Early Activation of c-Jun N-terminal Kinase and p38 Kinase Regulate Cell Survival in Response to Tumor Necrosis Factor α*

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Fas ligand and tumor necrosis factor α (TNF) bind to members of the TNF receptor superfamily. Stimulation by Fas ligand results in apoptosis, whereas TNF induces multiple effects including proliferation, differentiation, and apoptosis. Activation of the c-Jun N-terminal kinase (JNK) and p38 kinase pathways is common to Fas and TNF signaling; however, their role in apoptosis is controversial. Fas receptor cross-linking induces apoptosis in the absence of actinomycin D and activates JNK in a caspase-independent manner. In contrast, TNF requires actinomycin D for apoptosis and activates JNK and p38 kinase with biphasic kinetics. The first phase is transient, precedes apoptosis, and is caspase-independent, whereas the second phase is coincident with apoptosis and is caspase-dependent. Inhibition of early TNF-induced JNK and p38 kinases using MKK4/MKK6 mutants or the p38 inhibitor SB203580 increases TNF-induced apoptosis, whereas expression of wild type MKK4/MKK6 enhances survival. In contrast, the Mek inhibitor PD098059 has no effect on survival. These results demonstrate that early activation of p38 kinase (but not Mek) are necessary to protect cells from TNF-mediated cytotoxicity. Thus, early stress kinase activation initiated by TNF plays a key role in regulating apoptosis.

Fas ligand (FasL) and tumor necrosis factor α (TNF) bind to members of the TNF receptor superfamily (1). Fas/CD95/ FasL and tumor necrosis factor α (TNF) bind to members of the TNF receptor superfamily (1). Fas/CD95/APO-1 receptor oligomerization induced by FasL or by agonist antibodies results in apoptosis in a variety of cells, including T, B, and NK cells, macrophages, and fibroblasts (2). TNF binds to two ubiquitously expressed receptors, TNF receptor I (TNFR1/ p55) and TNF receptor II (TNFRII/p75), that do not share any homology within their cytoplasmic domains (3). Unlike FasL, TNF elicits a wide range of cellular effects, including apoptosis, proliferation, differentiation, inflammation, and chemotaxis (4).

FasL and TNF activate apoptotic signaling pathways through a similar mechanism. Fas and TNF interact either directly or indirectly with the adapter protein FADD/MORT1, which recruits caspase 8 to the receptor complex (reviewed in Ref. 3). The resulting cascade of caspase activation causes cleavage of cytosolic, cytoskeletal, and nuclear proteins and leads to apoptosis (5). TNFRI and TNFRII also associate with molecules that do not interact with Fas, TRAF2 binds directly to TNFRII and is indirectly associated with TNFRI via TRADD (3). TRAF2 mediates rapid activation of NF-κB, JNK, and p38 kinases in response to TNF (6–8). The Raf-Mek-Erk (MAPK) cascade can be activated through FAN, a protein that binds to TNFRI and activates neutral sphingomyelinase (9).

Although JNK and p38 kinase pathways are activated by both Fas and TNF receptor oligomerization (10–13), it is not clear whether this occurs through a common mechanism. JNK and p38 kinase are key mediators of the inflammatory response and are activated by cytokines, growth factors, and a variety of cellular stresses, including UV and ionizing radiation, hyperosmolarity, and heat shock (reviewed in Ref. 14). JNKs are activated by phosphorylation on Tyr and Thr by the dual specificity kinases, MKK4/SEK1 (15, 16) and the newly identified MKK7 (17). Similarly, p38 kinases are activated by MKK3 and MKK6/SAPKK3; MKK6 is the predominant activator of p38α (18–20). Downstream effectors of the stress pathways include the transcription factors c-Jun, ATF-2, and Elk-1, which are phosphorylated and activated in response to the JNK cascade, whereas ATF-2, Elk-1, Max, and the kinases MAPKAPK-2/3 and MNK1/2 (21, 22) are activated by the p38 cascade (reviewed in Ref. 14).

The role of the stress-activated kinases during apoptosis induced by various stimuli is controversial. Several published reports propose that overexpression of constitutively active forms of stress kinase regulators, such as MEKK1 (23–26), ASK1 (27), and MKK6b (28), result in apoptosis. Conversely, stable cell lines expressing dominant negative mutants of MKK4 (29) or JNK1 (30) inhibit apoptosis stimulated by various agents, suggesting that stress kinases play a proactive role during apoptosis. In contrast, other reports suggest that stress kinases do not promote apoptosis. For example, transient expression of catalytically inactive MEKK1, MKK4, or c-Jun mutants does not impair Fas- or TNF-induced apoptosis (6, 31) but can inhibit γ-radiation-induced and UV-C radiation-induced apoptosis (24). Furthermore, thymocytes from mice deficient in MKK4 are more susceptible to Fas- and CD3-induced apoptosis than wild type thymocytes, suggesting that the JNK pathway may protect cells from apoptosis (32). These widely varying conclusions highlight the importance of understanding differences in stress kinase signaling in response to various apoptotic stimuli.

This report defines an important new role for JNK and p38 kinases during TNF-induced apoptosis. Our results indicate that JNK and p38 kinases are activated through distinct mechanisms during Fas- and TNF-induced apoptosis. While Fas receptor cross-linking induces apoptosis in the absence of actinomycin D (Act. D) and activates JNK in a caspase-dependent manner, TNF requires Act. D for apoptosis and activates JNK and p38 kinase with biphasic kinetics. The first phase is transient, precedes apoptosis, and is caspase-independent, whereas
the second phase is coincident with apoptosis and is caspase-dependent. Inhibition of early TNF-induced JNK and p38 kinase by expression of dominant negative mutants of MKK4, MKK6, or the p38 inhibitor SB203580 increased TNF-induced apoptosis, whereas expression of wild type MKK4 and MKK6 enhanced cell survival in the presence of TNF. In contrast, the Mek inhibitor PD98059 had no effect on survival. In addition, neither JNK nor p38 kinase is required during Fas- or TNF-induced apoptosis; rather, late phase activation is a response to caspases. These results demonstrate that early activation of JNK and p38 kinase (but not Mek) mediates cell survival signals during exposure to TNF.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents—**KYM-1 cells were obtained from the ATCC (Rockville, MD) and were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Summit Biotechnologies, Ft. Collins, CO), 8 mM l-glutamine, penicillin/streptomycin (50 μg/ml each); Irvine Scientific, Santa Ana, CA). NIH-3T3 cells (ATCC) were maintained in Dulbecco’s minimal essential medium (high glucose), 10% calf serum (Summit Biotechnologies) supplemented as above. L929-cyt16 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum (Summit Biotechnologies), 8 mM l-glutamine, penicillin/streptomycin (50 μg/ml each), and 1% non-essential amino acids (Life Technologies, Inc.). L929 cells were starved in Dulbecco’s minimal essential medium (0.1% bovine serum albumin, penicillin/streptomycin, 10% Opti-MEM) and KYM-1 cells in AIM-V medium (Life Technologies, Inc.) for 30 min at 37 °C followed by 5 μg/ml goat anti-rat IgG+IgM (The Jackson Laboratory, West Grove, PA). TNF-induced apoptosis was initiated with 20 ng/ml TNF (murine, Boehringer Mannheim; human, Chiron) ± 0.5 μg/ml Act. D (Sigma). The caspase inhibitor Z-VAAD-FMK (Kamiya, Seattle, WA) was preincubated with cells for 1 h prior to antibody cross-linking or TNF addition. Cell extracts were prepared after the appropriate incubation periods by rinsing cells in cold phosphate-buffered saline followed by lysis in Triton lysis buffer (20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 15% glycerol, 1% Triton), Complete inhibitor mixture (Boehringer Mannheim), 1 μM pepstatin (Boehringer Mannheim), 1 mM sodium vanadate, and 2 mM β-glycerophosphate). Extracts were then cleared at 14,000 rpm for 10 min and quick frozen. Protein concentration was determined by the BioRad Protein Assay (Pierce) using bovine serum albumin as a standard.

**Caspase Activity Assay—**Caspase 3 activity was measured in vitro by incubating 10 μg of cell extract with 20 μM Z-DEVD-afc (fluorescent substrate (Kamiya) in 100 μl of Triton lysis buffer supplemented with 1 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 2 mM dithiothreitol (33)). Substrate cleavage was allowed to occur for 20 min at room temperature, and released fluorescence was measured in a 96-well plate fluorometer (excitation, 400 nm; emission, 505 nm). The assay was found to be linear at maximum caspase activation under these conditions.

**Plasmid Constructs—**Flag-tagged MKK4, JNK1, and GST-jun (1–79) cDNAs were a generous gift from R. Davies. Flag-tagged MKK4, MKK4KR, and ΔNTRAP2 were inserted into the CMV promoter-driven expression vector pCG (8, 34). cDNA encoding IκBα (a gift from S. Haskill) was PCR amplified and inserted in-frame into the pCGF vector (pCG modified to include an N-terminal Flag tag). IκBα phosphorylation sites Ser-32 and Ser-36 were mutated to Ala (IκBαSR) (35) using the Quickchange PCR mutagenesis kit (Stratagene, La Jolla, CA). Full-length MKK6 was cloned from an expressed sequence tag (GenBank® accession no. R42128, Genome Systems, St. Louis, MO) by PCR amplification and inserted in-frame into pCGF. MKK6 mutants to replace Lys-82 with Met (MKK6KM) (18) were made by two step PCR mutagenesis using express sequence tag R42125 as a template and inserted into pCGF. JNK1 and p38α cDNAs were PCR amplified and inserted in-frame into pCGN (pCG modified to include an N-terminal HA tag). The nucleotide sequence of all PCR-generated inserts was verified by sequence analysis. Expression of each construct was verified in cell lysates by Western blotting using an anti-Flag antibody (Eastman Kodak Co.).

**Cell Transfections and TNF Sensitivity—**NIH-3T3 cells were co-transfected with a green fluorescent protein expression construct pEGFP-N1 (CLONTECH, Palo Alto, CA) and either pCDNA3 (vector) or the various test constructs described above, using LipofectAMINE transfection reagent (Life Technologies, Inc.). Following a 12-h expression period, murine TNF (0.5 ng/ml) was added to the culture medium for 10 h (Boehringer Mannheim). Transfected cells were viewed by fluorescent microscopy, and sensitivity to TNF was measured by counting the number of surviving transfected cells in 10 representative microscopic fields with and without TNF treatment. TNF-induced apoptosis was expressed as the decrease in cell viability in relation to an untreated control sample for each test plasmid. To assess JNK and p38 kinase activity, NIH cells were co-transfected with HA-JNK1 or HA-p38 and various test constructs as described above. Following a 16-h expression period, cells were starved in Dulbecco’s minimal essential medium supplemented with 0.3% calf serum for 24 h and then treated with 20 ng/ml murine TNF for 15 min. Cells were then washed twice in cold phosphate-buffered saline (PBS) and then lysed in Triton lysis buffer, and kinase activity was measured as described below.

**JNK and p38 Kinase Assays—**The activity of endogenous JNKs was assessed by solid phase pull-down kinase assays as described previously (36). Briefly, 100 μg of cell extract was incubated in the presence of 8 μg of recombinant GST-c-Jun (1–79) as substrate and GSH-Sepharose beads (Sigma) for 3 h at 4 °C. GST-Sepharose beads were then washed in JNK reaction buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, and 0.1 mM sodium vanadate). The in vitro kinase reaction was carried out in 30 μl of JNK reaction buffer supplemented with 20 μM [γ−32P]ATP (Amersham Pharmacia Biotech) for 20 min at 32 °C. Transfected JNK1 and p38α kinases were immunoprecipitated with anti-HA antibodies (Babco, Richmond, CA) and polyacrylamide gel electrophoresis transferred to nitrocellulose, and relative phosphorylation was quantitated using the Bio-Rad Molecular Imager System. The expression levels of JNK and p38α were verified by Western blotting using the anti-HA antibody (Babco).

**MTT Assay—**Approximately 5 × 10⁵ L929-cyt16 cells per well were plated into 96-well plates, allowed to grow for 36 h, and then pretreated for 30–60 min with MeSO, Act. D (0.5 μg/ml), or various concentrations of the inhibitors PD908059 (New England Biolabs, Beverly, MA) or SB203580 (Calbiochem) in starvation medium prior to TNF addition. Cells were incubated in TNF at 0, 1, 10, or 100 ng/ml in triplicate for 6 h, after which, 10 μl of WST-1 reagent (Boehringer Mannheim) was added for an additional 2 h. The optical density of each sample (in triplicate) was determined at 450 nm using a Thermomax microplate reader (Molecular Devices, Menlo Park, CA).

**Promoter–V–Apoptosis Assay—**L929-cyt16 cells were starved for 20–24 h in starvation medium and then pretreated with the inhibitors PD908059 and SB203580 for 30 min prior to addition of TNF (10 ng/ml; Boehringer Mannheim). After 5 h of TNF treatment, cells were rinsed once in phosphate-buffered saline, stained with the ApoAlert Annexin V apoptosis kit (CLONTECH) according to the manufacturer’s instructions, and then analyzed on a fluorescence-activated cell sorter (Epics, Coulter, Miami, FL).

**RESULTS**

The kinetics of Fas-induced JNK activity correlate with caspase activation and apoptosis. The relationship between Fas-induced apoptosis, caspases, and JNK activation was investigated in a murine fibroblast cell line stably expressing a chimeric receptor consisting of the extracellular and transmembrane domains of murine CD4 fused to the cytoplasmic domain of murine Fas (L929-cyt16) (37). Within 30 min after receptor cross-linking, using anti-CD4 and secondary cross-linking antibodies (Fig. 1A), the cells began to exhibit membrane blebbing and nuclear condensation. Nearly 90% of the cells were apoptotic within 90 min in either the presence or absence of Act. D (Fig. 1A). Therefore, Fas-induced apoptosis is neither enhanced nor inhibited by signaling pathways leading to new gene expression.

The kinetics of JNK and caspase activation were measured in cell extracts from L929-cyt16 cells made over a similar time course. Initiation of the Fas pathway activated DEVD-specific caspases with kinetics that preceded the appearance of membrane blebs (compare Fig. 1A and 1B). The kinetics of endoge-
nous JNK activation closely resembled those of caspase activation but were slightly delayed compared with them, beginning at approximately 30 min after antibody cross-linking. Similar results were also obtained using the agonist CH-11 anti-Fas antibody in Jurkat cells expressing endogenous Fas (data not shown). Therefore, caspase activity precedes JNK activation during Fas-induced apoptosis.

**The Caspase Inhibitor Z-VAD-FMK Inhibits Fas-induced JNK Activity**—To investigate whether JNK activation induced by Fas was caspase-dependent, L929-cyt16 cells were pre-
L929-cyt16 cells were treated with 20 ng/ml TNF and 0.5 μg/ml Act. D for various times and then photographed and scored for membrane blebbing as in Fig. 1. Whole cell extracts were made from TNF (20 ng/ml) treated L929 cyt-16 in the absence (A) or presence (B) of 0.5 μg/ml Act. D over a 6-h course of treatment. JNK and ICE protease activities were assessed as indicated previously and are represented as fold induction over untreated cells. A representative of three independent experiments is shown.

The kinetics of stress kinase activation and their dependence on transcription and caspase activation were also examined in response to TNF. L929-cyt16 cells were treated over a 6-h time course with TNF in the presence or absence of Act. D, and apoptotic cells were counted. In the absence of Act. D, 15% of cells died, whereas in the presence of Act. D, apoptosis increased to greater than 95% (Fig. 2). This is in contrast to Fas-induced apoptosis in the same cell line where apoptosis increased to greater than 95% (Fig. 2B). A similar profile was seen with p38 kinase activation in the same extracts (data not shown). Cells treated with Act. D only for 60 or 360 min displayed no enhancement of JNK, p38, or caspase activity (data not shown). Furthermore, in human KYM-1 cells, which are sensitive to TNF in the absence of biosynthetic inhibitors, two peaks of JNK activation were also observed (data not shown). These results demonstrate that early TNF-stimulated JNK and p38 kinase activity is independent of caspases, whereas late phase activity coincides directly with caspase activation. Furthermore, early stress kinase activation occurs in cells regardless of whether they ultimately undergo apoptosis, whereas late phase activity occurs only in cells during apoptosis.

**TNF-induced JNK Activation Occurs by Caspase-dependent and Caspase-independent Means**—To confirm the relationship between JNK and caspase activation during TNF-induced apoptosis, extracts were prepared from KYM-1 cells treated with TNF in the presence or absence of Z-VAD-FMK. Z-VAD-FMK did not affect the early phase of TNF-induced JNK activity at 15 min (Fig. 3), but it did inhibit late phase JNK at 4 h by almost 50%. Similar results were observed in L929-cyt16 cells. Early JNK was not affected by caspase inhibition; however, late phase JNK could not be assessed because of the obvious cytotoxicity of ZVAD-FMK after 3 h of TNF treatment (data not shown). Taken together, our data demonstrate that early phase JNK activation is caspase-independent, whereas late phase JNK is partially caspase-dependent.

**Inhibition of JNK and p38 Kinase Pathways Sensitize Cells to TNF-induced Apoptosis**—The results described above suggest that TNF induces an early peak of stress kinase activation that does not promote apoptosis. To investigate whether the early phase of JNK and p38 kinase protects cells from apoptosis, we inhibited each pathway by transiently expressing dominant negative mutants of MKK6 (MKK6KM) or MKK4 (MKK4KR) (15, 18). NIH-3T3 cells were used because L929-cyt16 cells were difficult to transfect to high efficiency. The expression levels from each construct were equalized as indicated by Western blotting using an anti-Flag antibody (Fig. 4A). Treatment of cells with TNF alone (0.5 ng/ml) for 10 h induced cytotoxicity in approximately 30% of cells transfected with vector (Fig. 4A). However, in cells expressing MKK6KM or MKK4KR, TNF-induced apoptosis increased to 60%, suggesting that JNK and p38 pathways are required for survival in response to TNF. Consistent with this view, expression of either MKK6-wt or MKK4-wt reduced apoptosis to 15 and 25%, respectively, indicating that JNK and p38 pathways enhance cell survival during TNF exposure. A similar increase in TNF-induced apoptosis (30% over vector control) was also observed using a super-repressor IκBα containing phosphorylation site mutations (IκBαSR) that prevent inducible NF-κB DNA binding activity (35) (Fig. 4A). These data are consistent with those reported by others (41, 42). In vitro kinase assays indicate that overexpression of either MKK6-wt or MKK4-wt stimulates both JNK and p38 kinases; similarly, expression of MKK6KM or MKK4KR inhibits both JNK and p38 kinase (Fig. 4, C and D). Therefore, in transient expression analysis, we cannot distinguish between the role of JNK and p38 kinase. Although it appears that IκBα-SR expression inhibits JNK and p38 kinase activity, it visibly impairs expression of the HA-JNK and HA-p38 reporter constructs and therefore may indirectly affect kinase activity.

Overexpression of an N-terminally truncated TRAF2...
DNTRAF2 inhibits NF-κB, JNK, and p38 signaling pathways in response to TNF (Fig. 4, C and D) (6–8). In our hands, inhibition of all three pathways by expression of DNTRAF2 sensitized 70% of transfected cells to TNF cytotoxicity (Fig. 4A), a 10% increase over inhibition of the JNK and p38 pathways. This suggests that JNK, p38, and NF-κB pathways may coordinately activate protective signals mediated by TNF.

Chemical Inhibitors of p38 but Not MEK Sensitize Cells to TNF-induced Apoptosis—In addition to JNK and p38, the MAPK cascade is activated in response to TNF (8, 9). We made use of specific kinase inhibitors to investigate the role of Mek during TNF-induced apoptosis and to address the specific protective role of p38 kinase in L929-cyt16 cells. The inhibitor PD098059 is specific for Mek1/Mek2 (43), whereas SB203580 specifically inhibits p38α kinase (44, 45). L929-cyt16 cells were pretreated with each inhibitor for 30 min and then with various concentrations of TNF for several hours. TNF sensitivity was then assessed in two different assays: 1) cell viability in a MTT assay (46) (Fig. 5A and B), and 2) Annexin V binding, an early marker of apoptosis (47) (Fig. 5C). The p38 inhibitor SB203580 increased TNF-induced cell death in a dose-dependent manner (Fig. 5A and C). Endogenous JNK activity was not affected by SB203580 at either concentration or by PD098059 (data not shown), demonstrating that p38 kinase is required for protective signals during TNF exposure. The MEK inhibitor PD098059 did not affect TNF-induced apoptosis even at 30 μM, a concentration at which TNF-induced phosphorylation of Erk1/Erk2 was completely inhibited (data not shown). These results demonstrate that the MAPK cascade is not involved in a protective response to TNF (Fig. 5B and C).

To address the requirement for p38 kinase during Fas- and TNF-induced apoptosis, L929-cyt16 cells were pretreated with SB203580, and then either Fas- or TNF-mediated apoptosis was triggered. Caspase activity was slightly enhanced with increasing doses of p38 inhibitor for both Fas- and TNF-induced apoptosis (Fig. 6). This clearly demonstrates that p38 is not required for either form of apoptosis. Interestingly, the p38 inhibitor enhances caspase activity in cells treated with TNF.
and Act. D, suggesting that p38 may mediate posttranscriptional protective responses. Together, these results demonstrate that \( p38 \) is not required for Fas- or TNF-induced apoptosis but is an important mediator of an early TNF response, necessary for cell survival.

**DISCUSSION**

This report outlines differences in stress kinase activation between Fas and TNF and defines an important protective role for JNK and p38 kinases during TNF signaling. Our results demonstrate that early p38 kinase but not Mek signaling pathways are necessary for cell survival in the presence of TNF (Figs. 4 and 5). Previous work shows that TNF-induced NF-\( \kappa \)B is also necessary for cell survival (6, 41, 42, 48). More recent results indicate that cells from TRAF2-deficient mice activate NF-\( \kappa \)B but not JNK activity in response to TNF and are more susceptible to apoptosis than cells from wild type mice (49, 50). Both reports suggest the existence of TRAF2-dependent signaling pathways distinct from NF-\( \kappa \)B that also protect cells from apoptosis. JNK and p38 kinase may be such protective pathways. Although the use of dominant negative mutants in transient expression assays does not permit us to discriminate between the activities of JNK and p38, it is likely that their activities overlap because of common downstream effectors (14). However, the specificity of the SB203580 inhibitor allows us to define a specific requirement for p38 kinase as a mediator of protective signals. Our data suggest that although early stress kinase activation is necessary, it may not be sufficient to confer complete protection against TNF-induced apoptosis (Fig. 4).

Most cultured cell lines require inhibitors of transcription or translation for a cytotoxic response to TNF (Fig. 3) (39, 40, 51, 52), suggesting that protective mechanisms may involve new gene induction. The NF-\( \kappa \)B regulated genes \( A20 \) (53), manganese superoxide dismutase (54) and \( \text{c-IAP2} \) (55) are up-regulated following TNF treatment and protect cells from apoptosis. Although studies show that JNK, p38 kinase, and NF-\( \kappa \)B pathways diverge downstream of TRAF2 (6, 8, 56), each pathway leads to activation of transcription factors, such as c-Jun, ATF-2, and NF-\( \kappa \)B, which can interact and may cooperate during transcription (57, 58). Surprisingly, the MAPK pathway does not contribute to these survival mechanisms (Fig. 5), suggesting that downstream effectors of the MAPK pathway, some of which are common to JNK and p38 (14), are not involved in protective gene induction. However, our results also suggest that protective mechanisms for TNF may be posttranscriptionally regulated (Fig. 6). The recently identified p38 substrates Mnk1 and Mnk2 can phosphorylate eIF-4E (22), an essential step during translation initiation (59). In addition, p38 kinase enhances translation of specific messages containing the AUUUA repeat motif in their 3'-untranslated region (45). Therefore, early coordinate activation of JNK, p38 kinase, and NF-\( \kappa \)B pathways is a key regulatory mechanism for TNF-induced apoptosis.

Our results demonstrate that JNK and p38 kinases are ac-
activated through distinct mechanisms during Fas- and TNF-induced apoptosis. Fas-induced JNK activation is entirely caspase-dependent in L929-cyt16 cells (Fig. 1). Fas-mediated JNK, MKK6b, and p38 kinase activation is also caspase-dependent in the lymphoid cell lines SKW 6.4 (60) and Jurkat (13, 24, 28, 31). In contrast, this is the first demonstration that early induction of JNK and p38 activity by TNF is independent of caspases and apoptosis, whereas late phase JNK and p38 correlate with caspase activation, are partially caspase-dependent, and occur only during apoptosis (Figs. 2 and 3 and data not shown). The lack of early stress kinase activation during Fas signaling may be explained by evidence linking TRAF2 to TNF but not Fas receptor complexes (61, 62).

There are several mechanisms by which caspases may activate stress response pathways. Caspases cleave and activate D4-GDI/Ly-GDI (63), hPAK65/PAK2 (64, 65), and MEKK-1 (26) and can generate ceramide (66, 67); these are four upstream stimulators of JNK and p38 kinases. Our results show that late phase TNF-stimulated JNK activity cannot be completely inhibited by Z-VAD-FMK (Fig. 3). This phase of JNK may be independently stimulated by oxygen radicals released from the mitochondria, which in L929 cells begins after 1 h of TNF treatment (68). Mitochondrial breakdown may also explain why late phase JNK activation slightly precedes caspase activation (Fig. 2). Our results demonstrate that neither JNK nor p38 activity is required for Fas- or TNF-induced apoptosis (Figs. 4–6). This is in agreement with recent results indicating that short term inhibition of JNK or p38 pathways cannot prevent TNF- or Fas-induced apoptosis in fibroblasts and T cells (6, 13, 31, 69). Therefore, late phase Fas- and TNF-induced stress kinase activation is a response to caspase activation and does not contribute to cell death.

Although Fas and TNF share homology within their cytoplasmic domains, as well as some common associated molecules, our results show that signaling to apoptosis through the two receptors is clearly distinct in at least three ways. First, the kinetics of Fas-induced apoptosis in L929-cyt16 cells (Fig. 1). Fas-mediated JNK, MKK6b, and p38 kinase is mediated through TRAF2, which binds directly to TNFRII and via TRADD to TNFRI. These three signaling cascades and perhaps others may coordinate protective signals, preventing apoptosis and allowing a wide range TNF-mediated cellular effects. If any one of the protective signals is inhibited, the apoptotic pathway will proceed, presumably by FADD recruiting and activating a cascade of caspases, which then result in late phase stress kinase activation and apoptosis. In contrast, the absence of early protective signals, such as NF-κB, JNK, and p38 following Fas receptor oligomerization may suggest why Fasl leads directly to caspase activation and apoptosis.

Fig. 7. Early and late stage signaling pathways regulating Fas- and TNF receptor-mediated apoptosis. TNF binds to TNFRI, causing receptor oligomerization and the initiation of multiple signaling pathways. Early transient activation of NF-κB, JNK, and p38 kinase is mediated through TRAF2, which binds directly to TNFRII and via TRADD to TNFRI. These three signaling cascades and perhaps others may coordinate protective signals, preventing apoptosis and allowing a wide range TNF-mediated cellular effects. If any one of the protective signals is inhibited, the apoptotic pathway will proceed, presumably by FADD recruiting and activating a cascade of caspases, which then result in late phase stress kinase activation and apoptosis. In contrast, the absence of early protective signals, such as NF-κB, JNK, and p38 following Fas receptor oligomerization may suggest why FasL leads directly to caspase activation and apoptosis.

The early TNF-induced JNK and p38 Kinase Regulate Cell Survival
bolic inhibitors (Fig. 1A) (72, 73). TNF-stimulated stress kinase activation and NF-κB may lead to protective gene expression, suggesting a mechanism by which Act. D can enhance TNF-mediated but not Fas-mediated apoptosis in L929-cyt16 cells (Figs. 1 and 2). In fact, evidence suggests that early TNF signaling may induce survival mechanisms that prevent TNF-induced but not Fas-induced apoptosis (73). Together, these data outline clear distinctions in the mechanism regulating Fas- and TNF-induced apoptosis, a mechanism that, for TNF, involves early coordinate activation of JNK, p38 kinase, and NF-κB pathways.

In summary, this study characterizes differences in signaling mechanisms between Fas and TNF that regulate apoptosis (Fig. 7). We have identified a new role for JNK and p38 kinase as essential components of an early response to TNF that, in combination with NF-κB, mediate survival signals that protect cells from apoptosis. Furthermore, MAPK is not involved in this protective response. Because the onset of TNF-induced apoptosis is delayed, early activation of protective signals may have time to act in the absence of caspase activation, possibly by regulating new gene expression. Activation of JNK and p38 kinase during Fas-induced apoptosis and during the late phase of TNF-induced apoptosis is not required for cell death but rather is a stress response to caspase activation. Tight regulation of survival versus apoptotic signals may contribute to the ability of TNF to elicit a wide range of cellular effects. The identification of genes involved in survival mechanisms may reveal the therapeutic potential for TNF in cancer treatment.

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REFERENCES
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