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Lack of Requirement of STAT1 for Activation of Nuclear Factor-κB, c-Jun NH_2-Terminal Protein Kinase, and Apoptosis by Tumor Necrosis Factor-α

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Abstract Tumor necrosis factor (TNF) is one of the most potent activators of nuclear transcription factor NF-κB, c-Jun N-terminal protein kinase (JNK), and apoptosis in a wide variety of cells. The biological effects of TNF are mediated through sequential interactions of various cytoplasmic proteins with intracellular domains of TNF receptors. Whether signal transducer and activator of transcription-1 (STAT1), which mediates interferon (IFN) signaling, also plays any role in the TNF-mediated activation of NF-κB, JNK, and apoptosis has not been established. Here, we report our investigation of the role of STAT1 in TNF signaling using STAT1-deficient U3A and STAT1-stably transfected U3A-PSG91 cells. IFNα inhibited the proliferation of STAT1-expressing U3A-PSG91 cells but had no effect on STAT1-negative U3A cells. TNF alone, even up to 10 nM, had no effect on the proliferation of either U3A-PSG91 or U3A cells. Irrespective of STAT1 status, TNF induced cytotoxic effects in the presence of cycloheximide (CHX) in both cell types. Additionally, TNF-induced caspase-3 and caspase-8 activation and TNF-induced PARP cleavage were unaffected by the presence or absence of STAT1. TNF activated NF-κB, consisting of p50 and p65, in both U3A and U3A-pSG91 cells in a dose- and time-dependent manner, but the degree and rate of activation were slightly lower in U3A cells, as were IκBα degradation and NF-κB-dependent reporter gene expression. STAT1 was, however, required for IFNα-mediated downregulation of TNF-induced NF-κB activation. TNF activated JNK in both cell types, but dose and time of exposure required for optimum activation differed slightly. Thus, overall our results indicate that STAT1 plays a minimal role in TNF-mediated cellular responses.J. Cell. Biochem. 84: 803–815, 2002. © 2002 Wiley-Liss, Inc.

Key words: TNF; IFN-α; STAT1; JNK; NF-κB; apoptosis

Signal transducer and activator of transcriptions (STATs) are a family of transcription factors that are activated by most cytokines and mediate rapid induction of genes, leading to immune responses, cell growth and differentiation, cell survival, and apoptosis [Schindler, 1998; Shuai, 2000]. These proteins are latent in the cytoplasm and become activated through tyrosine phosphorylation. To date, seven different STATs have been identified in mammals [Darnell, 1997], of which STAT1 is reported to induce apoptosis in several primary and tumor cells [Chin et al., 1996, 1997; Kumar et al., 1997; Ohmori et al., 1997; Xu et al., 1998; Kirou, 2000]. Treatment with interferons (IFNs) causes activation of two receptor-associated tyrosine kinases (Janus kinases) Jak1 and Jak2, which in turn activate STAT1 by phosphorylation of Tyr^{701}. Activated STAT1 homodimerizes through a reciprocal interaction between phosphotyrosine 701 of one STAT1 and the src-homology 2 (SH2) domain of its partner. STAT1 dimers translocate to the nucleus and there activate genes containing

Abbreviations used: STAT1, signal transducer and activator of transcription-1; CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; IκB, inhibitory subunit of NF-κB; IFNα, interferon-α; JNK, c-jun NH_2-terminal kinase; NF-κB, nuclear transcription factor-κB; PARP, poly (ADP)-ribose polymerase; SEAP, secretory alkaline phosphatase; TNF, tumor necrosis factor.

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gamma-activated sequence (GAS) elements. STAT1 is itself activated by a large number of cytokines, including epidermal growth factor [Chin et al., 1996], interleukin-9 [Demoulin et al., 1996], granulocyte colony stimulating factor [Sato et al., 1997], and IFNs [Lopez-Collazo et al., 1998; Xu et al., 1998; Leon and David, 2000].

Another cytokine, tumor necrosis factor (TNF) binds to virtually all nucleated cells via its receptors, TNFR1 (also called p55 or p60), and TNFR2 (also called p75 or p80) [for references see Aggarwal and Natarajan, 1996]. The interaction of TNF and its receptor leads in almost all cell types to activation of a nuclear transcription factor NF-κB and a c-Jun N-terminal protein kinase (JNK) and in some cell types to activation of apoptosis [for references see Darnay and Aggarwal, 1999]. The activation of NF-κB is mediated through the sequential recruitment by the cytoplasmic domain of TNFR1 of TRADD (TNF-R1-associated death domain protein), TRAF2 (TNFR-associated factor 2), RIP (receptor-interacting protein) and CIKS (NF-κB-inducing kinase), leading to the activation of IKK-signalosome and hence to IκBα phosphorylation, polyubiquitination, and degradation [Chen et al., 1995; Hsu et al., 1996; Shu et al., 1996; Malinin et al., 1997; Devin et al., 2000; Zhang et al., 2000]. JNK activation by TNF is mediated through sequential interaction of TRADD, TRAF2, and CIKS with TNFR1 [Leonardi et al., 2000; Li et al., 2000]. The pathway leading to TNF-induced apoptosis involves sequential recruitment of Fas-associated death domain (FADD), FADD-like ICE (FLICE, also called caspase-8) and executioner caspases by TRADD [Ashkenazi and Dixit, 1998].

What role does STAT1 plays in TNF signaling is not fully understood. The physiological role of STAT1 has been made evident by knockout studies, where STAT1-deficient mice are impaired in their ability to respond to IFN and became susceptible to infections from bacterial and viral pathogens [Durbin et al., 1996; Meraz et al., 1996]. Mice with homozygous deletion of STAT1 are predisposed to certain malignancies and developed tumors more rapidly than their wild-type littermates [Kaplan et al., 1998]. In addition, p53-null mice that are defective for STAT1 signaling develop a broader tumor spectrum than p53-null counterparts [Kaplan et al., 1998]. These results suggest that STAT1 has a role in tumor suppression and that loss of STAT1 signaling enhances oncogenesis.

Although IFNα and TNF function through two independent pathways, they are reported to act synergistically to carry out diverse biological functions [Jahnke and Johnson, 1994; Lechleitner et al., 1998; Sekine et al., 2000]. The above findings suggested a cooperation between TNF and IFNα signaling pathways. Given STAT1's role in IFN signaling, there is a possibility that it plays similar roles in TNF signaling. Indeed two recent reports indicate that STAT1 may be associated with the TNF receptor complex [Guo et al., 1998; Wang et al., 2000]. Because the relationship between the two is not yet established, we investigated in detail the role of STAT1 in TNF signaling using U3A cells, a variant of human fibroblasts that does not express STAT1 ($STAT1^{-/-}$) [McKendry et al., 1991; Muller et al., 1993], and U3A-PSG91 cells, which have been stably transfected with STAT1 cDNA. Our results indicate that STAT1 is optional for TNF-induced activation of NF-κB, JNK, and apoptosis.

MATERIALS AND METHODS

Materials

Bacterially produced TNFα was a generous gift from Genentech, Inc., (South San Francisco, CA), and clinical grade IFNα (Roferon A) was kindly supplied by Dr. Moshe Talpaz of the M. D. Anderson Cancer Center (Houston, Texas). Caspases-3 and -8 assay kits were bought from R&D Systems (Minneapolis, MN). Rabbit polyclonal antibodies to IκBα, p50, p65, JNK1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to STAT1 and β-actin antibodies were obtained from (Znika, Canada) and Sigma, (St. Louis, MO), respectively. PARP antibody was procured from New England Biolabs (Beverly, MA), and all tissue culture media, reagents, and oligonucleotides were obtained from Life Technologies (Grand Island, NY). T4 polynucleotide kinase was purchased from New England Biolabs, and ECL reagents from Amersham Pharmacia Biotech (Piscataway, NJ). Goat anti-mouse IgG polyclonal and goat anti-rabbit IgG polyclonal horse radish peroxidase (HRP)-conjugated antibodies were from BioRad (Hercules, CA) and Transduction Laboratories (Lexington, KY), respectively. γ-^{32}P-labeled ATP (7000 mCi/mmole) and 5 methyl ^{3}H-thymidine were from

ICN (Costa Mesa, CA) and Amersham Pharmacia Biotech, respectively.

Cell Culture

STAT1-deficient human fibroblasts U3A and STAT1 reintroduced stable cell lines (U3A-PSG91) have been described [McKendry et al., 1991; Muller et al., 1993; Chin et al., 1996]. U3A cells were cultured in DMEM, containing 10% FBS and 250 μg/ml hygromycin B (CalBiochem, SanDiago), for U3A-PSG91 cells 500 μg/ml G418 was added (Life Technologies). Cells were subcultured every 3 days.

NF-κB Activation Assays

TNF-treated cells were harvested and washed in phosphate-buffered saline (PBS) by low-speed centrifugation. Nuclear extracts were prepared according to Chaturvedi et al. [2000], with some modification. Briefly, cells were washed with cold PBS and suspended in 0.4 ml of lysis buffer (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; 2.0 μg/ml leupeptin; 2.0 μg/ml aprotinin; and 0.5 mg/ml benzamidine). The cells were allowed to swell on ice for 30 min, after which 12.5 μl of 10% NP-40 was added. The tube was then gently vortexed for 10 s. The homogenate was centrifuged for 3 min in a microfuge. The nuclear pellet was resuspended in 25 μl of ice-cold nuclear extraction buffer (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF; 2.0 μg/ml leupeptin; 2.0 μg/ml aprotinin and 0.5 mg/ml benzamidine), and the tube was incubated on ice for 1 h with intermittent vortexing. This nuclear extract was then centrifuged for 5 min in a microfuge at 4°C, and the supernatant was frozen at −70°C. The protein content was measured by the method of Bradford.

To measure DNA binding of NF-κB, electrophoretic mobility shift assays (EMSA) were performed by incubating 8 μg of nuclear extract with 16 fmoles of ^{32}P end-labeled 45-mer double-stranded NF-κB oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5′-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCCAG GGAGGCGTGG- 3′) in the presence of 2 μg of poly (dI-dC) in a binding buffer (25 mM HEPES pH 7.9; 0.5 mM EDTA; 0.5 mM DTT; 1% NP-40; 5% glycerol and 50 mM NaCl) for 20 min at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on a 6.6% native polyacrylamide gel using buffer containing 50 mM Tris-200 mM glycine pH 8.5, and 1 mM EDTA buffer. After being run at 50 mA for 2 h at room temperature, the gel was dried at 85°C for 1.5 h. Quantitation and visualization of radioactive bands were carried out by a phosphorimager (Molecular Dynamics, Sunnyvale, CA), using 'Image-quant' software.

c-Jun NH_2-Terminal Kinase Assay

The c-Jun kinase assay was performed by a modified method as described earlier [Kumar and Aggarwal, 1997]. Briefly, whole-cell extracts were prepared from TNF-treated cells, and 100 μg cytoplasmic extracts were treated with anti-JNK1 antibodies. The immune complexes were precipitated with protein A/G-Sepharose beads (Pierce, USA). The kinase assay was performed using washed beads as source of enzyme and glutathione S-transferase-Jun (1–79) as substrate (2 μg/sample) in the presence of 10 μCi [^{32}P]ATP per sample. The kinase reaction was carried out by incubating the above mixture at 30°C in kinase assay buffer for 15 min. The reaction was stopped by boiling beads in SDS sample buffer. Finally, protein was resolved on 10% SDS–PAGE gel. The radioactive bands of the dried gel were visualized and quantitated by phosphorImager as mentioned previously.

Western Blot Analysis of STAT1 and IκBα

Sixty micrograms of whole cell protein was resolved on 7.5% SDS–PAGE gel. The protein was transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with STAT1 antibody (1:1,000). For IκBα, 30 μg cytoplasmic protein extracts, prepared as described [Chaturvedi et al., 1999], was resolved on 10% SDS–PAGE gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane, blocked with 5% non-fat milk, and probed with IκBα antibodies (1:3,000). The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by ECL reagent.

Apoptosis Assay

To determine the antiproliferative effects of IFNα or TNF, 2×10^3 U3A or U3A-PSG91 cells in 0.2 ml were taken in triplicate wells of 96-well plates and exposed to various concentrations of either IFNα or TNF for different days. Cell viability was then determined by the modified

tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described earlier [Hansen et al., 1989] or by the incorporation of tritiated thymidine. To determine the cell viability by thymidine incorporation, cells were pulsed with 0.5 μCi ^{3}H-TdR for 6 h, trypsinized, harvested on a glass wool filter, and counted by a Matrix 9600 direct β-counter (Packard Instruments company, Downer Grove, IL). To determine the cytotoxic effect of TNF, 20,000 cells in 0.1 ml were exposed to various concentrations of TNF (0–10 nM) in the presence of 10 μg/ml of cycloheximide (CHX) for 24 h. After incubation, cell viability was determined by the MTT assay.

The apoptotic effects of TNF in the presence of CHX were determined by activation of caspase as examined by cleavage of poly(ADP-ribose) polymerase (PARP) [Haridas et al., 1998]. Forty micrograms whole cell extracts were resolved on 7.5% polyacrylamide gel, transferred to nitrocellulose membrane, blocked with 5% non-fat milk protein, probed with PARP antibodies (1:3,000), and detected by ECL reagent.

The activation of caspase-3 and caspase-8 was also assayed by using specific synthetic substrates according to the manufacturer's protocol. In brief, 100 μg of cell lysate was incubated with 50 μl of reaction buffer and 5 μl of either caspase-3 colorimetric substrate (DEVD-pNA) or caspase-8 colorimetric substrate (IETD-pNA) at 37°C for 3 h. The optical density of the reaction mixture was read on a microplate reader at 405 nm wavelength.

NF-κB SEAP Reporter Assay

The NF-κB-SEAP reporter gene expression assay was based on our earlier report [Darnay et al., 1999]. In brief, 0.18×10^6 U3A-PSG91 and U3A cells/1.5 ml were plated in each well of a 6-well plate and incubated for 16–18 h. Cells were transiently transfected for 8 h with the expression vector (1.5 μg pCMV), pNF-κB-SEAP2 (0.5 μg), and pCMV-β-gal (0.5 μg) by the calcium phosphate method. After transfection, cells (duplicate wells) were washed and incubated with medium or with medium containing 1 nM TNF for 24 h. The culture supernatant was removed and assayed for SEAP activity. The culture supernatant (25 μl) was mixed with 30 μl of 5 × buffer (500 mM Tris Cl, pH 9, and 0.5% bovine serum albumin) in a total volume of 100 μl in a 96-well plate, and the heat-labile endogenous alkaline phosphatase deactivated by heating the mixture at 65°C for 30 min. The plate was chilled on ice for 2 min, 50 μl of 1 mM 4-methylumbelliferyl phosphate was added to each well, the plates incubated at 37°C for 2 h, and fluorescence read on a 96-well fluorescent plate reader (Fluoroscan II, Lab Systems, Needham, Heights, MA) with excitation set at 360 nm and emission at 460 nm. The average (± SEM) number of relative fluorescent light units for each transfection was determined.

To determine the efficiency of transfection, the transfected cells were fixed with 0.5% glutaraldehyde and stained with 20 mM each of $K_3Fe(CN)_6$ and $K_4Fe(CN)_6.3H_2O$, 1 mM $MgCl_2$, and 1 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) until suitable blue color cells appeared [Stanger et al., 1995]. The number of blue cells in each of five different fields per well were counted and compared.

RESULTS

In experiments designed to define the role of STAT1 in TNF signaling pathways leading to the activation of NF-κB, JNK, and apoptosis, we used STAT1-deficient U3A cells and STAT1-reconstituted U3A-PSG91 cells. Because IFNα is known to downregulate TNF-induced NF-κB activation and potentiate TNF-induced apoptosis [Manna et al., 2000], the role of STAT1 in IFN-α-mediated downregulation of TNF-induced NF-κB activation was also investigated.

STAT1 is Required for the Antiproliferative Effects of IFNα

We first examined the expression of STAT1 protein in both U3A-PSG91 and U3A cells by Western blot analysis. STAT1 was expressed in the reconstituted U3A-PSG91 cells but not in deficient U3A cells (Fig. 1A). Furthermore, as revealed by Western blot analysis, STAT1 was phosphorylated in a time-dependent manner on treatment of cells with IFN-α (Fig. 1B). STAT1 has been shown to be required for the antiproliferative effects of IFNα [Bromberg et al., 1996]. To demonstrate that U3A-PSG91, but not U3A cells, contain functional STAT1, we treated both cell types with different concentrations of IFNα for different days and then assayed cell viability (Fig. 1C). IFNα inhibited the proliferation of U3A-PSG91 cells in a time- and dose-dependent manner but had no effect on U3A cells, indicating that STAT1 is needed for the antiproliferative effects of IFNα.

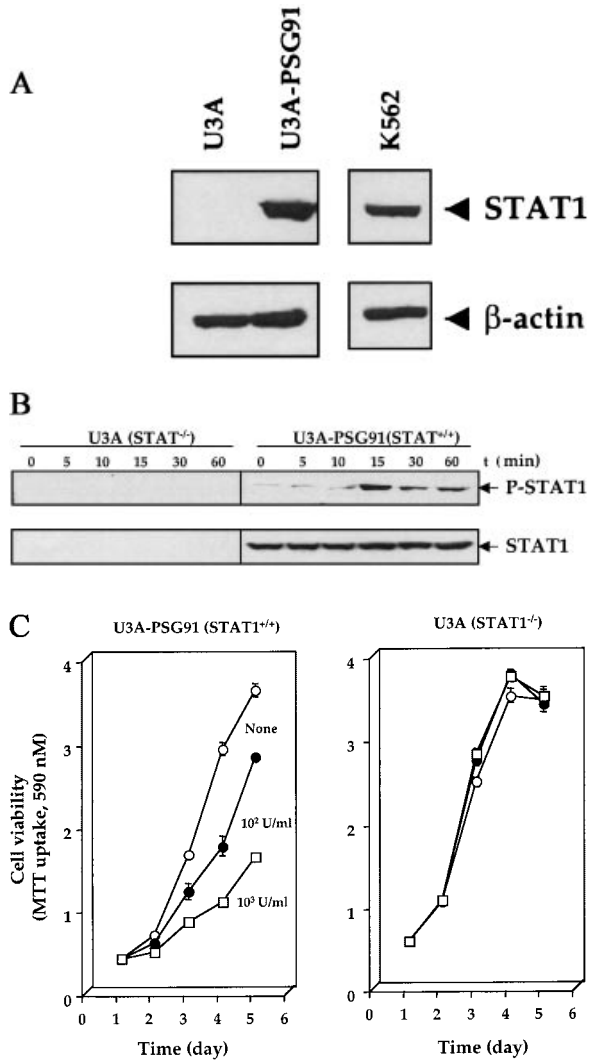


Fig. 1. **A**: Western blot analysis of STAT1 protein. Fifty micrograms of protein (whole cell extracts) from U3A-PSG91, U3A, and K562 (positive control) cells was resolved on 6% SDS–PAGE gel, electrotransferred to a nitrocellulose membrane, and probed with STAT1 antibody. **B**: Kinetics of IFN-α induced phosphorylation of STAT1 protein. One million U3A or U3A-PSG91 cells were treated with IFN-α (1,000 U/ml) for different times, prepared the whole cell extracts, resolved on 10% SDS–PAGE gels, and then probed with antibodies against either phospho-STAT-1 (upper panel) or STAT-1 (lower panel). **C**: Proliferation of U3a-PSG91 and U3a cells in the presence of IFNα. Two thousand cells of each type in 200 μl were incubated with medium containing 0, 10^2, and 10^3 U/ml IFNα for 1–5 days. The viability of the cells was determined by MTT assay, as indicated in Materials and Methods.

STAT1 is Not Required for the Cytotoxic Effects of TNF

To examine the role of STAT1 in the antiproliferative effects of TNF, we treated both U3A-PSG91 and U3A cells with 10 nM TNF for a range of days and then measured cell viability by the MTT method. TNF had no effect on the proliferation of either cell type even after a 5-day exposure (Fig. 2A,B).

When cells are treated with TNF in low serum medium or in the presence of a protein synthesis inhibitor, TNF produces cytotoxic effects. To determine if STAT1 is required for these cytotoxic effects, cells were treated with different concentrations of TNF in the presence of 1% FBS. As shown in Figure 2C, U3A-PSG91 cells

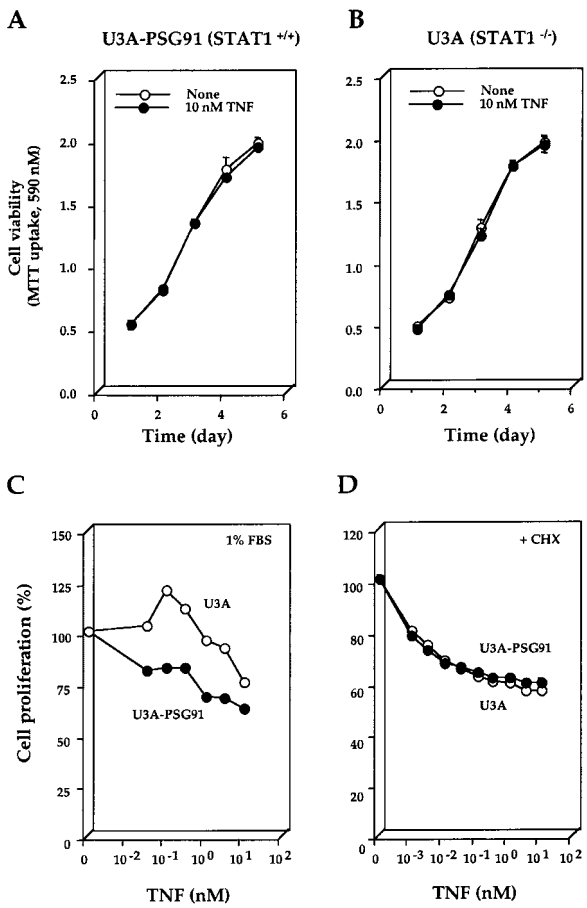


Fig. 2. **A** & **B**: Cellular proliferation in the presence of TNF. Two thousand U3A-PSG91 and U3A cells per 200 μl medium were incubated for 1–5 days in the presence of 10 nM TNF. Relative cell viability was determined by MTT assay at each time point. **C**: Antiproliferative effect of TNF in low serum containing medium. 2×10^3 cells of each type in 200 μl were incubated in 1% FBS-containing medium in the presence of various concentrations of TNF (0-10 nM) for 72 h. Cells were pulsed with [^{3}H]TdR, and incorporated thymidine was determined in a β-counter. **D**: Cytotoxic effects of TNF in the presence of CHX. 2×10^3 cells (U3A-PSG91 and U3A) per 100 μl medium were incubated in the presence of 10 μg/ml CHX and various concentrations of TNF (0-10 nM) for 24 h. Cell viability was determined by MTT assay.

were more sensitive to TNF than U3A cells. A slight increase in proliferation of U3A cells, not U3A-PSG91 cells, was observed at low concentration of TNF. When cells were treated with different concentrations of TNF in the presence of 10 μg/ml CHX, the cytotoxic effects were comparable in U3A-PSG91 and U3A cells (Fig. 2D), suggesting that STAT-1 is not required for the cytotoxic effects of TNF.

STAT1 is Not Required for the TNF-Induced Caspase Activation

The effect of STAT1 on TNF-induced cytotoxicity was examined in the presence of CHX. Whether TNF or CHX treatment affects either the levels of STAT1 protein or phosphorylation of STAT1 is not known and thus was investigated. As shown in Figure 3A, treatment of cells with TNF for different times had no affect either on STAT1 phosphorylation (upper panel) or on STAT1 protein (lower panel). Similarily, treatment with CHX also had no effect on both STAT1 protein and its phosphorylation (Fig. 3B).

There are reports which indicate that STAT1 activation mediates caspase activation and apoptosis [Chin et al., 1996, 1997; Lee et al., 1999]. Apoptosis induction by TNF is also mediated through the activation of caspase, which cleaves the PARP substrate. To examine the function of STAT1 in TNF-induced PARP cleavage, we treated U3A-PSG91 and U3A cells with different concentrations of TNF in the presence of CHX for 6 h. Western blot analysis indicated that PARP degradation in these cells was essentially equivalent, indicating that apoptosis induction was STAT1 independent (Fig. 3C).

TNF-mediated apoptosis is caused by the activation of caspases-3 and -8. To determine whether activation of these caspases was STAT1 dependent, we treated both cell types with TNF and CHX for 3, 6, and 12 h, and relative activation of these enzymes was analyzed by spectrophotometry. TNF activated caspase-3 in both cell types to a similar extent, with peak activation occurring at 12 h after treatment (Fig. 3D). The activity of caspase-8 was marginally higher in U3A-PSG91 cells. These findings suggest that STAT1 does not play any role in modulating TNF-induced apoptosis induction or caspase activation.

TNF-Induced NF-κB Activation and IκBα Degradation are Differentially Regulated in STAT1-Containing and STAT1-Deficient Cells

Activation of NF-κB is one of the earliest events initiated by TNF, occurring within a few minutes of its application. To determine whether STAT1 is required for TNF-induced NF-κB activation, we treated U3A-PSG91 cells and U3A cells with various concentrations of TNF for 30 min and measured NF-κB activation by EMSA. Figure 4A indicates that TNF activated NF-κB in both cell types in a dose-dependent manner, with maximum NF-κB/DNA binding activity occurring at 10 nM TNF. However, overall NF-κB activation was a little higher in U3A-PSG91 cells (5-fold) than in U3A cells (3-fold), thus suggesting that STAT1 may play some role in the NF-κB activation by TNF. Under these conditions, in either cell lines TNF had no affect on STAT1 phosphorylation as shown in Figure 3A.

We also examined the effect of STAT1 on the kinetics of TNF-induced NF-κB activation in both cell types. Cells were treated with 0.1 nM TNF for different times and nuclear extracts were prepared and analyzed by EMSA. This experiment revealed that maximum NF-κB activation by 0.1 nM TNF was comparable in both cells, but the rate of activation was slower in U3A cells than in U3A-PSG91 cells, suggesting that the absence of STAT1 may decrease the rate of NF-κB activation by TNF (Fig. 4B).

To confirm that NF-κB activated by TNF in both cell types consisted of p50 and p65 subunits, nuclear extracts prepared from TNF-treated cells were incubated with anti-p65 (*Anti-p65*) or anti-p50 antibodies (*Anti-p50*) and then analyzed by EMSA. Either antibody supershifted the NF-κB/DNA complex, whereas preimmune sera (PIS) had no effect (Fig. 4C). Thus, NF-κB induced by TNF in U3A-PSG91 and U3A cells contained both the p50 and p65 (RelA) subunits. The specificity of the TNF-induced NF-κB/DNA complex was further confirmed by demonstrating that the binding was disrupted in the presence of a 100-fold excess of unlabeled kB-oligonucleotide (Fig. 4C, *Cold oligo*) but not by mutant oligonucleotide (*Mutant oligo*).

Since TNF-induced degradation of IκBα is a critical step in the pathway leading to NF-κB activation, we examined whether the difference

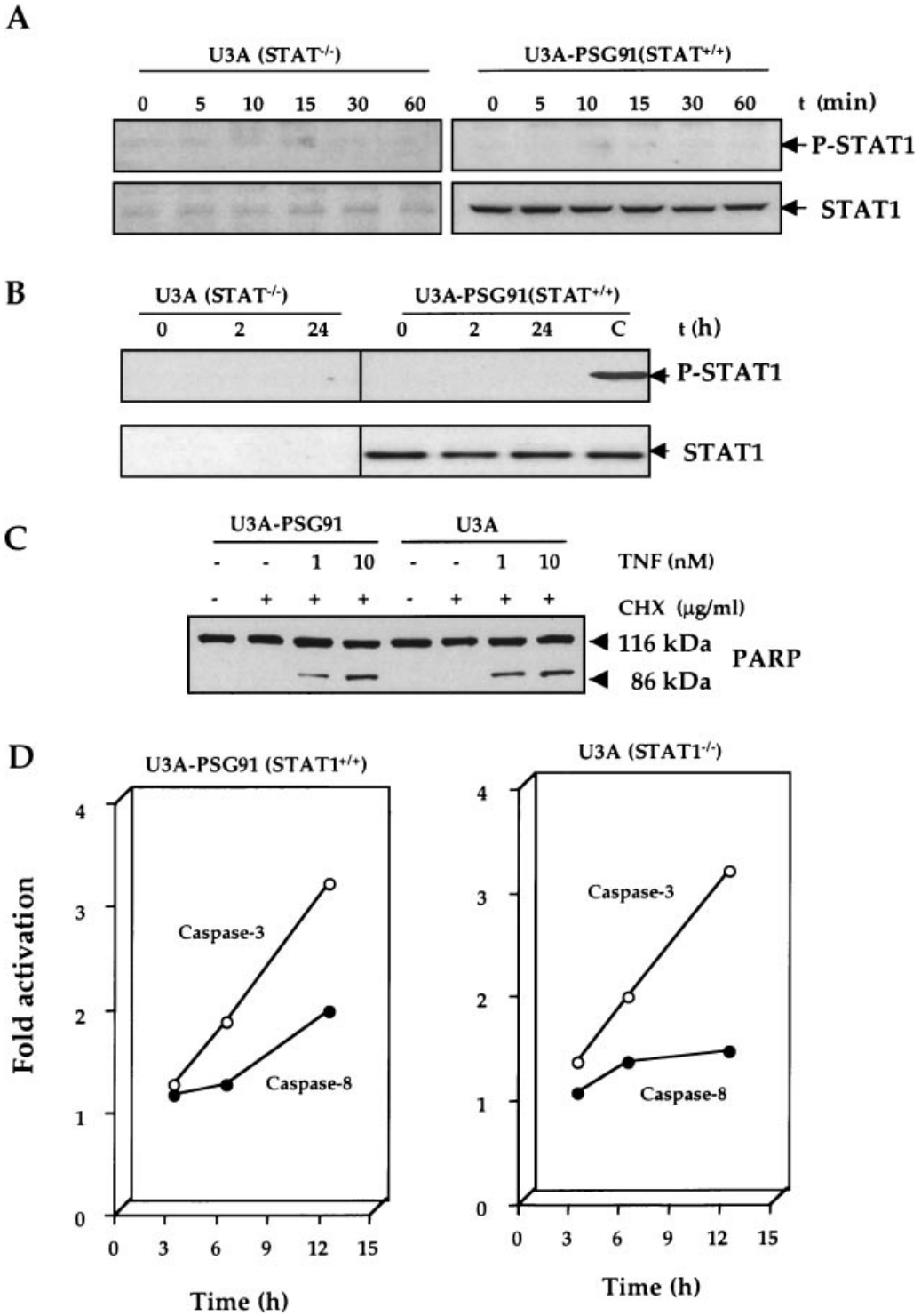
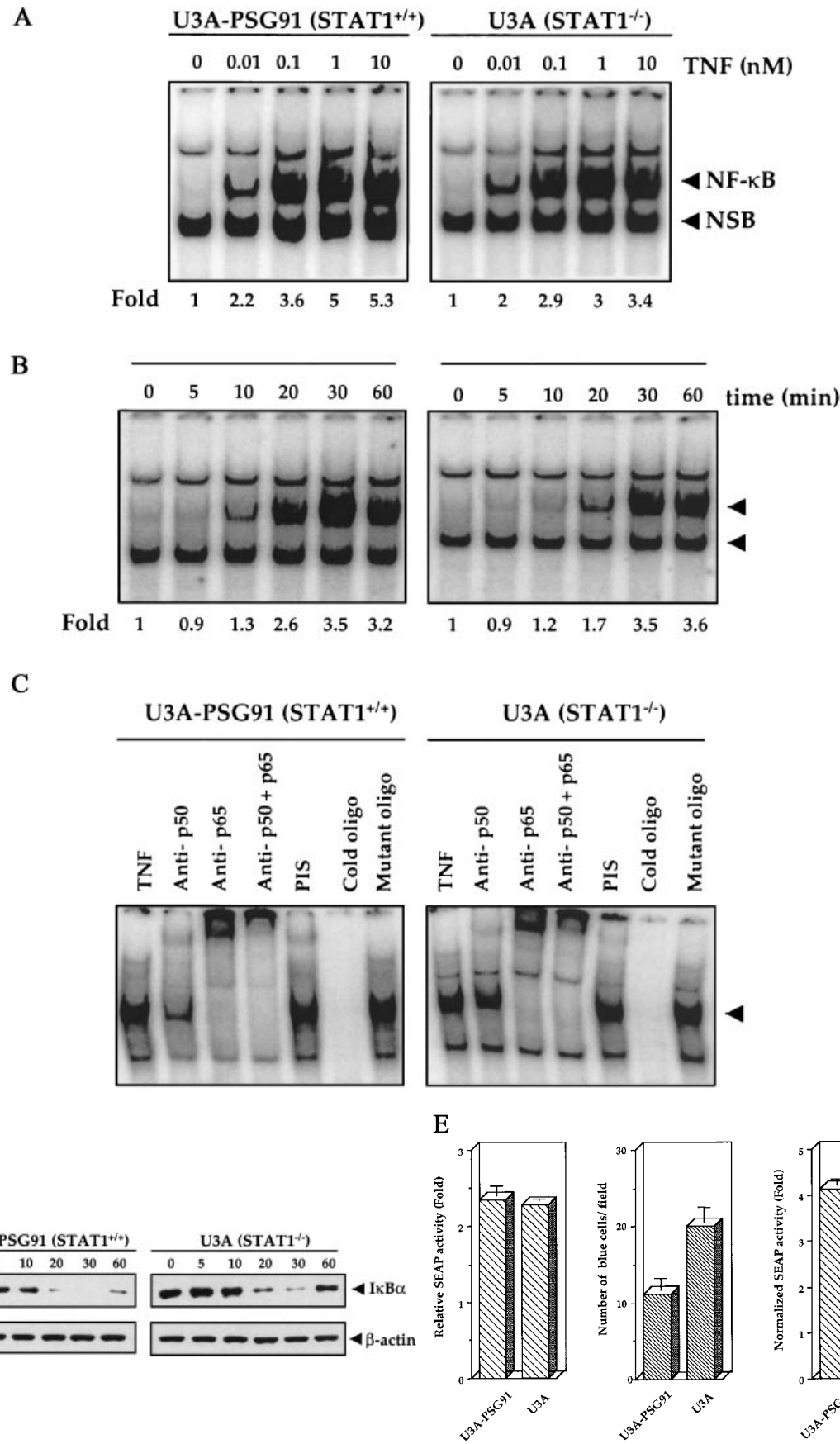


Fig. 3. **A**: TNF does not induce phosphorylation of STAT1 protein. One million U3A or U3A-PSG91 cells were treated with 0.1 nM TNF for different times, prepared the whole cell extracts, resolved on 10% SDS–PAGE gels, and then probed with antibodies against either phospho STAT-1 (upper panel) or STAT-1 (lower panel). **B**: CHX affects neither the phosphorylation of STAT1 nor levels of STAT1 protein. One million U3A or U3A-PSG91 cells were treated with CHX (10 ug/ml) for different times, prepared the whole cell extracts, resolved on 10% SDS–PAGE gels, and then probed with antibodies against either phospho STAT-1 (upper panel) or STAT-1 (lower panel). **C**, is a control from IFN-α-treated cells. C: Cleavage of PARP by TNF in U3A-PSG91 and U3A cells. Two million cells per milliliter were pretreated with none and 10 μg/ml CHX for 2 h, followed by treatment with 0, 1, and 10 nM TNF for 6 h as indicated. Forty micrograms whole cell proteins were analyzed in Western blot by PARP antibodies. **D**: Activation of caspases-3 and -8 in U3A-PSG91 and U3A cells. Two million cells per milliliter were pretreated with 10 μg/ml CHX for 2 h, followed by treatment with 10 nM TNF for 3, 6, and 12 h. One hundred micrograms of whole-cell protein was used for caspase assays, as described in Materials and Methods. Medium control indicates cells treated with CHX alone.


A
U3A-PSG91 (STAT1+/+)
U3A (STAT1-/-)
0 0.01 0.1 1 10
0 0.01 0.1 1 10
TNF (nM)
NF-κB
NSB
Fold 1 2.2 3.6 5 5.3
1 2 2.9 3 3.4
B
0 5 10 20 30 60
0 5 10 20 30 60
time (min)
Fold 1 0.9 1.3 2.6 3.5 3.2
1 0.9 1.2 1.7 3.5 3.6
C
U3A-PSG91 (STAT1+/+)
U3A (STAT1-/-)
TNF
Anti- p50
Anti- p65
Anti- p50 + p65
PIS
Cold oligo
Mutant oligo
D
U3A-PSG91 (STAT1+/+)
U3A (STAT1-/-)
0 5 10 20 30 60
IκBα
β-actin
E
Relative SEAP activity (Fold)
Number of blue cells/ field
Normalized SEAP activity (Fold)
U3A-PSG91
U3A

in rate of NF-κB activation correlates with IκBα degradation. Cytoplasmic extracts from the above experiment (Fig. 4B) were analyzed for IκBα by Western blot. The result showed that IκBα is degraded faster in U3A-PSG91 cells than in U-3A cells (Fig. 4D), which corresponds with the kinetics of NF-κB activation (Fig. 4B). Thus, the results suggest that TNF-induced IκBα degradation and subsequent NF-κB activation is somewhat slower in STAT1-deficient cells than control cells and that STAT1 may participate in optimum TNF-induced NF-κB activation.

Several reports suggest that binding of NF-κB proteins to the DNA as examined by EMSA is not sufficient for NF-κB-dependent gene expression [Darnay et al., 1999]. Therefore we investigated the role of STAT1 in NF-κB dependent gene expression. TNF activated NF-κB-dependent reporter gene expression in both cell types, but the normalized level of activation was lower in STAT1-deficient U3A cells (Fig. 4E), further suggesting the contribution of STAT1 in TNF signaling.

STAT1 is Essential in IFNα-Mediated Downregulation of NF-κB Induced by TNF

Previously we have reported that IFNα can downregulate TNF-induced NF-κB activation [Manna et al., 2000]. We investigated whether this effect is mediated through STAT1. U3A-PSG91 and U3A cells were pre-exposed to different concentrations of IFNα for 48 h, then treated with 0.1 nM TNF for 30 min, and examined for NF-κB/DNA binding by EMSA. IFNα decreased the TNF-induced NF-κB activation in U3A-PSG91 cells (from 3.6- to 1.9-fold) in a dose-dependent manner, but not in U3A cells (Fig. 5). These results suggest that IFNα downregulates TNF-mediated NF-κB activation through the activation of STAT1.

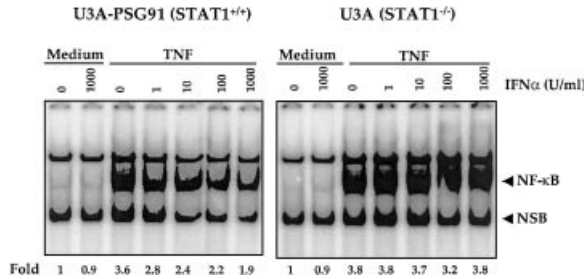


Fig. 5. TNF-induced NF-κB activation in U3A-PSG91 cells is suppressed by IFNα. Two hundred fifty thousand U3A-PSG91 and U3A cells per 3 ml were treated with 0-1,000 U/ml IFNα for 2 days, followed by induction with none and 0.1 nM TNF for 30 min. Nuclear extracts were prepared and assayed for NF-κB, as described in Materials and Methods. NSB, non-specific binding.

TNF-Induced JNK Activation is STAT1 Independent

Because JNK is activated by TNF through a pathway that is similar to NF-κBs, we investigated the role of STAT1 in TNF-induced JNK activation. We first treated both cell types with 1 nM TNF for different times and then examined them for JNK activation by the immune-complex kinase assay [Kumar and Aggarwal, 1999]. TNF induced JNK activation in both U3A-PSG91 and U3A cells in a time- and dose-dependent manner (Fig. 6). Treatment of cells with 0.1 nm TNF activated low levels of JNK in U3A-PSG91 cells but not in U3A cells. Lack of appreciable difference in JNK activation was observed between the two cell types, indicates that the pathway leading to JNK activation is independent of STAT1.

DISCUSSION

In this report, we demonstrated that deletion of STAT1 has minimal effect on TNF-induced signaling, including activation of NF-κB, JNK,

Fig. 4. TNF-induced NF-κB activation. **A**: Dose response for NF-κB activation. Two million cells per milliliter were treated with 0–10 nM TNF for 30 min, and nuclear extracts were prepared and assayed for NF-κB. **B**: Time course activation of NF-kB. Two million cells per milliliter were treated with 0.1 nM TNF for 0–60 min, and nuclear extracts were prepared and assayed for NF-κB. **C**: Activated NF-κB in U3A-PSG91 and U3A cells is composed of p50 and p65 subunits. Nuclear extracts prepared by treating cells with 0.1 nM TNF were incubated at 37°C for 15 min either alone, or with anti-p50 antibodies, or anti-p65 antibodies, or a mixture of anti-p50 and anti-p65 antibodies, or pre-immune sera, or unlabeled oligo, or mutant oligo, and then assayed for NF-κB as described in Materials and Methods. **D**: Time course degradation of IκBα. Two million cells per milliliter were treated with 0.1 nM TNF for 0–60 min, the cytoplasmic extracts were prepared, and 30 μg protein analyzed by Western blot using IκBα-specific antibodies (upper panel). The same blot was stripped and reprobed with β-actin antibodies (lower panel). **E**: NF-κB-dependent reporter gene expression. U3A-PSG91 and U3A cells were transiently transfected with 2.5 μg total plasmid DNA in duplicate as described in Materials and Methods. Cells were induced with none or 1 nM TNF for 24 h. The culture supernatants were assayed for SEAP activity, and the cells were stained with X-Gal to determine the efficiencies of transfection, as described in Materials and Methods. NSB, non-specific binding.

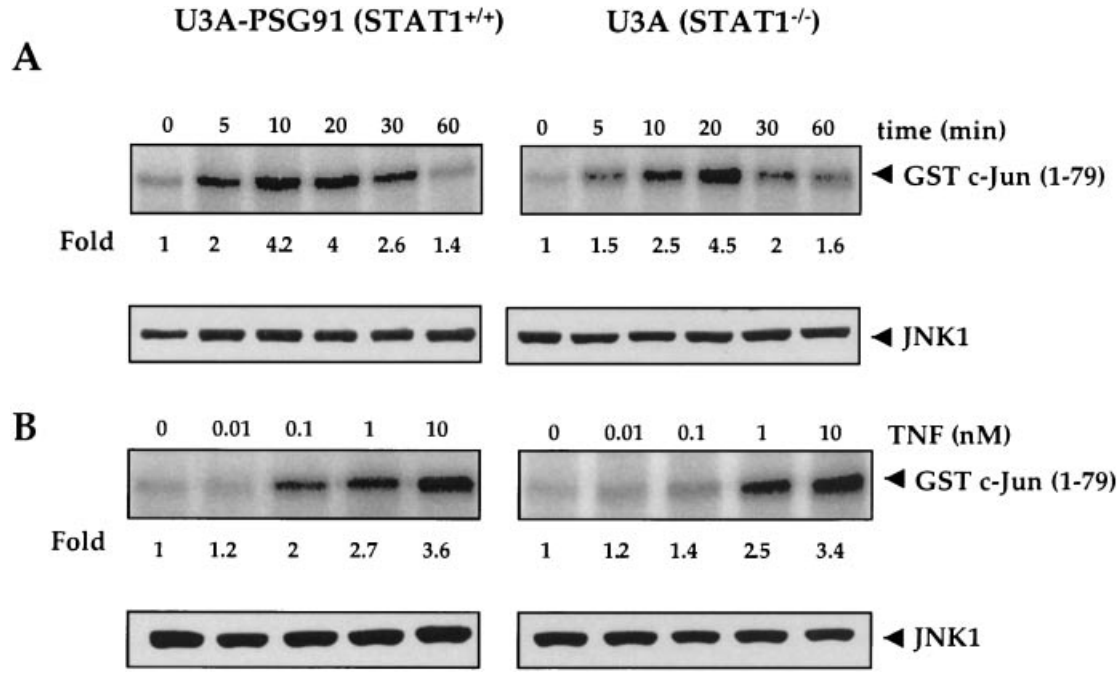


Fig. 6. TNF-induced JNK activation is STAT1 independent. **A**: Time course activation of JNK. Two million cells per milliliter were treated with 1 nM TNF for 0–60 min. One hundred micrograms of whole-cell protein was treated with JNK1 antibodies and then immunoprecipitated with protein A/G sepharose. The beads were washed and subjected to kinase assay as described in Materials and Methods. Forty micrograms of the same protein extracts were probed with JNK1 antibodies (lower panel). **B**: Dose response for JNK activation. Two million cells per milliliter were treated with 0–10 nM TNF for 20 min. One hundred micrograms of whole-cell protein was treated with JNK1 antibodies and then immunoprecipitated with protein A/G sepharose. The beads were washed and subjected to kinase assay as described in Materials and Methods (upper panel). Forty micrograms of the same protein extracts were probed with JNK1 antibodies (lower panel).

and apoptosis. STAT1 was found, however, to be required for downregulation of TNF-induced NF-κB activation by IFNα.

Our results show the cytotoxic activity of TNF in human fibroblast cells is independent of STAT1 status. Similar results were recently reported by Morita et al. [2000] who, using mouse embryo fibroblasts, found that the TNF-induced growth-inhibition curves of STAT1-null and STAT1-expressing cells were almost superimposable. Other reports, however, have showed that TNF is more cytotoxic to STAT1-bearing cells than to STAT1-deficient cells [Kumar et al., 1997; Xu et al., 1998]. To resolve this difference, we exposed the cells according to the protocol of Kumar et al. [1997]. Cells were treated with TNF (20 ng/ml) in the presence of actinomycin D (20 ng/ml) for 18 h, and then examined the viability by either trypan blue dye exclusion method or by the MTT method. These results showed no significant difference in the sensitivity between the STAT1 plus and STAT1 minus cells (data not shown). Thus our results are similar to that of Morita et al. [2000] and differ from Kumar et al. [1997]. Xu et al. [1998] exposed the cells to TNF in the presence of low serum (0.5%) conditions and found 40% killing in STAT1-deficient cells and 60% in STAT1-bearing cells. We also observed that in low serum (1%)-containing medium, TNF was more cytotoxic to STAT1-expressing cells than to STAT1-deficient cells (Fig. 2C). Despite the probable role of STAT1 in TNF-mediated cytotoxicity, we did not find any significant difference in activation of either caspase-3 or caspase-8. Activation of executioner caspase-3 and degradation of its substrate PARP were identical in both STAT1-deficient and reconstituted cells. Our demonstration that STAT1 lacks a role in TNF-induced caspase activation is similar to that of Morita et al. [2000] and Xu et al. [1998] but different from that of Kumar et al. [1997]. Interestingly, Morita et al. [2000] found that STAT1-induced STAT inhibitor-1 (SSI-1, also called suppressor of cytokine signaling-1, SOCS-1), but not

STAT1, suppresses TNF-induced cell death in fibroblasts.

Our results show that STAT1 plays a minimal role in TNF-induced NF-κB activation. The kinetics of NF-κB activation is faster in STAT1-bearing cells than STAT1-deficient cells. We also found that STAT1 had minimal effect on TNF-induced IκBα degradation. Our results differ from that of Wang et al. [2000], who reported that TNF-induced IκBα degradation and NF-κB activation were enhanced in STAT1-deficient cells. However, no dose-response or time course of NF-κB activation was reported by Wang et al. [2000]. They also did not quantitate their results. The difference in the results could be due to the level of expression of STAT1 protein. Our EMSA results are, however, in agreement with the TNF-induced NF-κB-dependent reporter gene expression. We found that the absolute SEAP activities in these cells were comparable, whereas the transfection efficiency in U3A ($STAT^{-/-}$) cells was almost 2-fold higher than that in U3A-PSG91 ($STAT^{+/+}$) cells. Overall, the normalized NF-κB reporter activity was higher in STAT1-bearing cells. Wang et al. [2000] showed that STAT1 is present in the TNF-activated complex consisting of TNF receptor 1, TRADD, and FADD, and that STAT1 competes with RIP and TRAF2 for binding with TRADD, thereby preventing TNF-induced NF-κB activation. They also showed that IκBα was completely degraded in STAT1-deficient cells. In accordance with NF-κB activation results, however, we found greater and faster IκBα degradation in STAT1-bearing cells. Wang et al. [2000] concluded that STAT1 prevents NF-κB activation by blocking interaction of TRAF2 with TRADD. The TRAF2 knockout mice experiment, however, indicated that TRAF2 is not critical for TNF-induced NF-κB activation [Lee et al., 1997], thus raising doubts about the hypothesis of Wang et al. [2000].

That the binding of TRAF2 to TRADD is unaffected by the presence or absence of STAT1 is further evident from our studies with TNF-induced JNK activation. TRAF2 has been shown to be indispensable for TNF-induced JNK activation [Lee et al., 1997]. Our results show a comparable JNK activation in both STAT1-bearing and STAT1-deficient cells. Similar to the JNK result, we also found that TNF-induced p38MAPK activity, another TRAF2-dependent activity, was comparable in both STAT1-null and STAT1-reconstituted cells (unpublished data).

Previously, Manna et al. [2000], Gribaudo et al. [1995], and Chan et al. [1999] have shown that IFNs downregulate TNF-induced NF-κB activation and NF-κB-dependent gene expression. What role STAT1 plays in IFN-mediated downregulation of NF-κB was not understood. Our results here demonstrate that IFN-α-mediated downregulation of NF-κB is STAT1-dependent. At this stage we postulate the existence of signaling molecules of the JAK-STAT pathway that inhibit the TNF-induced NF-κB activation pathway. Collectively, this study demonstrates that STAT1 plays a minimum role in TNF signaling pathway leading to activation of apoptosis, JNK and NF-κB, but is involved in IFN-α induced suppression of TNF-mediated NF-κB activation.

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