments at 0, 2, 4, and 6 h where 100% is the maximum cleaved caspase-8 obtained. The mean ± S.D. is shown, p < 0.001 at 4 and 6 h, p < 0.01 at 0 and 2 h. C, transfected with FADD-FLAG (FADD-FL). The graph shows the average of three experiments at 0, 2, 4, and 6 h where 100% is the maximum cleaved caspase-8 obtained. The mean ± S.D. is shown, p > 0.1 at all time points.

or anti-Fas antibody. Overexpression of 5/6-kinase protected cells against TNFα-induced caspase-8, caspase-3, and BID cleavage. In addition, a decrease in expression of 5/6-kinase rendered cells more susceptible to TNFα-induced cell death, through a mechanism independent of NF-κB activation. Further, overexpression studies of signaling proteins suggested
that 5/6-kinase may exert its inhibition of the caspase cascade through TRADD.

We showed previously (10) that 5/6-kinase could phosphorylate IκBα. It was hypothesized that 5/6-kinase may inhibit apoptosis through the regulation of NF-κB. This phosphorylation event was predicted to decrease the stability of IκBα and therefore enhance the activation of NF-κB, which then feeds back to block the apoptotic pathway. However, our results from IκBα and NF-κB functional assays show that 5/6-kinase does not significantly alter the NF-κB pathway. NF-κB nuclear translocation, transcriptional activity, and levels of anti-apoptotic proteins regulated by NF-κB did not change significantly. However, NF-κB targets many different genes, and we cannot definitely exclude the possibility of 5/6-kinase affecting another NF-κB-regulated gene as a further mechanism for apoptosis inhibition.

The formation of DISC is an early and important stage of both Fas and TNF-R1 signaling (15). There are several anti-apoptotic factors that can inhibit both death-receptor pathways. Inhibitors of apoptosis, such as inhibitor of apoptosis protein 1, are recruited to the DISC and block caspase activation by interfering with the prodomain or the catalytic domain of caspases. (see Ref. 31, and for review see Ref. 32). In addition, FLIP (FLICE/caspase-8 inhibitor protein), a caspase-8-like protein that lacks the death effector domain, functions as a dominant negative inhibitor of caspase-8 and affects both Fas and TNFα signaling (33, 34). In contrast to 5/6-kinase, these known inhibitors of caspase-8 activation affect both Fas- and TNFα-mediated cell death and therefore differ from the TNFα-specific protection by 5/6-kinase. Another possible target of 5/6-kinase anti-apoptotic activity is silencer of death domain (SODD) (35). SODD specifically interacts with TNF-R1 and competes with TRADD for binding to the receptor. Overexpression of SODD inhibits TNFα signaling, resulting in decreased NF-κB activation and apoptosis. However, 5/6-kinase specifically affects the apoptotic arm of TNF signaling. Therefore an effect on SODD is not likely a mechanism by which 5/6-kinase inhibits apoptosis.

We propose that 5/6-kinase can interfere the interaction of FADD and TRADD. Because only the apoptotic pathway is affected, inhibition of TNF-R1 and TRADD activation is not likely mechanisms for the 5/6-kinase protective effect; both are required for the NF-κB arm that is unaffected. In addition, we have shown that 5/6-kinase-mediated protection can be abrogated by FADD overexpression, thus FADD must act downstream of 5/6-kinase. The most likely mechanism is that 5/6-kinase inhibits the recruitment of FADD to TRADD. It is possible that 5/6-kinase phosphorylates a specific domain required for apoptosis but not for NF-κB activation. Furthermore, phosphorylation of FADD could inhibit its recruitment to TRADD but play no role for clustering upon overexpression.

There are two possible mechanisms for which 5/6-kinase could exert its protective effect(s). 5/6-Kinase may exhibit protein kinase activity toward subunits within the DISC. Phosphorylation has only been recently reported to affect DISC function. For instance, FADD is phosphorylated on Ser-194 in a cell cycle-dependent manner (36). Phosphorylation of FADD can enhance apoptosis induced by etoposide (37). Also, PKC II may regulate cellular FLIP expression and phosphorylation in response to Fas (38). It is conceivable that 5/6-kinase could selectively phosphorylate a component within DISC and alter its function specific to TNFα signaling. Another possibility is an isoinositol phosphate product of 5/6-kinase could affect binding and function of DISC. Based on in vitro studies by Wilson and Majerus (5), 5/6-kinase is responsible for generating isoinositol 1,3,4,5-tetakisphosphate and inositol 1,3,4,6-tetakisphosphate, precursors for inositol 1,3,4,5,6-pentakisphosphate and inositol hexakisphosphate. Elevation of cellular inositol phosphates or depletion of substrates, such as inositol 1,3,4-trisphosphate, could affect the formation of DISC and would represent a novel link between inositol phosphate and cell death signaling pathways. Future studies are needed to dissect the protein and inositol phosphate kinase activities of 5/6-kinase and determine the activity required for TNFα-protection.

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REFERENCES

1. Majerus, P. W. (1996) 
2. Irvine, R. F., and Scheff, M. J. (2001) 
3. Ohman, A. R., Stahlberg, A., Wente, S. R., and York, J. D. (2000) 
4. Verheij, J. W., Wilson, M. P., Kuselova, M. V., Majerus, P. W., and Wente, S. R. (2002) 
5. Wilson, M. P., and Majerus, P. W. (1996) 
6. Chang, S. C., Miller, A. L., Feng, Y., Wente, S. R., and Majerus, P. W. (2002) 
7. Hansen, C. A., von Dalhi, S., Bredt, D., and Williams, J. (1988) 
8. Yang, X., and Shears, S. B. (2000) 
9. Wilson, M. P., Sun, Y., Can, L., and Majerus, P. W. (2001) 
10. Sun, Y., Wilson, M. P., and Majerus, P. W. (2002) 
11. Schwechheimer, C., Serino, G., Callis, J., Crosby, W. B., Lypapina, S., Deshaies, R. J., Gray, W. M., Estelle, M., and Deng, X. W. (2001) 
12. Lymarina, S., Cope, G., Shevchenko, A., Serino, G., Tuzhe, Z., Zhou, C., Wolf, A. D., Wei, N., Shevchenko, A., and Deshaies, R. J. (2001) 
13. Tomoda, K., Kubota, Y., Arata, Y., Mori, S., Maeda, M., Tanaka, T., Yoshida, M., Yoneda-Kato, N., and Kato, J. Y. (2002) 
14. Karin, M., and Lin, A. (2002) 
15. Koschel, F. C., Heilbarth, S., Behrman, I., Germer, M., Pawlita, M., Krammer, P. H., and Peters, K. H. (1995) 
16. Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) 
17. Kuida, K., Haydar, T. F., Kuan, C. Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. (1998) 
18. Yoshida, H., Kong, Y. G., Yoshida, R., Elia, A. J., Hakem, A., Hakem, R., Benninger, J. M., and Mak, T. W. (1998) 
19. Rothwarf, D. M., and Karin, M. (1999) 
20. Gobbo, S., and Karin, M. (2002) 
21. Song, W. X., Edelstein, L. C., Chen, C., Bash, J., and Gelinas, C. (1999) 
22. Kemp, C. J., Sun, S., and Gurley, K. E. (2000) 
23. Mizu, S., Stowell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) 
24. Nicholos, D. W. (1999) 
25. Deckere, P., Isenberg, D., and Muller, S. (2000) 
26. Deshmukh, M., Vlasakolas, J., Decker, T. L., Lampe, P. A., Shivers, B. D., and Johnson, E. M., Jr. (1996) 
27. Varfolomeev, E. E., Schuchmann, M., Luria, J., Chiannilkulhai, N., Beckmann, J. S., Matsuoka, K., and Lapidot, T. (2002) 
28. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) 
29. Shu, H. B., Takeuchi, M., and Goddell, D. V. (1996) 
30. Shi, Y. (2002) 
31. Miecz, O., Lens, S., Gaude, O., Aleviopoulos, K., and Tschopp, J. (2001) 
32. Zong, W. X., Edelstein, L. C., Bash, J., and Gelinas, C. (1999) 
33. Vidal, S., and Sibille, C. (1999) 
34. Sun, Y., Wilson, M. P., and Majerus, P. W. (2002) 
35. Jiang, Y., Woronicz, J. D., Liu, W., and Goeddel, D. V. (1999)