Translation Inhibitors Sensitize Prostate Cancer Cells to Apoptosis Induced by Tumor Necrosis Factor-related Apoptosis-inducing Ligand (TRAIL) by Activating c-Jun N-terminal Kinase*

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Nand K. Sah‡§, Anupama Munshi‡, John F. Kurland‡, Timothy J. McDonnell‡, Bing Su†, and Raymond E. Meyn‡**

From the Departments of ‡Experimental Radiation Oncology, ‡Molecular Pathology, and †Immunology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in several human tumors both in vitro and in vivo, however, some tumors remain resistant for poorly understood reasons. Using a quantitative DNA fragmentation assay for apoptosis, we have shown that human prostate cancer cells are resistant to a wide range of TRAIL doses up to 500 ng/ml. However, translation inhibitors, such as anisomycin, cycloheximide, emetine, harringtonine, and puromycin, unlike several transcription inhibitors, significantly sensitized PC3-neomycin (PC3-neo) cells to TRAIL-induced apoptosis. These effects were inhibited in PC3 cells engineered to express bel2 (PC3-bel2). Translation inhibitors led to activation of c-Jun N-terminal kinase (JNK), which plays a role in this sensitization process because inhibition of JNK activation resulted in protection against TRAIL plus translation inhibitor-induced apoptosis. JNK activation may be required for this process, but it is not sufficient because activation of JNK using an MEKK2 expression vector did not mimic the sensitizing effect of translation inhibitors. Other stress-activated protein kinases, such as ERK and p38, play an insignificant role in determining the apoptotic sensitivity. We conclude that activation of JNK is required for sensitization of PC3 cells to TRAIL-induced apoptosis by translation inhibitors in cells that are otherwise TRAIL-resistant. However, in addition to JNK activation, other aspects of translation inhibition such as the suppressed activity of apoptosis-inhibitory proteins or activation of other signal transduction pathways must also be involved.

Tumor necrosis factor α-related apoptosis-inducing ligand (TRAIL),† also known as Apo2L (1–2), is synthesized, similar to other tumor necrosis factor (TNF) superfamily members, as a membrane-bound apo-protein that can be cleaved to generate soluble TRAIL. TRAIL is being actively investigated as a cancer therapeutic agent, because different types of tumor cells are vulnerable to apoptotic death by soluble TRAIL, whereas normal cells are relatively insensitive to this effect (3–5). The reason for this discriminatory apoptotic function of TRAIL has been ascribed to restricted receptor distribution. There are five known receptors for TRAIL: (i) TRAIL-R1, DR4; (ii) TRAIL-R2, DR5; (iii) Decoy receptor-1, TRAIL-R3, TRID, LITE (6, 7); (iv) Decoy receptor-2, TRUNDD, TRAIL-R4 (8); and (v) osteoprotenrin (OPG) (9). DR4 and DR5 are the only functional receptors known so far that can induce apoptosis upon ligation with TRAIL, because they possess a complete cytoplasmic death domain, which interacts downstream with Fas-associated death domain protein and caspase 8 (10). The decoy receptors have either a deleted or inactive death domain and are, therefore, unable to transduce an apoptotic signal upon interaction with TRAIL. OPG is a secreted protein with a similar decoy function. These decoy receptors were thought to abstract TRAIL from productive engagement with DR4 and DR5, thus explaining why TRAIL preferentially kills some cell types but not others. However, the relative expression of the active or decoy receptors does not generally correlate with the relative TRAIL responsiveness of cells, and the biochemical and molecular pathways that govern the propensity for TRAIL-induced apoptosis are still poorly understood.

As a TNF superfamily member, TRAIL shares many common features with TNF and Fas/CD95-induced apoptosis. Important to note, however, are the observations that TRAIL may follow a faster kinetics of apoptosis induction than TNF and Fas/CD95 and that TNF and Fas/CD95 seldom preferentially discriminate between tumor and normal cells for inducing apoptosis, whereas TRAIL does (11). However, it is also noteworthy that not all tumor cells respond equally well to TRAIL cytotoxicity. Recently, we have shown that the human prostate cancer cell lines, PC3, DU145, and LNCaP, which are resistant to low concentrations of TRAIL, may be sensitized to TRAIL-induced apoptosis by cycloheximide (CHX), a protein synthesis inhibitor (12). Overexpression of bel2 completely blocked induction of apoptosis by CHX and TRAIL in these cells. This effect is similar to what has been observed in many previous reports on TNF, FAS, and TRAIL (13–17). The conventional explanation for this phenomenon has been that CHX suppresses the synthesis of a short-lived protein that normally interferes with death receptor-mediated apoptosis (12, 14, 18). FLICE inhibitory protein is an example of one such protein (12, 18). However, it has been known for some
time that, in addition to their ability to inhibit translation, protein synthesis inhibitors also activate c-Jun N-terminal kinases (JNKs).

The JNKs are classic examples of stress-activated protein kinases. A number of stress stimuli, including UV radiation (19), heat and osmotic shock (19), protein synthesis inhibition (20), inflammatory cytokines (21), growth factor withdrawal, and chemotherapeutic drugs (13, 22), induce potent and preferential activation of JNKs. Several reports show that JNK may regulate apoptosis (23–25). Using prostate cancer as a model system, we have investigated the role of JNK in the sensitizing effects of protein synthesis inhibitors for TRAIL-induced apoptosis. Our observations demonstrate that activation of the pro-apoptotic JNK pathway is necessary but not sufficient for these effects.

**Fig. 1.** TRAIL induces dose-dependent induction of apoptosis in combination with CHX in the PC3-neo but not in the PC3-bcl2 cells. A, cells were harvested for apoptosis-assay (DNA fragmentation) following treatment with and without 0–500 ng/ml of TRAIL for 6 h. B, PC3-neo and PC3-bcl2 cells were treated with 0–50 ng/ml of TRAIL plus CHX for 6 h before harvest for apoptosis-assay. C, Western blot showing levels of bcl2 expression in the sensitive (PC3-neo) and resistant (PC3-bcl2) cells following treatment with CHX (10 μg/ml) plus TRAIL (15 ng/ml) for 1–6 h. β-Actin levels in all these treatments depict equal loading.

**Fig. 2.** Other translation inhibitors also exert comparable sensitization. Five structurally different translation inhibitors (5 μM) in combination with 15 ng/ml TRAIL for 6 h induce closely comparable levels of apoptosis in the PC3-neo and PC3-bcl2 cells. Aniso, anisomycin; CHX, cycloheximide; Harringtonine; and Puro, puromycin.

**Fig. 3.** Transcription inhibitors exert marginal effect on TRAIL-induced apoptosis. Three structurally unrelated transcription inhibitors in combination with TRAIL (15 ng/ml) induce marginal apoptosis in PC3-neo cells. Cells were pre-treated for 24 h with various transcription inhibitors prior to treatment with TRAIL for 6 h. Act D, actinomycin D (20 μg/ml); a-ama, α-amanitin (2 μg/ml); DRB, dichlorobenzimidazole riboside (100 μM); and T, TRAIL (15 ng/ml).
EXPERIMENTAL PROCEDURES

Cell Lines and Recombinant Human TRAIL—The neomycin control (PC3-neo) and the bcl2-transfected variant (PC3-bcl2) of the PC3 human prostate cancer cell line were obtained from Timothy J. McDonnell (The University of Texas M.D. Anderson Cancer Center) and maintained in a selection medium of RPMI 1640 supplemented with 10% fetal bovine serum, 2 mML-glutamine, 10,000 units/ml penicillin-streptomycin, and 200 μg/ml G418 (Invitrogen). The recombinant bioactive human TRAIL without a disulfide-linked homotrimer was purchased from R&D Systems (Minneapolis, MN).

Antibodies—Antibody to the phospho-specific form of JNK was purchased from Promega (Madison, WI). Antibodies to phospho-specific forms of p38 and pERK2 were obtained from Cell Signaling Technology (New England Biolabs, Beverly, MA). Total p38 and pERK2 antibodies were obtained from Cell Signaling Technology. JNK, c-Jun, and phospho-specific c-Jun antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to bcl-2 and β-actin were purchased from DAKO (Carpenteria, CA) and Sigma (St. Louis, MO), respectively.

Chemicals—L-JNKI1 (JNK inhibitor) was purchased from Alexis Biochemicals (San Diego, CA). CHX, anisomycin, emetine, harringtonine, puromycin, α-amanitin, actinomycin D, DRB, and curcumin were obtained from Sigma. SB203580 was obtained from Calbiochem (San Diego, CA). Transfection reagent FuGene6 was purchased from Roche (Indianapolis, IN).

DNA Fragmentation Assay—This apoptosis-specific assay is a modified method of Sellins and Cohen (26). Briefly, [14C]thymidine-labeled cells were treated for up to 6 h with TRAIL to induce apoptosis. At the end of the treatment, cells were harvested, washed thrice with phosphate-buffered saline, and lysed in 0.5 ml of lysis buffer containing 10 mM Tris, 1 mM EDTA, and 0.2% Triton X-100, pH 7.5. The lysed pellet was incubated on ice for 20 min. The chromatin fraction was separated by centrifugation at 14,000 × g for 10 min at room temperature. The supernatant containing fragmented DNA and the pellet containing unfragmented DNA were solubilized separately in 1 ml of Soluene (Packard, Meriden, CT) overnight in a 60 °C water bath. Radioactivity was determined in a liquid scintillation counter (Packard Instruments, Downers Grove, IL). Percent DNA fragmentation was calculated as the percent radioactivity in the supernatant divided by total radioactivity.

Western Blot Analysis—Following treatments, the cells were harvested and the pellet washed in cold phosphate-buffered saline. The cells were lysed by vortexing in a lysis buffer containing 50 mM HEPES, pH 7.0, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10% glycerol, and 10 μg/ml each of aprotinin and leupeptin. The lysate was incubated at 4 °C for 20 min on ice before centrifugation at 14,000 × g for 10 min at 4 °C for 15 min. The supernatant was assayed for protein concentration by Bio-Rad protein reagent (27). Equal amounts of protein were resolved on 8–12% polyacrylamide gels and then transferred...
to Immobilon nylon membrane (Millipore, Bedford, MA). After the membrane was washed in TBS-T (20 mM Tris, 150 mM NaCl, and 0.05–0.1% Tween 20 at pH 7.4), it was incubated in a blocking buffer as per the manufacturer's suggestions. The membrane was incubated with primary antibody in a suitable buffer for 1 h at room temperature or overnight at 4 °C. After several washes, the membrane was incubated with a compatible secondary antibody tagged with horseradish peroxidase for 1 h at room temperature, washed in TBS-T, and developed by enhanced chemiluminescence (ECL Plus, Amersham Biosciences, Piscataway, NJ) on a Storm 860 PhosphorImager (Amersham Biosciences).

**RESULTS**

**CHX Sensitizes PC3-neo Cells to Dose-dependent Induction of Apoptosis by TRAIL**—Treatment of PC3 cells with TRAIL alone for 6 h produced negligible apoptosis in both the PC3-neo and PC3-bcl2 cells (Fig. 1A). A dose of TRAIL as high as 500 ng/ml induced only about 5% apoptosis in the PC3-neo control cells and about 2% in the bcl2 line following a 6-h treatment. However, a 6-h combination treatment with CHX (10 μg/ml) dramatically sensitized PC3-neo cells to apoptosis by TRAIL in a dose-dependent manner using comparatively low doses (Fig. 1B). A dose as low as 5 ng/ml of TRAIL was able to induce about 27% apoptosis, which increased to 75% at 20 ng/ml. The bcl2-overexpressing cells were resistant by comparison but were also sensitized by CHX. The same range of TRAIL doses (5–20 ng/ml) produced a maximum of about 10–15% apoptosis in the bcl2-overexpressing cells. The levels of bcl2 expression in the PC3-neo and its bcl2-overexpressing variant remained approximately the same in each cell line with and without treatment with CHX and TRAIL (Fig. 1C).

**Other Translation Inhibitors Also Sensitize PC3 Cells to TRAIL-induced Apoptosis**—We determined whether effects similar to those produced by CHX could be obtained with other translation inhibitors, i.e. anisomycin, emetine, harringtonine, and puromycin when used in combination with TRAIL for 6 h. Each of these inhibitors potently sensitized PC3-neo cells to TRAIL-induced apoptosis. As shown in Fig. 2, equimolar concentrations (5 μM) of the translation inhibitors sensitized the PC3-neo cells to TRAIL-induced apoptosis in a dose-dependent manner using comparatively low doses (Fig. 2A). A dose as low as 5 ng/ml of TRAIL was able to induce about 27% apoptosis, which increased to 75% at 20 ng/ml. The bcl2-overexpressing cells were resistant by comparison but were also sensitized by CHX. The same range of TRAIL doses (5–20 ng/ml) produced a maximum of about 10–15% apoptosis in the bcl2-overexpressing cells. The levels of bcl2 expression in the PC3-neo and its bcl2-overexpressing variant remained approximately the same in each cell line with and without treatment with CHX and TRAIL (Fig. 1C).
PC3-neo cells to TRAIL-induced apoptosis, whereas bcl2 overexpression exerted a significant resistance to all of these agents. These results indicate that the ability of translation inhibitors to sensitize PC3 cells to TRAIL toxicity is not limited to CHX. None of the translational inhibitors induced significant apoptosis on their own (Fig. 2).

**Transcription Inhibitors Exert Marginal Effect on TRAIL-induced Apoptosis**—TRAIL can activate NF-κB, a pro-survival transcription factor, which, in turn, can transcriptionally activate anti-apoptotic genes, such as bcl2, bclXL, cIAPs, and XIAP (28–32). If these or other transactivated genes suppress TRAIL-induced apoptosis, then transcription inhibitors should also be able to sensitize PC3 cells. Cells were pre-treated with various transcription inhibitors for 24 h followed by a 6-h exposure to TRAIL. As shown in Fig. 3, actinomycin D (20 μg/ml), which is a classic transcription inhibitor, α-amanitin (2 μg/ml), and dichlorobenzimidazole riboside (DRB) (100 μM), when used at their optimal doses to inhibit transcription, induced about 4, 9, and 25% apoptosis, respectively, when combined with TRAIL, as compared with 76% with CHX plus TRAIL. These observations suggest that inhibition of the transcription of putative TRAIL-mediated or constitutively expressed apoptosis inhibitory factors does not contribute to the sensitization of PC3 cells to TRAIL-induced apoptosis.

**CHX and Anisomycin Activate JNK**—We performed additional experiments to further understand the mechanism by which protein synthesis inhibitors sensitize cells to TRAIL. It has been known for some time from reports in the literature that both CHX and anisomycin activate JNK (13, 17, 20). Activation of JNK by CHX (10 μg/ml) was evident within 30 min (Fig. 4A) in PC3-neo cells. Anisomycin (5 μM) also followed a similar time course of JNK activation in PC3-neo cells (Fig. 4B). In the PC3-bcl2 cells, a similar kinetics of JNK activation by CHX and anisomycin was observed, but the level of activation was significantly suppressed compared with that in PC3-neo cells. c-Jun, the classic downstream target of active JNK, was also observed to be activated with similar kinetics by CHX and anisomycin in PC3-neo and PC3-bcl2 cells (Fig. 4, A and B). We also observed activation of c-Jun by emetine, harringtonine, and puromycin (data not shown). The fact that emetine, harringtonine, and puromycin activate JNK and/or c-Jun albeit to differing degrees has been previously reported (17, 33, 34). The basal levels of JNK and c-Jun remained more or less the same in all the above-mentioned treatments with or without CHX and anisomycin. JNK was not activated by TRAIL alone (Fig. 4C) but was activated by the combination of TRAIL plus CHX to a degree similar to that seen with CHX alone.

**CHX and Anisomycin Also Activate p38 Kinase**—We also examined p38 kinase, another member of the family of stress-activated protein kinases. As shown in Fig. 5 (A and B), both CHX and anisomycin potently activated p38 kinase within 30 min of incubation in PC3-neo as well as PC3-bcl2 cells. The total p38, however, remained unchanged with or without treatment with CHX and anisomycin. ATF2, the downstream target of active p38, was also examined and was found to be activated with a kinetics similar to that of p38 by both CHX and anisomycin. There was no activation of p38 by TRAIL alone (Fig. 5C), however, p38 was activated by the combination of TRAIL plus CHX.

**SB203580 Inhibits the Activation of ATF2 by p38 but Has Only a Marginal Effect on Induction of Apoptosis**—p38 kinase, which was activated significantly by CHX and anisomycin, was examined for its possible involvement in the ability of protein synthesis inhibitor-mediated sensitization of apoptosis. SB203580 specifically inhibits the downstream activation of ATF2 by p38. As shown in Fig. 6A, CHX plus TRAIL induced about 54% apoptosis in the PC3-neo cells, whereas pretreatment of these cells for 1 h with SB203580 (20 μM) marginally lowered apoptosis by about 5%. However, this concentration of SB203580 exerted significant suppres-
The data in Fig. 7 (and A) was seen when anisomycin was used in place of CHX (Fig. 7 plus TRAIL-mediated apoptosis by about 90%. A similar effect correlated with an effect on JNK activation but not with p38

Curcumin, a natural product purified from the turmeric (Curcuma longa) plant, is known to inhibit protein kinases in a nonspecific manner (36, 37). We used curcumin to further investigate the role of JNK and p38 in TRAIL-mediated cell death. The results (Fig. 7A) demonstrated that curcumin protects against CHX plus TRAIL-induced apoptosis. Pretreatment of the cells for 1 h with 25 μM curcumin suppressed CHX plus TRAIL-mediated apoptosis by about 90%. A similar effect was seen when anisomycin was used in place of CHX (Fig. 7B). The data in Fig. 7 (A and B) are consistent with a role for protein kinases in CHX and anisomycin-mediated apoptotic sensitization. We, therefore, tested whether curcumin inhibits anisomycin-mediated activation of JNK and p38 by Western blot analyses. As shown in Fig. 7C, pretreatment with 25 μM of curcumin for 1 h exerted a significant suppression of anisomycin-induced JNK-activation in PC3-neo cells. Interestingly, curcumin was not able to suppress the lower level of anisomycin-induced JNK activation seen in the PC3-bcl2 cells. This suggests that anisomycin activates JNK in PC3 cells by two different routes, one that is inhibited by curcumin and bcl2 and one that is not inhibited by either. In contrast, p38 kinase, which was also activated significantly by anisomycin in both PC3-neo and PC3-bcl2 cells, was not significantly modulated by curcumin. Thus, the ability of curcumin to suppress the apoptotic sensitization mediated by CHX and anisomycin correlated with an effect on JNK activation but not with p38 activation.

The Peptide L-JNKI1 Selectively Inhibits JNK and Suppresses Apoptosis—Because curcumin activity is not specific for JNK, we used L-JNKI1, a peptide that specifically binds to and inhibits JNK activity, to further test the role of activated JNK. As shown in Fig. 8 (A and B), preincubation with L-JNKI1 for 24 h partially protected PC3-neo cells from anisomycin plus TRAIL-induced apoptosis in a dose- and time-dependent manner.

Western blot analysis, presented in Fig. 8C, demonstrated that L-JNKI1 exerts a dose-dependent inhibition of anisomycin-activated JNK and p-c-Jun. However, in the PC3-bcl2 cells, anisomycin (5 μM) induced minimal activation of JNK, which remained unaffected following treatment with L-JNKI1.

Western blot analysis results presented in Fig. 9A demonstrated that 5 μM anisomycin activated JNK and c-Jun upon treatment for 1 h, as was shown previously. Transfection of the PC3-neo cells with 0.5 μM of dn-JNK DNA for 72 h suppressed CHX plus TRAIL-induced apoptosis by about 58% as compared with the same treatment but with an inactive control vector (normalized to 100) (Fig. 9A). In a separate experiment with anisomycin in place of CHX, transfection with dn-JNK suppressed apoptosis by 44% (Fig. 9B).

Western blot analysis results presented in Fig. 9C demonstrate that 5 μM anisomycin activated JNK and c-Jun upon treatment for 1 h, as was shown previously. Transfection of the PC3-neo cells with 0.5 μM of dn-JNK for 72 h suppressed the activation of JNK and c-Jun by anisomycin consistent with a
role for JNK activation in the sensitization of PC3 cells to TRAIL-induced apoptosis by protein synthesis inhibitors. Specific Activation of JNK Using an MEKK2 Expression Vector Sensitizes Cells to TRAIL-induced Apoptosis—The previous experiments demonstrated that activation of JNK is required for sensitization to TRAIL-induced apoptosis by translation inhibitors. However, this left questions unanswered: whether activation of JNK is sufficient by itself to sensitize cells to TRAIL or there are other aspects of these translation inhibitors that contribute to the overall sensitization process. To address these questions, we pre-treated PC3-neo and PC3-bcl-2 cells with a MEKK2 expression vector for various periods of time and then assessed activation of JNK and p-c-Jun by Western blot analysis and TRAIL-induced apoptosis on the basis of DNA fragmentation. The results, shown in Fig. 10, indicate that JNK was maximally activated by 24 h following transfection of PC3-neo cells with the MEKK2 vector and that activation was sustained for at least 72 h. Activation of p-c-Jun was also observed by 24 h, peaking by 48 h and then declining by 72 h. Although the MEKK2 vector did not induce substantial apoptosis by itself, it did sensitize the PC3-neo cells to TRAIL-induced apoptosis. The levels of apoptosis, however, did not reach those achieved in the TRAIL plus CHX controls. The time course was also different; the PC3-neo cells required 24, 48, and 72 h of JNK activation by MEKK2 to achieve 20, 30, and 60%, respectively, of the apoptosis level seen in the TRAIL plus CHX control. The MEKK2 vector also activated JNK and p-c-Jun in the PC3-bcl2 cells but to a lesser degree compared with the PC3-neo cells, and there was minimal restoration of TRAIL-induced apoptosis in this situation.

**DISCUSSION**

Our results indicate that PC3 cells are relatively resistant to TRAIL as a single agent even at doses as high as 500 ng/ml (Fig. 1A). Previous reports and our own studies (data not shown) show that TRAIL, like TNFα, activates NF-κB, a strong pro-survival factor capable of transactivating several antiapoptotic genes, including bcl2, bcl-xL, XIAP, and cIAP (28–32). Thus, inhibitors of eukaryotic transcription would be expected to sensitize PC3 cells to TRAIL-mediated apoptosis, if their resistance was due to either constitutive or induced expression of apoptosis-inhibitory factors. We tested various structurally unrelated transcription inhibitors, including actinomycin D, α-amanitin, and DRB. Although they were not totally without effect, none of these well known agents except DRB induced more than 10% apoptosis in combination with TRAIL (Fig. 3). DRB produced about 25% apoptosis with TRAIL, which may also be related to its ability to interfere with several additional cellular functions, such as inhibition of casein kinase II and p70S6 kinase and induction of G1/S arrest (38).

In contrast to the transcription inhibitors, five unrelated translation inhibitors potently sensitized PC3 cells to TRAIL-induced apoptosis (Fig. 2). In PC3-bcl2 cells, although a small amount of apoptosis was restored, bcl2 overexpression effectively suppressed cell death by TRAIL in combination with translation inhibitors. The disparity between transcription in-
hibitors and translation inhibitors with regard to their ability to facilitate TRAIL-induced apoptosis in PC3-neo cells raises a question about the role of apoptosis-inhibitory factors in TRAIL resistance. These findings do not rule out such factors, because it is possible that a stable transcript for the factor exists in the cells that would require only translation to be expressed. However, an alternative explanation would be that inhibitory factors have either no or only a minor role and that the translation inhibitors sensitize cells to TRAIL via some other mechanism. The latter possibility is further evidenced by the fact that interferon-α, which suppresses protein synthesis significantly in the PC3 cells, produced only marginal apoptosis in combination with TRAIL (data not shown). It has been established in the literature that inhibition of translation by CHX and anisomycin is perceived by the cells as a stress that leads to activation of the stress-activated protein kinases, particularly JNK (13, 17, 20). We, therefore, investigated whether activation of these kinases by translation inhibitors could explain the ability of these agents to sensitize PC3 cells to TRAIL. We found that ERK was poorly activated by CHX and anisomycin (data not shown), and, therefore, a role for ERK was not pursued further. In contrast, p38 kinase and its downstream transcription factor, ATF2, were potently activated by CHX and anisomycin. However, the fact that inhibition of activated p38 by SB203580 did not suppress apoptosis suggests that p38 is not significantly involved in the translation inhibitor-sensitized TRAIL-induced apoptotic pathway of PC3 cells. This was further supported by the observation that curcumin, which inhibited apoptosis almost completely, had only a negligible effect on activated p38 kinase. On the other hand, translation-inhibitor sensitization to TRAIL-induced apoptosis was significantly suppressed by JNK inhibitors suggesting a role for activated JNK in this sensitization. Inhibitors with different specificities for JNK were tested: curcumin (a nonspecific protein kinase inhibitor), L-JNKI1 (a specific inhibitor of JNK), and a dn-JNK construct. Curcumin suppressed induction of apoptosis almost completely, whereas L-JNKI1 and dn-JNK exerted a partial suppression, suggesting that, although JNK plays an important role, there may be other elements of the signal transduction cascade triggered by translation inhibition involved as well. Our observations comprising the general involvement of JNK in the apoptotic pathway are adequately supported by several previous reports (24, 39–43).

The specific JNK inhibitors partly suppressed the ability of the translation inhibitors to sensitize to TRAIL-induced apoptosis indicating a requirement for JNK activation in this context.

Fig. 9. Expression of dominant negative (dn) JNK down-regulates active JNK (p-JNK), active c-Jun (p-c-Jun), and induction of apoptosis due to CHX/anisomycin plus TRAIL. A, using FuGene6, PC3-neo cells were transfected with 0.5 μg of the inactive control vector or the active dn-JNK construct, incubated at 37 °C for 72 h followed by treatment with CHX (C) (10 μg/ml) plus TRAIL (T) (15 ng/ml) for 6 h. The cells were harvested and subjected to apoptosis assay. Apoptosis with the inactive control (vect + C + T) was normalized to 100. B, the same as in A, but with anisomycin (A) (5 μM) in place of CHX. C, using FuGene6, PC3-neo cells were transfected with 0.5 μg of the dn-JNK plasmid and incubated at 37 °C for 72 h followed by treatment with anisomycin (A) (5 μM) for 1 h. The cells were harvested and subjected to Western blot analysis, as described in Fig. 7C, for active JNK (p-JNK), active c-Jun (p-c-Jun), and β-actin (for loading control).
effect. Thus, the question remains: is activation of JNK alone sufficient to sensitize PC3 cells to TRAIL. We tested this possibility through the use of an MEKK2 expression vector that has previously been shown to potently activate JNK (44, 45). The results (Fig. 10) indicated that, although pretreatment of the PC3-neo cells with this vector activated JNK by 24 h, TRAIL-induced apoptosis was only minimally restored compared with the level of apoptosis seen when TRAIL and CHX are combined for only 6 h as shown in Fig. 1. This comparison suggests that specifically activating JNK does not mimic the sensitizing effect of translational inhibitors. Activation of JNK is apparently at least partially required for this effect but is not sufficient to reproduce it. We suggest, therefore, that in addition to activation of JNK other effects mediated by the translation inhibitors are also required and that the suppressed synthesis of apoptosis-inhibitory proteins cannot be ruled out.

The mechanism by which activated JNK sensitizes cells to TRAIL-induced apoptosis is not fully understood, but at least three different possibilities can be envisioned. First, transactivation of certain genes through phosphorylation of the c-Jun transcription factor has been shown to play an important role in the apoptotic pathway in sympathetic neurons and PC12 cells. Fas ligand and Nur77 (46–48) are among the genes that are positively regulated through JNK activation. However, the involvement of c-Jun as a mediator of the sensitizing effect being discussed here would be ruled out, because it would have to produce its effect in the presence of the translation inhibitors that activated it. Furthermore, expression of a dn-c-Jun construct exerted a negligible effect on the apoptotic sensitivity of PC3 cells due to the translation inhibitors that activated it. Second, activated JNK has been shown to phosphorylate several proteins, including bcl-xL (49), bcl2 (50, 51), BAD (52), ATF2, Elk1 (53), and p53 (54). Many of these targets have identified roles in the regulation of apoptosis. The biological function of phosphorylated bcl2 may be either pro- or anti-apoptotic depending on the site phosphorylated. A recent report (55) shows that bax is essential for the apoptotic signal transduction by JNK. PC3 cells have a robust expression of bax. But their total bax levels in the cytoplasmic as well as mitochondrial fractions remain unaffected by treatment with CHX and/or TRAIL. Third, a recent report (48) found a direct activation of mitochondrial apoptotic machinery by JNK in cardiac myocytes without the engagement of c-Jun and caspase 8. Several recently identified mitochondrial proteins may be the potential targets of JNK for mitochondrial apoptotic engagement (56), but the specific mitochondrial protein targeted for apoptotic activation by JNK is yet to be identified. A recently discovered (57) mitochondrial protein, apoptosis-inducing factor is a case in point. However, unlike several other proapoptotic proteins, including TRAIL, apoptosis-inducing factor does not engage caspases for final execution of cells (58) thereby ruling out its involvement in the present scenario. We speculate that the phosphorylated forms of BAD and bax as potential targets of active JNK in PC3 cells may play significant roles in the mitochondrial apoptotic engagement.

In PC3-bcl2 cells, although a small amount of apoptosis was restored, overexpression of bcl2 in these cells blocked protein synthesis inhibitor sensitization of TRAIL-induced apoptosis (Fig. 2). Moreover, the protein synthesis inhibitors CHX and anisomycin were unable to activate JNK in these cells correlating with this inhibition of apoptosis (Fig. 4). The exact mechanism by which protein synthesis inhibitors activate JNK is not well understood, but bcl2 has previously been reported to inhibit the ability of other stimuli to activate JNK as well (59). It is also possible that these agents signal JNK activation by

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mechanisms independent of their ability to inhibit protein synthesis. Anisomycin has been shown to be able to activate JNK at concentrations that are below those necessary to produce any detectable inhibition of protein synthesis (13). In most cases, signaling through the JNK pathway is mediated by reactive oxygen species and is, therefore, a very redox-sensitive process (60). Becl2 has potent antioxidant properties that may function to inhibit the reactive oxygen species that mediate JNK activation (35, 61). The fact that becl2 expression blocked JNK activation but not activation of p38 following treatment with protein synthesis inhibitors (Fig. 5) suggests that redox regulation of these two pathways may be different. Further evidence to illustrate the potent ability of becl2 to suppress JNK activation at the level of or downstream of MEKK2 was shown in Fig. 10.

In brief, our data support the conclusion that activation of JNK is a major component of the mechanism of sensitization of PC3 cells to TRAIL-induced apoptosis by translational inhibitors. However, because activation of JNK alone is not sufficient to reproduce this sensitization, other aspects associated with translation inhibition are also important, and their identification will be critical for designing strategies for enhancing the response of tumor cells to TRAIL.

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