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Type 1 TNF Receptor Forms a Complex with and Uses Jak2 and c-Src to Selectively Engage Signaling Pathways That Regulate Transcription Factor Activity¹

Roxana Pincheira,² Ariel F. Castro, Osman Nidai Ozes, Prema S. Idumalla, and David B. Donner²

The type 1 TNFR (TNFR1) contains a death domain through which it interacts with other death-domain proteins to promote cellular responses. However, signaling through death-domain proteins does not explain how TNFR1 induces the tyrosine phosphorylation of intracellular proteins, which are important to cellular responses induced by TNFR1. In this study, we show that TNFR1 associates with Jak2, c-Src, and PI3K in various cell types. Jak2 and c-Src constitutively associate with and are constitutively active in the TNFR1 complex. Stimulation with TNF induces a time-dependent change in the level of Jak2, c-Src, and PI3K associated with TNFR1. The tyrosine kinase activity of the complex varies with the level of tyrosine kinase associated with TNFR1. TNFR1/c-Src plays a role in activating Akt, but not JNK or p38 MAPK, whereas TNFR1/Jak2 plays a role in activating p38 MAPK, JNK, and Akt. TNFR1/c-Src, but not TNFR1/Jak2, plays an obligate role in the activation of NF- κ B by TNF, whereas TNFR1/Jak2, but not TNFR1/c-Src, plays an obligate role in the activation of STAT3. Activation of TNFR1 increased the expression of vascular endothelial growth factor, p21^{WAF1/CIP1}, and manganese superoxide dismutase in MCF7 breast cancer cells, and increased the expression of CCL2/MCP-1 and IL-1 β in THP-1 macrophages. Inhibitors of Jak2 and c-Src impaired the induction of each of these target proteins. These observations show that TNFR1 associates with and uses nonreceptor tyrosine kinases to engage signaling pathways, activate transcription factors, and modulate gene expression in cells. *The Journal of Immunology*, 2008, 181: 1288–1298.

Tumor necrosis factor is a multifunctional cytokine that promotes immunity and inflammation, modulates the growth, differentiation, and viability of transformed and nontransformed cells, and potentially affects cell metabolism (1, 2). Cellular responses to TNF are initiated by the type 1 TNFR (TNFR1)³ and the type 2 TNFR (2–4). Most TNF actions are elicited by TNFR1, which contains a death domain that fosters protein-protein interactions, particularly with other death-domain proteins (2–4). The first of these, the TNFR-associated death-domain protein (TRADD), recruits the Fas-associated death-domain protein and procaspase 8 into a complex that initiates an apoptotic caspase cascade. TRADD also binds and uses the receptor-interacting protein (RIP) and TNFR-associated factor (TRAF)-2 to activate NF- κ B, which induces gene products that promote immunity and cell viability (2–4).

TNFR1 does not contain intrinsic tyrosine kinase activity or any motif that suggests biological activity, yet tyrosine phosphoryla-

tion reactions are induced by TNF and necessary for its biologic effects (5–9). Such phosphorylations associate with alterations of cellular sensitivity to TNF-mediated cytotoxicity (8, 10). In addition, inhibitors of protein tyrosine kinases suppress TNF-stimulated DNA fragmentation (11), activation of NF- κ B, and expression of endothelial cell adhesion molecules (12, 13). The priming of neutrophils by TNF is also accompanied by tyrosine phosphorylations, and these phosphorylations participate in the transduction of signals that direct the cells to undergo a respiratory burst (5–7). Our laboratory has shown that TNF induces the tyrosine phosphorylation of a group of cytoplasmic proteins, including PI3K and insulin receptor substrate-1 (14), that promote activation of NF- κ B (15–17), and modulate the responsiveness of cells to insulin (18).

The proximal steps through which TNFR1 induces tyrosine phosphorylation reactions important to diverse cellular responses are poorly understood. Canonical TNFR1/TRADD signaling does not provide a mechanism through which tyrosine phosphorylations can be induced by TNFR1. Cytokine receptors that do not possess tyrosine kinase activity use associated nonreceptor tyrosine kinases to initiate signaling (19). Many such receptors associate with Jak and Src family kinases; in these instances, members of each family of kinases activate and are necessary for optimal signal transduction (19). These observations, considered together with the diverse nature of TNF-induced tyrosine phosphorylations, raise the possibility that TNFR1 might associate with and use Src and Jak kinases. We previously showed that a GST fusion protein that contained the cytoplasmic domain of TNFR1 precipitated Jaks from a cell-free system or adipocyte lysates (20). Our previous studies also showed that TNFR1 induces phosphorylation of STAT proteins and PI3K (14, 20), but did not determine whether these were

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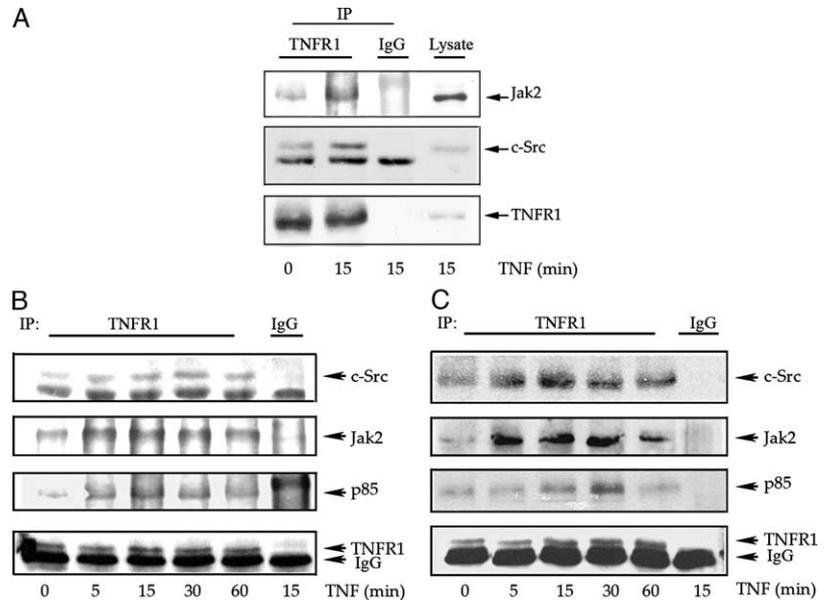
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³ Abbreviations used in this paper: TNFR1, type 1 TNFR; TRADD, TNFR-associated death-domain protein; RIP, receptor-interacting protein; TRAF, TNFR-associated factor; VEGF, vascular endothelial growth factor; MnSOD, manganese superoxide dismutase; IKK, I κ B kinase.

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FIGURE 1. Endogenous Jak2, c-Src, and PI3K form a complex with TNFR1. *A*, MCF7 cells were incubated in the absence or presence of 1 nM TNF for 15 min. Endogenous TNFR1 was immunoprecipitated from 5 mg of cell lysate and a Western blot was probed with Abs to TNFR1, Jak2, and c-Src. Mouse IgG was a negative control for immunoprecipitation. As a positive control for identification of TNFR1, Jak2, and c-Src, 100 μ g of cell lysate protein was fractionated by SDS-PAGE and the blot was probed with Abs to each protein. Mouse IgG True Blot, which does not react with light or H chain Ig, was used as the secondary Ab for this experiment. *B*, HEK293 or, C, H1299 cells were incubated in the absence or presence of 1 nM TNF for various times. TNFR1 was immunoprecipitated from cell lysates and a control immunoprecipitation was conducted with IgG. Western blots were probed with anti-c-Src, Jak2, the p85 regulatory subunit of PI3K and TNFR1; 55-kDa TNFR1 was just above the IgG H chain. Consequently, it was most readily detected and distinguished from IgG on lightly exposed autoradiographs. These experiments were repeated three to five times with similar results.



substrates for the Jaks or members of other families of nonreceptor protein tyrosine kinases (20).

In this study, we show that TNFR1 constitutively forms a complex with endogenous c-Src, PI3K, and Jak2. TNF induces the recruitment of additional c-Src, PI3K, and Jak2 into the complex and these are used by TNFR1 to engage signaling cascades, activate transcription factors, and alter gene expression. Jak2 and c-Src associated with TNFR1 are constitutively active, which suggests that the receptor complex can self-activate.

Materials and Methods

Reagents

Recombinant human TNF was a gift from Genentech. Abs to p-Akt, Akt, p38 MAPK, and p-p38 MAPK were obtained from Cell Signaling; anti-Rel A and anti-p-c-jun were obtained from Upstate Biotechnology. The Ab used to immunoprecipitate TNFR1 was obtained from Santa Cruz Biotechnology. The agonist TNFR1 Ab and the soluble extracellular domain of TNFR1 were obtained from R&D Systems. An ELISA kit for the assay of CCL2/MCP-1 and IL-1 β in cell supernatants was obtained from eBioscience.

Plasmids

Kinase-dead c-Src is a substitution of arginine for lysine at position 297. The vector control for KD-c-Src is pUSE amp(-) (Upstate Biotechnology). KD-Jak2, a substitution of lysine by glutamic acid in motif II of the kinase domain, was a gift from Dr. M. Harrington (Indiana University, Indianapolis, IN). The vector control for KD-Jak2 is pKR5.

Cell culture and transfections

HEK293 kidney epithelial cells, H1299 lung adenocarcinoma, and MCF7 human breast carcinoma cells were cultured in DMEM supplemented with 10% (v/v) FBS, 1% glutamine, and 1% penicillin/streptomycin. SYF cells and SYF cells reconstituted with Jak2 were cultured in DMEM, 1.5 g/L sodium bicarbonate, 10% FBS, and 1% penicillin/streptomycin. γ 2A cells and γ 2A cells reconstituted with Jak2 were obtained from P. Sayeski (University of Florida, Gainesville, FL). γ 2A cells were cultured in DMEM, 1% glucose, 10% FBS, 1% penicillin/streptomycin, 100 μ g/ml zeocin, and 200 μ g/ml G418. THP-1 monocytes from the American Type Culture Collection were grown in RPMI 1640, 10% FBS, 1 mM sodium pyruvate, 0.05 mM 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Differentiation of the cells into macrophages was induced by culture in complete medium plus 100 nM PMA for 2 days. HEK293 cells were transfected with KD c-Src or KD-Jak2 and other vectors by the calcium phosphate precipitation method.

Cell stimulation

MCF7, HEK293, and HeLa cells were serum-starved for 24 h before stimulation with TNF or the agonist TNFR1 Ab. Cells were treated with 10 μ M

PP2 for 30 min or 50 μ M AG490 for 1 h before activation of TNFR1. Lysates were fractionated by SDS-PAGE and Western blots were prepared. For THP-1 macrophages, cells were treated with PP2 or AG490, as described above, and then incubated with TNF for 24 h at 37°C. Supernatants were then collected and stored at -70°C until biological assay for cytokine production was performed using an ELISA kit.

In vitro kinase assays

Src kinase. TNFR1 immunoprecipitates were washed twice with lysis buffer and three times with kinase assay buffer (30 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM MnCl₂, and 1 mM DTT). The reaction was conducted in 20 μ l of kinase buffer, 0.5 μ g of Sam68, 10 μ M ATP for 30 min at 30°C. A Western blot was then probed with an Ab to phosphotyrosine.

JNK kinase. A c-Jun fusion protein linked to agarose beads was used to pull down JNK from cell lysates. The reaction was conducted for 30 min at 30°C in kinase assay buffer (25 mM Tris (pH 7.5), 5 mM β -glycerolphosphate, 2 mM DTT, 0.1 mM Na₂VO₄, 10 mM MgCl₂) to which ATP (200 μ M) and c-jun were added. An Ab to phospho-c-jun (Ser⁶³) assayed JNK activation by Western blot analysis.

Immunoprecipitation and immunoblotting

To immunoprecipitate TNFR1, cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.2% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, 0.15 U/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin A, and 1 mM sodium orthovanadate during a 20-min incubation at 4°C. After centrifugation to remove debris, 2–5 mg of supernatant was pre-cleared with protein A/G agarose and incubated with anti-TNFR1 for 1 h at 4°C before 50 μ l of protein A/G agarose was added and incubated at 4°C for 2–16 h. Samples were centrifuged for 10 s (12,000 \times g, 4°C), washed at least three times in lysis buffer, suspended in Laemmli buffer, boiled for 5 min, and centrifuged for 10 s. Equal amounts of protein from the supernatants were fractionated by SDS-PAGE on 7.5% polyacrylamide gels and transferred to Immobilon-P. Western blots were probed with Abs and proteins were detected by ECL.

NF- κ B DNA binding

EMSA were conducted using 6 μ g of protein from cells lysed in 40 mM HEPES (pH 7.0), 100 mM KCl, 1% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 1 μ g of aprotinin, 1 μ g of pepstatin A, and 1 μ g of leupeptin. Cell protein was incubated with a double-stranded [³²P] κ B probe (5'-GTT GAGGACTTTCCACAG-3') in 1 \times Tris-EDTA, 1 mM KCl, 10% glycerol, 1 mM DTT, and 1 mg/ml polydeoxycytosine-deoxyinosine for 30 min at room temperature. DNA/protein complexes were fractionated on native 5% polyacrylamide gels, dried, and exposed to film.

Gene reporter assays

HEK293 cells were transiently cotransfected with a 5 \times NF- κ B reporter and an Rous sarcoma virus β -gal plasmid using the calcium phosphate

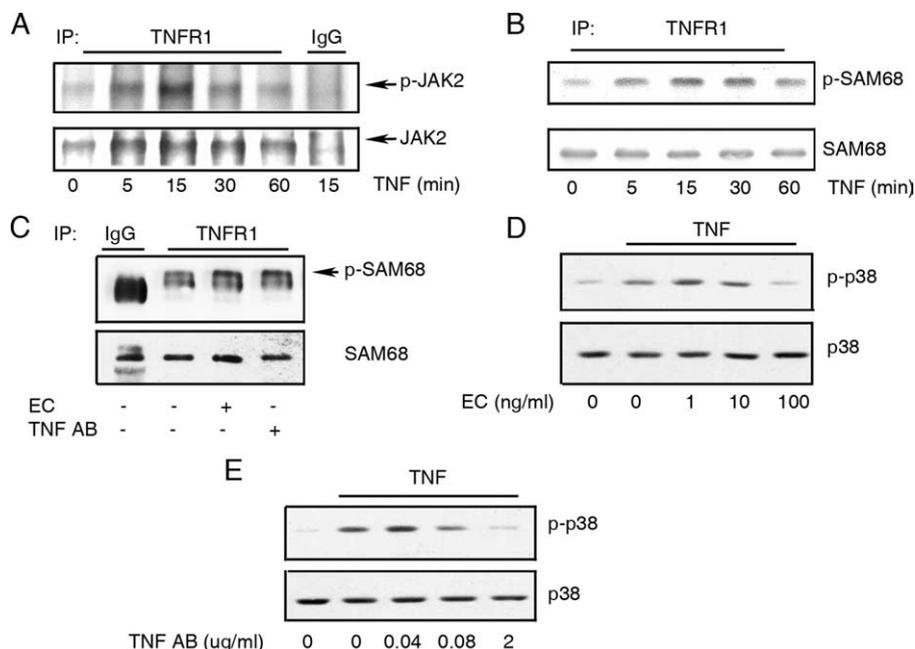
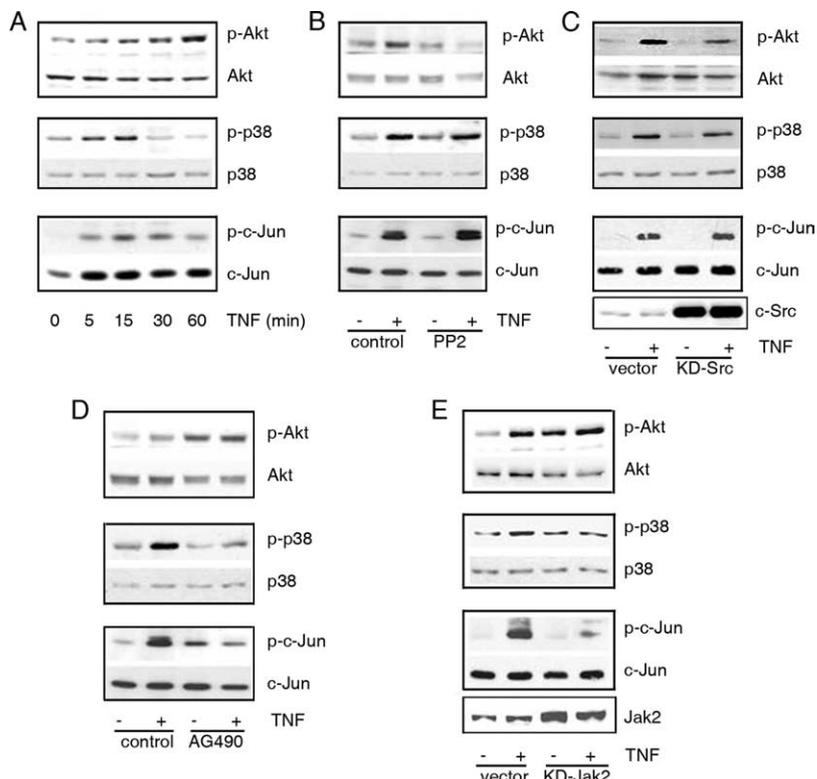


FIGURE 2. Jak2 and c-Src are active in the TNFR1 complex. HEK293 cells were serum-starved for 24 h, stimulated with 1 nM TNF for the indicated times, and TNFR1 was immunoprecipitated. *A*, A Western blot was probed with Abs directed against phospho-Jak2 (Y1007/Y1008) (*top*) or Jak2 (*bottom*). *B*, c-Src activity in the TNFR1 immunoprecipitates was measured using an in vitro kinase assay in which Sam68 was the substrate for active c-Src. Phosphorylation of Sam68 was determined by probing a Western blot with an Ab to phosphotyrosine. *C*, HEK293 cells were treated with TNF in the absence or presence of 100 ng/ml of the soluble extracellular domain of TNFR1 (EC), or with 2 μ g/ml of a neutralizing Ab to TNF (TNF AB). TNFR1 was then immunoprecipitated from cell lysates. The *first lane* shows an immunoprecipitation with mouse IgG. c-Src activity was determined as in *B*. *D*, Cells were incubated with various amounts of the soluble extracellular domain of TNFR1 and stimulated with TNF; phosphorylation of p38 MAPK was assayed by Western blot analysis. *E*, Cells were incubated with various amounts of a neutralizing TNF Ab and stimulated with TNF; phosphorylation of p38 MAPK was assayed by Western blot analysis. These experiments were repeated three times with similar results.

method. SYF cells, SYF cells reconstituted with c-Src, γ 2A cells, and γ 2A cells reconstituted with Jak2 were transfected using Effectene reagent (Qiagen). The amount of transfected DNA was held constant by addition of

vector control plasmid. After 48 h, cells were incubated with 1 nM TNF or vehicle for 6 h and luciferase activity was divided by β -gal activity to normalize for variances in transfection efficiencies.

FIGURE 3. Jak2 and c-Src transmit TNFR1 signals to downstream enzymes. *A*, Serum-starved HEK293 cells were incubated with TNF for various times before phosphorylation of Akt and p38 MAPK was assayed by Western blotting. JNK activity was determined by an in vitro kinase assay in which *c-jun* was the substrate. JNK-induced phosphorylation of *c-jun* was assayed by probing an immunoblot with an Ab directed against phospho-*c-jun* (Ser⁶³). *B*, Serum-starved HEK293 cells were incubated in the absence or presence of 10 μ M PP2 for 30 min and then with 1 nM TNF. Akt phosphorylation was assayed after 60 min and phosphorylation of p38 MAPK and JNK activity was assayed after a 15-min incubation with TNF. *C*, HEK293 cells were transfected with vector or KD-Src. Twenty-four hours later, the cells were serum-starved for 24 h and then stimulated with 1 nM TNF for 15 and 60 min. Phosphorylation of Akt and p38 MAPK, and JNK activity, were assayed. *D*, Serum-starved HEK293 cells were incubated in the absence or presence of 50 μ M AG490 for 1 h and then with 1 nM TNF. Phosphorylation of Akt was assayed after 60 min and phosphorylation of p38 MAPK, and JNK activity, were assayed after a 15-min incubation with TNF. *E*, Cells were transfected with vector or KD-Jak2. Twenty-four hours later, cells were serum-starved for 24 h and were then treated with 1 nM TNF for 15 and 60 min. Phosphorylation of Akt and p38 MAPK, and JNK activity were assayed. These experiments were repeated at least three times each producing results similar to those shown.



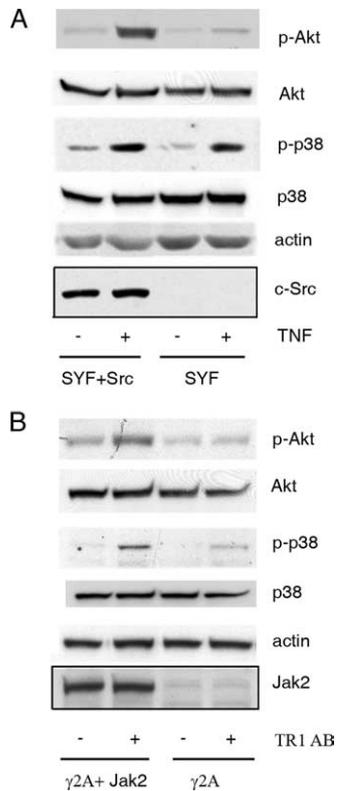


FIGURE 4. Activation of Akt and p38 MAPK by TNF in Src- and Jak-deficient cells. *A*, Serum-starved SYF cells or SYF cells reconstituted with c-Src were incubated in the absence or presence of 1 nM TNF for 30 min. Western blots prepared from cell lysates after SDS-PAGE were probed with Abs directed against p-Akt, Akt, phospho-p38 MAPK, p38 MAPK, and actin. The blot was also probed with anti-c-Src to validate the phenotypes of the cells. *B*, Serum-starved γ 2a cells and γ 2A cells reconstituted with Jak2 were incubated in the absence or presence of TNFR1 agonist Ab for 30 min. Western blots were prepared and probed with Abs directed against p-Akt, Akt, phospho-p38 MAPK, p38 MAPK, and actin. The blot was also probed with anti-Jak2 to validate the phenotype of the cells. The results shown are representative of experiments repeated three times.

Reproducibility of the data

Immunoprecipitations and Western blotting were repeated at least three times. Gene reporter assays and ELISA were repeated three times in duplicate.

Results

Endogenous c-Src, PI3K, and Jak2 form a complex with TNFR1

To test whether TNFR1 forms a complex with nonreceptor protein tyrosine kinases, cells were incubated in the absence or presence of TNF, and TNFR1 was then immunoprecipitated (Fig. 1). In this experiment, and throughout this study, we tested for associations of endogenous proteins to ensure that the interactions we observed were not artifacts. Western blotting showed that c-Src and Jak2 constitutively associated with TNFR1 in MCF7 human breast cancer cells and incubation with TNF for 15 min increased the amount of c-Src and Jak2 in the receptor complex (Fig. 1A). As a negative control, we used an Ab to IgG, which failed to immunoprecipitate TNFR1, Jak2, or c-Src. Our finding that TNFR1 associates with c-Src and Jak2 in MCF7 cells led us to assay for the TNFR1/kinase complex in other cells. Constitutive association of c-Src and Jak2 with TNFR1 was also observed in HEK293 human embryonic kidney cells (Fig. 1B) and H1299 human lung adenocarcinoma cells (Fig. 1C). TNF induced a time-dependent increase of the amount

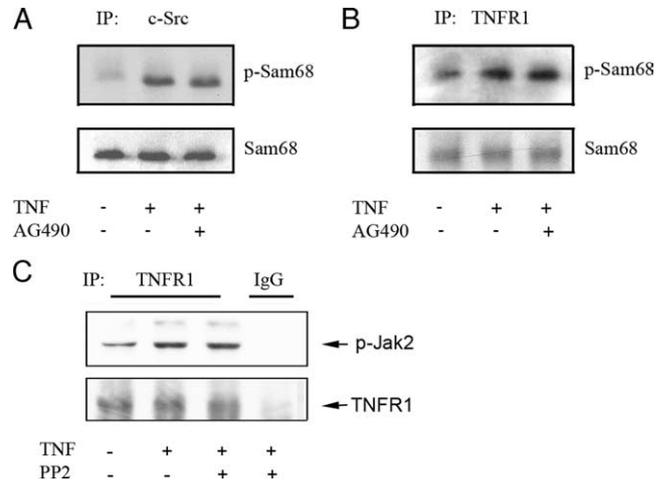


FIGURE 5. Jak2 and c-Src do not affect each other's activities in the TNFR1 complex. HEK293 cells were incubated in the absence or presence of AG490 and then stimulated with TNF for 15 min before (*A*) c-Src or (*B*) TNFR1 was immunoprecipitated. The activity of c-Src in the immunoprecipitates was then assayed using an in vitro kinase assay in which SAM68 was substrate. *C*, HEK293 cells were incubated in the absence or presence of PP2 and then with TNF for 15 min. TNFR1 was immunoprecipitated and a Western blot was probed with Abs to p-Jak2 and TNFR1. Mouse IgG was a negative control for immunoprecipitation. Mouse IgG True Blot was the secondary Ab used in this experiment. The results shown are representative of experiments repeated three times.

of Jak2 and c-Src associated with TNFR1 in both HEK293 and H1299 cells. Subsequently, the amount of Jak2 and c-Src associated with TNFR1 diminished, again approaching the constitutive level.

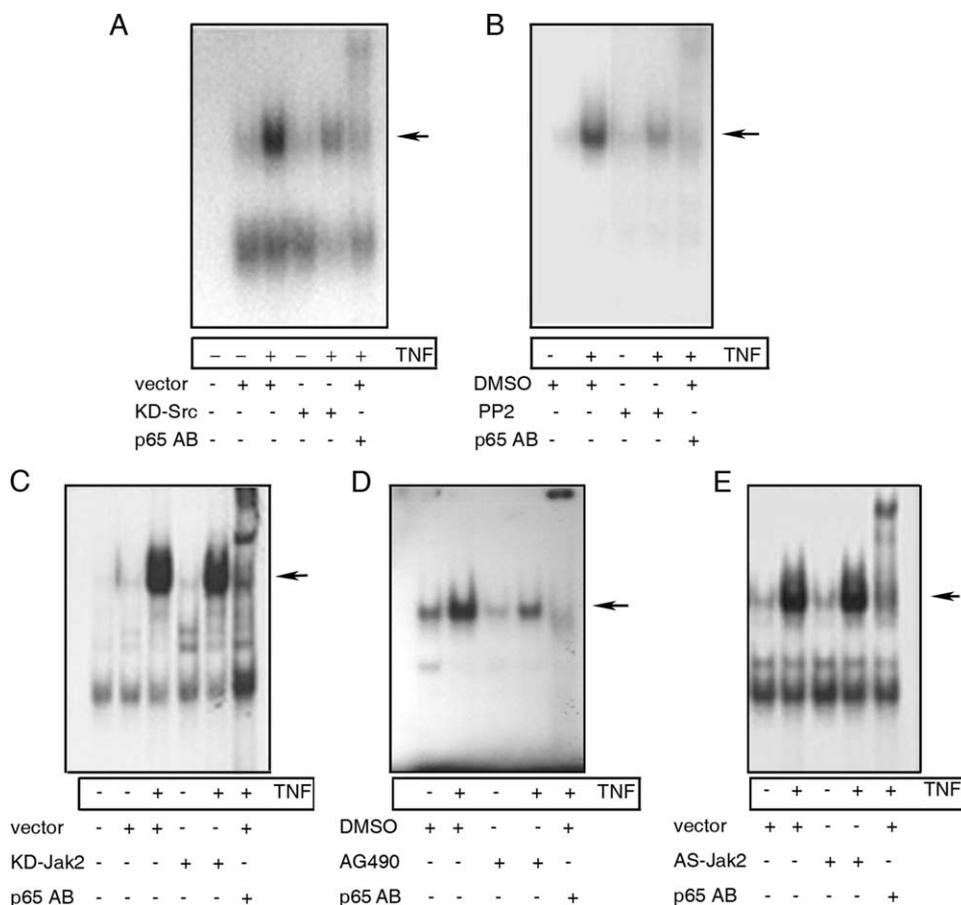
Src (21) and Jak kinases (22, 23) bind, phosphorylate, and activate PI3K, which lead us to assay for PI3K in the TNFR1 complex of HEK293 and H1299 cells. The p85 regulatory subunit of PI3K immunoprecipitated with endogenous TNFR1, and TNF induced recruitment of additional lipid kinase into the complex (Fig. 1, *B* and *C*). These observations show that TNFR1 associates constitutively with Jak2, c-Src, and PI3K in diverse cell types, and that TNF modulates the composition of the TNFR1 complex.

TNFR1-associated kinases are active in the absence and presence of TNF

The discovery that Jak2 and c-Src form a complex with TNFR1 led us to determine whether the TNFR1-associated kinases are active. To test this, TNFR1 was immunoprecipitated from untreated or TNF-treated HEK293 cells. Jak2 activity was then determined by probing a Western blot with an Ab to active (phospho) Jak2. c-Src activity was assayed using an in vitro kinase assay. Jak2 and c-Src were active in immunocomplexes from cells not exposed to TNF. Incubation with TNF for 15 min increased Jak2 and c-Src activity above the constitutive level; subsequently, the kinase activities diminished and approached the basal level (Fig. 2, *A* and *B*). Thus, Jak2 and c-Src are constitutively active when associated with TNFR1, and TNF coordinately changes the amount and activity of Jak2 and c-Src in the complex, suggesting that these events are related.

The demonstration that Jak2 and c-Src are active in immunocomplexes from cells not exposed to TNF was of considerable interest. To determine whether autocrine production of TNF or any protein that binds TNFR1 induced the constitutive activities of the tyrosine kinases, cells were treated with a neutralizing Ab to TNF

FIGURE 6. TNF-induced NF- κ B DNA binding requires c-Src but not Jak2 activity. *A*, HEK293 cells were transfected with vector or KD-Src or, *B*, pretreated with vehicle or 10 μ M PP2 for 30 min before incubation with 1 nM TNF for 30 min. *C*, HEK293 cells were transfected with vector or KD-Jak2; *D*, pretreated with AG490; or *E*, transfected with an antisense oligonucleotide to Jak2 or its vector control. Cells were then incubated with 1 nM TNF for 30 min and NF- κ B DNA binding was assayed by EMSA. Supershifting with an Ab to p65 showed that the slower migrating band in *A* and *C* was NF- κ B. The EMSAs were repeated at least twice, yielding results similar to those shown.



or the soluble extracellular domain of TNFR1 before basal c-Src activity was assayed using the highly sensitive *in vitro* kinase assay. c-Src was active in TNFR1 but not IgG immunoprecipitates, and its activity was not decreased by the neutralizing TNF Ab or

the extracellular domain of the receptor (Fig. 2C). Control experiments showed that activation of p38 MAPK by 1 nM TNF was inhibited by the concentration of soluble extracellular domain (100 ng/ml) (Fig. 2D) or neutralizing Ab (2 μ g/ml) (Fig. 2E) used in the

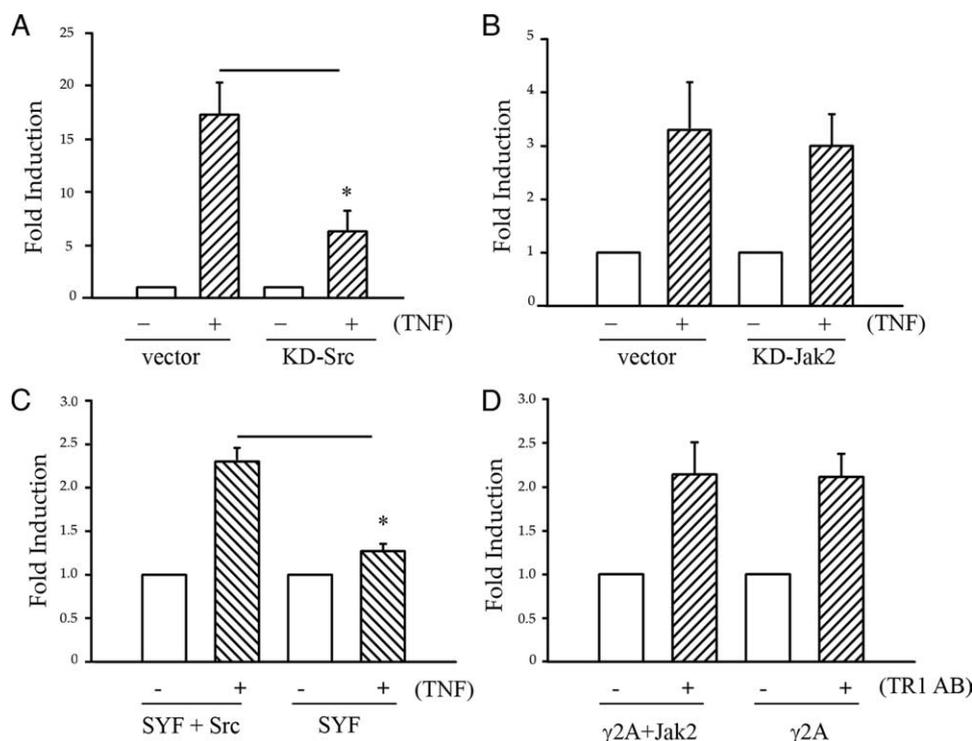


FIGURE 7. TNF-induced transactivation of NF- κ B requires c-Src but not Jak2. HEK293 cells were cotransfected with (*A*) vector (pUSE) or KD-c-Src or (*B*) vector (pKR5) or KD-Jak2, together with β -galactosidase (β -Gal) and an NF- κ B reporter plasmid. Forty-eight hours after transfection, the cells were serum-starved for 16 h, treated with 1 nM TNF for 6 h, and lysed. Luciferase activity normalized to β -Gal activity was determined and the results are the mean \pm SD of three experiments performed in duplicate. NF- κ B transactivation was different in *A* and *B* because the vector for KD-Src and KD-Jak2 differed. *C*, SYF cells deficient in or reconstituted with c-Src, or *D*, γ 2A cells reconstituted with or deficient in Jak2, were transfected with an NF- κ B reporter and β -Gal. Sixteen hours later, cells were stimulated with TNF or TNFR1 agonist Ab for 6 h and NF- κ B reporter activity was assayed. Luciferase activity normalized to β -Gal activity was determined and the results are the mean \pm SD of three experiments performed in duplicate. *, $p < 0.05$.

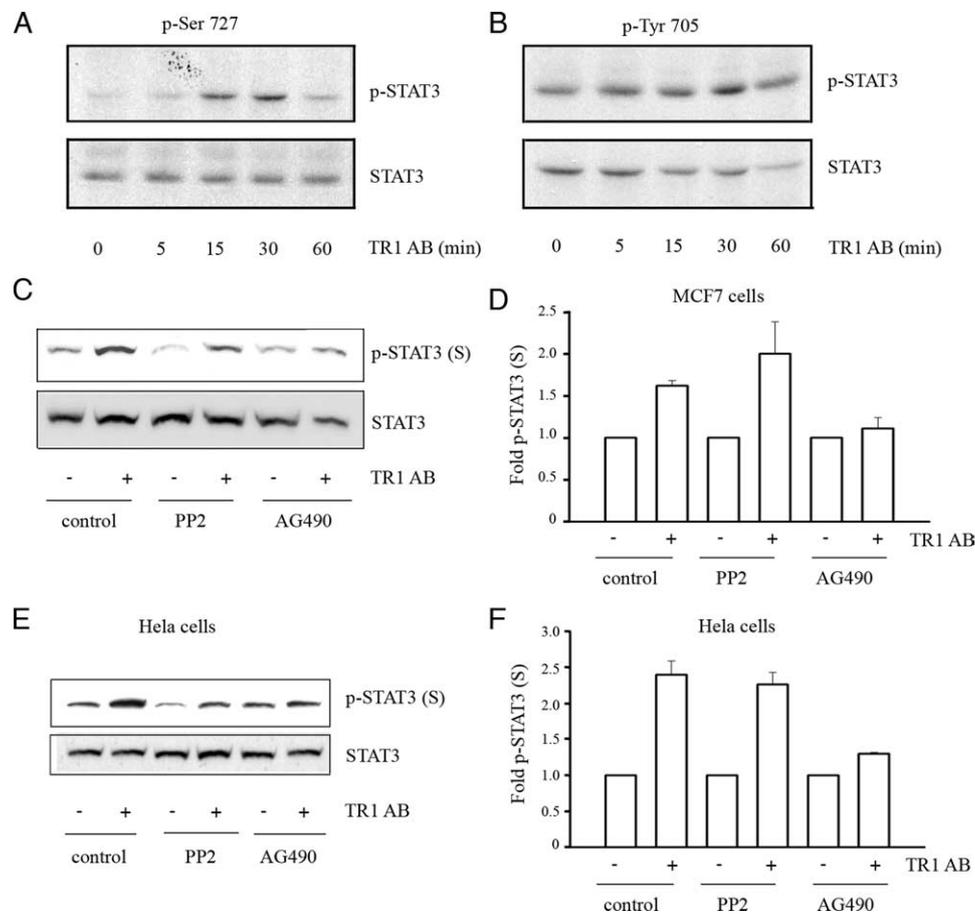


FIGURE 8. TNFR1-induced activation of STAT3 requires Jak2 but not c-Src. *A* and *B*, MCF7 cells were serum-starved for 24 h and then treated with agonist TNFR1 Ab for the indicated times. A Western blot was then prepared and probed with an Ab against phospho-S727 STAT3 (*A*), or an Ab against phospho-Y705 STAT3 (*B*). Membranes were stripped and probed for total STAT3. *C*, MCF7 cells were treated with 10 μ M PP2 (30 min) or 50 μ M AG490 (1 h) before incubation with an agonist TNFR1 Ab for 15 min. A Western blot was then probed with anti-phospho-S727 STAT3 and then with anti-STAT3. *D*, Densitometry was used to analyze the data from experiments conducted as described in *C* and the result illustrates the effect of PP2 and AG490 on TNFR1 agonist Ab-induced activation (serine phosphorylation) of STAT3. Data are the mean \pm SEM from three independent experiments. *E*, HeLa cells were treated with 10 μ M PP2 (30 min) or 50 μ M AG490 (1 h), and incubated with agonist TNFR1 Ab for 15 min. A Western blot was probed with anti-phospho-Ser⁷²⁷ STAT3 and anti-STAT3. *F*, Densitometry was used to analyze the data from experiments conducted as described in *D* and the results illustrate the effect of PP2 and AG490 on TNFR1 agonist Ab-induced activation (serine phosphorylation) of STAT3 in HeLa cells. Data are the mean \pm SEM from two independent experiments.

experiments described. Our observations show that Jak2 and c-Src are constitutively active in the TNFR1 complex and that such activity does not result from autocrine production of TNF or any protein that binds TNFR1.

TNFR1/Jak2 and TNFR1/c-Src engage signaling pathways

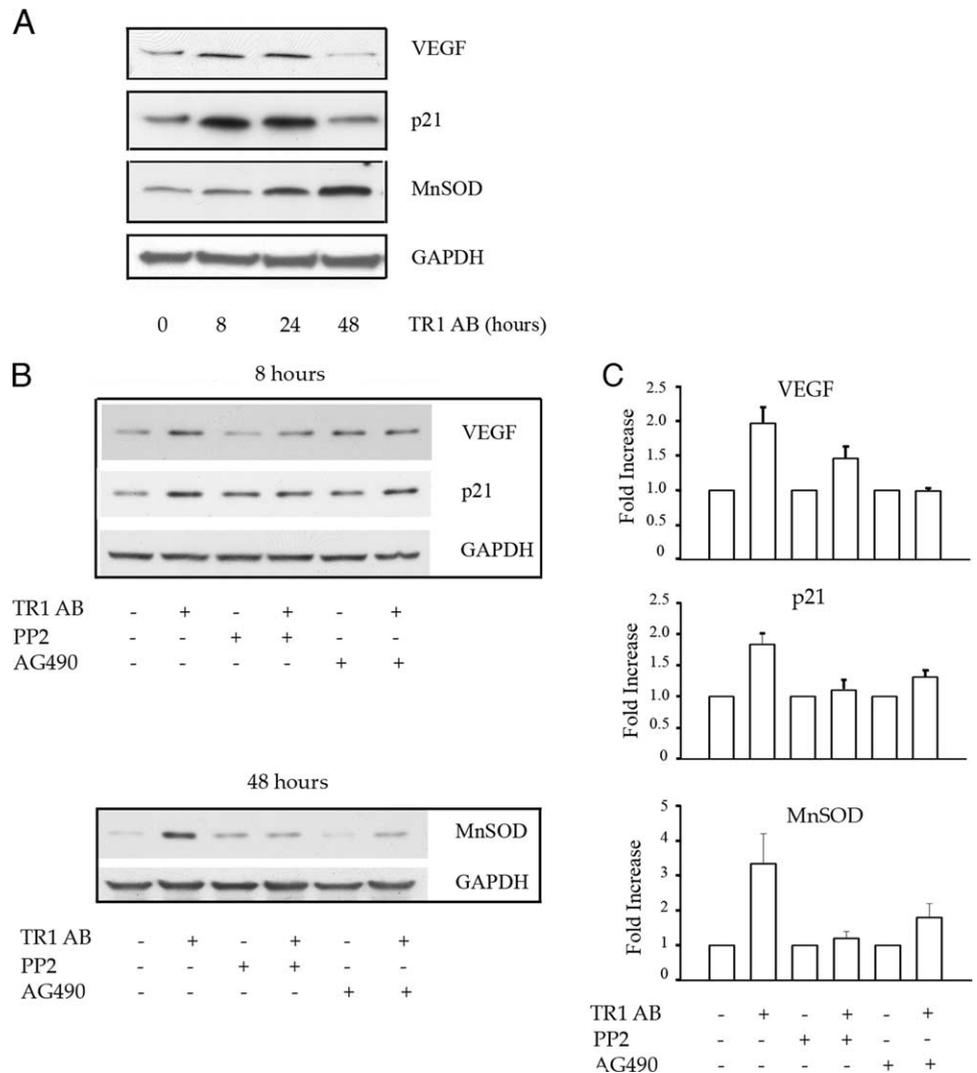
TNF activates Akt, p38 MAPK, and JNK in a time-dependent manner (Fig. 3A). Akt was maximally activated 60 min after cells were exposed to TNF, whereas p38 MAPK and JNK were maximally activated after 15 min. These times were used in subsequent assays of Akt, p38 MAPK, and JNK activation. To determine whether c-Src or Jak2 plays a role in activating these enzymes, cells were treated with c-Src or Jak2 inhibitors or transfected with KD-c-Src or KD-Jak2 (Fig. 3, B–E). HEK293 cells were used for these experiments because they transfect with high efficiency. Inhibition of c-Src with PP2 (Fig. 3B) or expression of KD-Src (Fig. 3C) diminished TNF-induced activation of Akt but not of p38 MAPK or JNK. Inhibition of Jak2, using AG490 (Fig. 3D), or expression of KD-Jak2 (Fig. 3E), diminished TNF-stimulated activation of p38 MAPK, JNK, and Akt. Interestingly, we repeatedly observed that AG490 or KD-Jak2 elevated the basal activity of Akt; also, TNF was unable to increase Akt activity above the high

basal level. These results show that TNFR1/c-Src is upstream of Akt, but not p38 MAPK or JNK, and that TNFR1/Jak2 is upstream of p38 MAPK, JNK, and Akt. Thus, TNFR1/Jak2 and TNFR1/c-Src permit the receptor to activate distinct as well as common targets.

Genetic models confirm that tyrosine kinases are active in TNFR1 signaling

To more definitively determine the role for each of the nonreceptor tyrosine kinases in TNFR1 signaling, we used genetic model cell lines deficient in c-Src or Jak2. Mouse embryo fibroblasts deficient in c-Src, Yes, and Fyn (SYF cells), or reconstituted with Src (SYF plus Src cells), were incubated in the absence or presence of human TNF, which exclusively binds TNFR1 in murine cells (24). TNF induced the phosphorylation of Akt in SYF plus Src cells and the level of such phosphorylation was diminished in c-Src-deficient cells (SYF cells) (Fig. 4A). Whereas phosphorylation of Akt was dependent on c-Src, activation of p38 MAPK was not (Fig. 4A). Experiments were also conducted with Jak2-deficient γ 2A cells and γ 2A cells reconstituted with Jak2. We used an agonist Ab to TNFR1 to specifically activate TNFR1 in these human cells. The agonist Ab activated p38 MAPK in cells reconstituted with

FIGURE 9. Jak2 and c-Src regulate TNF-dependent target genes in MCF7 cells. *A*, Cells were serum-starved for 24 h and then treated with an agonist TNFR1 AB for various times. Fifty micrograms of protein from cell lysates was assayed for expression of VEGF, p21^{WAF1/CIP1}, and MnSOD using Abs directed against each protein. *B*, Cells were incubated in the absence or presence of 10 μ M PP2 or 50 μ M AG490, and then with or without an agonist TNFR1 Ab for 8 h before expression of VEGF and p21^{WAF1/CIP1} were assayed by Western blot analysis. MnSOD expression was assayed after 48-h incubation with the TNFR1 AB. *C*, Densitometry analyzed data from experiments conducted as described in *B* and the result illustrates the effect of PP2 and AG490 on TNFR1 agonist Ab-induced gene expression. Data are the mean \pm SEM from three independent experiments.



Jak2 and such activation was greatly diminished in cells deficient in Jak2. Activation of Akt by TNF was also dependent on Jak2, being induced in cells reconstituted with Jak2, but not in cells deficient in Jak2 (Fig. 4B). Comparable observations were made when cells were treated with TNF (data not shown). The experiments with genetic models confirm that TNFR1/c-Src and TNFR1/Jak2 activate distinct and common downstream targets.

TNFR1/Jak2 and TNFR1/c-Src do not affect each other's activities

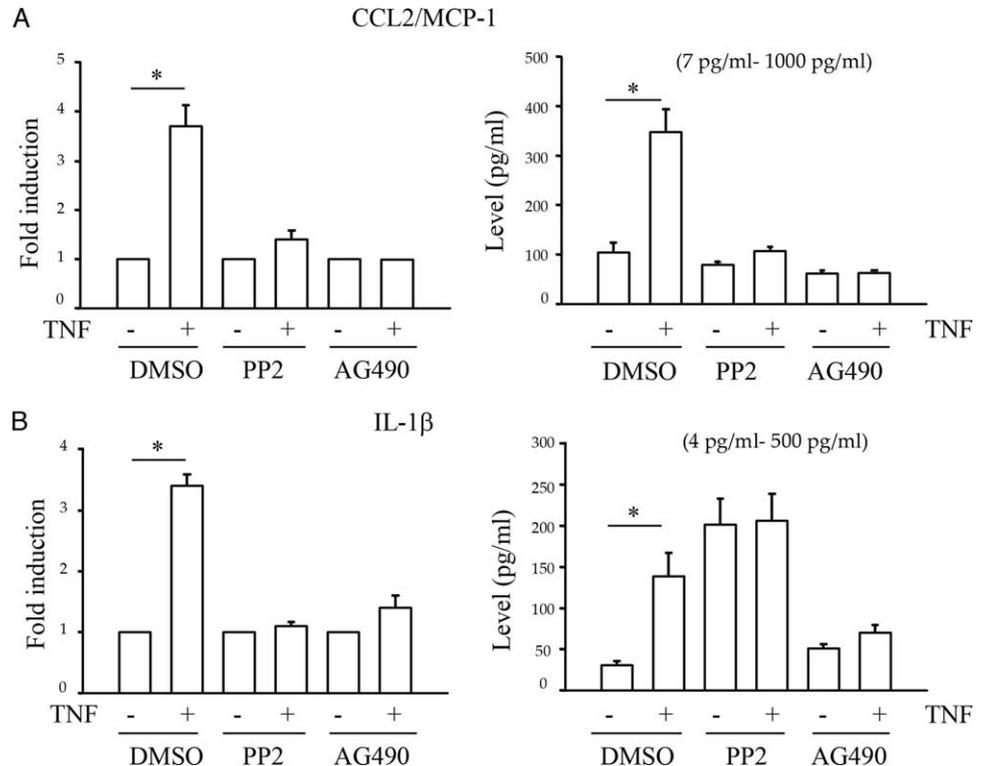
Jak2 and c-Src each play a role in activating Akt. Such an effect might be mediated by one tyrosine kinase affecting the other in the TNFR1 complex. To test whether TNFR1/Jak2 affects the activity of c-Src associated with TNFR1, HEK293 cells were incubated in the absence or presence of AG490 and then with TNF before c-Src or TNFR1 were immunoprecipitated. An *in vitro* kinase assay showed that inhibition of Jak did not affect c-Src activity (Fig. 5, A and B). To test whether TNFR1/c-Src affects the activity of Jak2 associated with TNFR1, cells were incubated in the absence or presence of PP2 and then with TNF before TNFR1 was immunoprecipitated. Western blot analysis showed that the activity (phosphorylation) of Jak2 in the TNFR1 complex was unaffected by the Src inhibitor (Fig. 5C). These results show that Jak2 and c-Src do not affect each other's activities in the TNFR1 complex.

TNFR1-associated kinases selectively regulate transcription factors

Having shown that the TNFR1 kinase complex can affect signaling pathways, we next determined whether the complex affects transcription factors. First, we studied NF- κ B, which is important to inflammation and cell survival. TNF-induced NF- κ B DNA binding in HEK293 cells was inhibited by KD-Src (Fig. 6A) or PP2 (Fig. 6B), but not by KD-Jak2 (Fig. 6C), AG490 (Fig. 6D), or Jak2 antisense (Fig. 6E). Gene reporter assays using KD-c-Src or KD-Jak2, and experiments in cells deficient in Src and Jak2, defined the significance of each tyrosine kinase to TNF-induced NF- κ B transactivation. In HEK293 cells, TNF-induced NF- κ B gene reporter activity was inhibited by KD-Src (Fig. 7A), but not by KD-Jak2 (Fig. 7B). SYF cells deficient in or reconstituted with Src (Fig. 7C), and γ 2A cells deficient in or reconstituted with Jak2 (Fig. 7D), also showed that c-Src, but not Jak2, mediates transactivation of NF- κ B by TNFR1.

Jak and Src kinases can each phosphorylate STAT family transcription factors (25–27). We therefore determined whether TNFR1-associated kinases would target a member of the STAT family, STAT3. In these experiments, we used MCF7 human breast cancer cells, a well-studied model for TNF action in cancer cells (28–32) in which TNFR1 associates with Jak2 and c-Src (Fig.

FIGURE 10. Jak2 and c-Src regulate TNF-dependent cytokine/chemokine production by THP-1 macrophages. Differentiated THP-1 macrophages were cultured in serum-free medium in the absence or presence of AG490 (1 h) or PP2 (30 min) and then incubated in the absence or presence of TNF for 24 h. (A) CCL2/MCP-1 or (B) IL-1 β in supernatants was assayed by ELISA. Results are expressed as fold-induction of each cytokine by TNF relative to control (left panels) and the amount of cytokine in supernatants, expressed in picograms per milliliter (right panels). Data are the mean \pm SEM from three independent experiments. *, $p < 0.001$. The linear range for each assay is shown above each of the panels on the right.



1A). To specifically initiate signaling through TNFR1, the cells were stimulated with an agonist Ab to human TNFR1 (TNFR1 AB). Full activation of STAT3 requires phosphorylation of serine 727 and tyrosine 705 (33, 34). TNFR1 AB, or TNF (data not shown), increased the phosphorylation of serine 727 (Fig. 8A) in MCF7 cells. Basal phosphorylation of tyrosine 705 was high in MCF7 cells and activation of TNFR1 did not increase tyrosine phosphorylation of STAT3 further (Fig. 8B). These observations indicate that TNFR1 AB promotes most of its effect by phosphorylation of serine 727 in STAT3, leading us to investigate this further. Inhibition of Jak2 abrogated the capacity of the TNFR1 AB to induce serine phosphorylation of STAT3 (Fig. 8C). However, inhibition of c-Src did not impair the capacity of TNFR1 AB to induce serine phosphorylation of STAT3 (Fig. 8C). Analysis of autoradiographs from three independent experiments showed that the TNFR1 AB increased serine phosphorylation of STAT3 by 1.8-fold. Inhibition of c-Src did not affect TNFR1 AB-induced phosphorylation, whereas inhibition of Jak2 abrogated it (Fig. 8D). We next tested whether the TNFR-associated tyrosine kinases affect STAT3 activity in a second cell type, HeLa cells (Fig. 8E), in which we previously demonstrated interaction of Jak2 with TNFR1 (20). As with MCF7 cells, in HeLa cells the TNFR1 AB increased the level of STAT3 serine phosphorylation (2.5-fold) and this capability was inhibited by pretreatment of cells with AG490, but not PP2 (Fig. 8, E and F). These results show that TNFR1/Jak2, but not TNFR1/c-Src, activates STAT3.

The TNFR1/tyrosine kinase complex affects gene expression

Our finding that Jak2 and c-Src are used by TNFR1 to affect transcription factors led us to test whether the nonreceptor tyrosine kinases also affect the expression of proteins induced by TNF. Because activated TNFR1 elicits diverse responses from cancer cells, we studied whether the TNFR1-associated kinases would affect the induction of vascular endothelial growth factor (VEGF), manganese superoxide dismutase (MnSOD), and p21^{WAF1/CIP1} in MCF7 breast cancer cells. The TNFR1 AB induced a time-depen-

dent increase of VEGF, p21^{WAF1/CIP1}, and MnSOD (Fig. 9A). AG490 and PP2 each inhibited the capacity of TNFR1 AB to induce these proteins (Fig. 9, B and C). Because TNF has important effects on immune cells, we determined whether Jak2 and c-Src mediate TNF-dependent cytokine production in THP-1 macrophages. Using an ELISA, we found that TNF induces the production and secretion of CCL2/MCP-1 (Fig. 10A) and IL-1 β (Fig. 10B). Pretreatment of the macrophages with AG490 or PP2 inhibited TNF-induced cytokine expression. Also, PP2 dramatically increased the basal level of IL-1 β secretion; however, TNF was unable to increase IL-1 β secretion above the elevated basal level. Our observations show that the TNFR1-associated tyrosine kinases affect the capacity of TNF to induce the expression of gene products in cancer and immune cells.

Discussion

Our study shows that endogenous c-Src and Jak2 constitutively associate with TNFR1. c-Src and Jak2 are already known to interact with and activate PI3K (21–23). Consequently, we sought to determine whether PI3K is a component of the endogenous TNFR1 complex. Our finding of an association of PI3K with TNFR1 brings PI3K into the TNFR1 complex and into proximity with Jak2 and c-Src. This proximity may permit each tyrosine kinase to activate PI3K, and its downstream target, Akt. These observations show that TNFR1 associates with members of two families of nonreceptor protein tyrosine kinases and a lipid kinase.

A novel observation reported here is that Jak2 and c-Src associated with TNFR1 are constitutively active. A preligand assembly domain in TNFR1 that mediates ligand-independent receptor aggregation may underlie the constitutive activity of Jak2 (35). Associations of TNFR1 monomers may bring associated Jak2 monomers into proximity and permit their activation by transphosphorylation. Aggregation of other cytokine receptors facilitates such activation of associated Jaks (26). c-Src activation results from dephosphorylation of tyrosine 527, and this is mediated by protein tyrosine phosphatases. One such phosphatase, SHP-1 (36),

associates with TNFR1 (37). The presence of an intrinsic preligand assembly domain and the activity of an associated tyrosine phosphatase may confer the capacity for self-activation onto the TNFR1 complex.

TNF modulates the amount and activity of Jak2 and c-Src associated with TNFR1 in a parallel manner. These observations lead us to conclude that changes in the amount of c-Src and Jak2 associated with TNFR1 underlie changes of the tyrosine kinase activity of the complex. By affecting the intensity of the signal emanating from the TNFR1/tyrosine kinase complex, TNF can induce changes of signaling that impact transcription factors and their target genes.

Jak2 and c-Src permit TNFR1 to engage distinct and common signaling pathways. c-Src is necessary for TNFR1-induced activation of Akt, but not p38 MAPK or JNK, whereas TNFR1/Jak2 plays a role in the activation of p38 MAPK, JNK, and Akt. As observed by Neria et al. (38) with endothelial cells, we found that inhibition of Jak2 increased basal Akt activity in 293 cells (Fig. 3, C and D). TNF was unable to activate Akt above the elevated basal level. A role for Jak2 in TNF-dependent Akt activation was also demonstrated in the Jak2-null human osteosarcoma cell line, γ 2A (Fig. 4B). In γ 2A cells, TNF-dependent Akt phosphorylation was dramatically decreased compared with γ 2A cells reconstituted with Jak2, the γ 2A plus Jak2 cell line. In previous work, we showed that TNFR1 promotes the tyrosine phosphorylation and activation of PI3K, and, through PI3K, Akt (14, 17). The present study shows that TNFR1/c-Src and TNFR1/Jak2 independently promote PI3K/Akt signaling. The PI3K/Akt pathway may be used by TNFR1 to modulate cellular responsiveness to insulin (18), and, as discussed further below, in conjunction with other processes, may impact NF- κ B (17, 18). The demonstration that TNFR1/Jak2 engages JNK and p38 MAPK is significant, as these MAPK family members activate AP-1, and through it, gene expression (3). Thus, the TNFR1-tyrosine kinase complex engages signaling pathways that affect transcription factors, gene expression, and metabolism.

The transcription factor NF- κ B promotes immunity and inflammation by inducing numerous genes, including those coding for cytokines, chemokines, and adhesion molecules (39). NF- κ B is also involved in the development and survival of cancers, in which it may be highly activated (40). TNF is among the most potent activators of NF- κ B, and, when acting through TNFR1, uses TRAF2 and RIP to activate the I κ B kinase (IKK) complex (39). The IKK complex phosphorylates, and induces the degradation of I κ B proteins, permitting NF- κ B to enter the nucleus, where it activates target genes. The present study shows that TNFR1/c-Src, but not TNFR1/Jak2, is necessary for activation of NF- κ B by TNFR1. This conclusion is based on the observation that PP2, KD-Src, or c-Src deficiency impaired the capacity of TNF to activate NF- κ B. In contrast, AG490, KD-Jak2, antisense to Jak2, or Jak2 deficiency had no effect on TNF-induced activation of NF- κ B. Thus, TNFR1/c-Src, as well as TRADD/TRAF2/RIP, is obligate for activation of NF- κ B.

Inhibition of Jak2 did not impair TNF-induced activation of NF- κ B despite affecting Akt, which phosphorylates IKK α in the IKK complex and plays a role in activation of NF- κ B by TNF and other cytokines (17, 41–43). In contrast, c-Src, which also affects Akt, did promote activation of NF- κ B by TNF. One explanation for these observations is that Akt and a second kinase targeted by c-Src, but not Jak2, is necessary for activation of NF- κ B. One candidate is the TGF- β -activated kinase 1, a MAPK kinase, which is activated by TNF (44) and recruited into the TNFR1 complex to activate NF- κ B (45). Whether TGF- β -activated kinase 1 (46, 47) or Akt (16) affects NF- κ B is dependent on cell type and context. In addition, c-Src, but thus far not Jak2, reportedly inter-

acts with and phosphorylates IKK β , thus affecting NF- κ B independently of Akt (48).

STATs are transcription factors that were originally identified in signaling pathways activated by nontyrosine kinase cytokine receptors (26). Tyrosine phosphorylation of STATs is an early step in their activation that is required for them to dimerize and bind to DNA. Phosphorylation of serine 727 in the transcriptional activation domain of STAT1 and STAT3 is required for complete activation of the factors (33, 34). In previous studies, we showed that TNF induces the tyrosine phosphorylation of STAT3 in adipocytes, which correlates with enhanced DNA binding of STAT3 (20). Whether TNF induces STAT3 serine phosphorylation was not investigated. We now show that TNF regulates STAT3 activity mainly through serine phosphorylation in MCF7 cells. The high basal level of STAT3 tyrosine phosphorylation found in these cells may preclude the necessity for TNF to affect STAT3 through tyrosine phosphorylation. In our previous work, we assumed, but did not demonstrate, that STATs were substrates of Jaks because both groups of proteins were phosphorylated in parallel and in conjunction with signaling through TNFR1. However, the demonstration that STATs are substrates for Src as well as Jak kinases (25–27), coupled with the demonstration that Jak2 and c-Src associate with TNFR1, made it important to characterize how TNFR1 acts on STAT proteins. We have now found that TNFR1/Jak2, but not TNFR1/c-Src, activates STAT3 in MCF7 and HeLa cells. It is possible that TNFR1/c-Src affects STAT3 through tyrosine, as well as serine phosphorylation, in cells in which TNF may induce this effect. Further study will be needed to address this issue. Because p38 MAPK and JNK induce serine phosphorylation of STAT3 (49) and are downstream of TNFR1/Jak2, they likely mediate the effect of TNFR1 on serine phosphorylation of STAT3. Thus, a Jak/Stat-signaling pathway emanating from TNFR1 has now been described.

Our finding that TNFR1 uses c-Src and Jak2 to affect NF- κ B and STAT3, respectively, led us to test whether the kinases would affect gene activity. We have shown such a change, because stimulation of MCF7 cells with TNF or a TNFR1 agonist Ab induces proangiogenic VEGF, the cell cycle modulator p21^{WAF1/CIP1}, and MnSOD, a vital antioxidant enzyme. Each protein is the product of a gene activated by NF- κ B (50–52) and STAT3 (53–56); consequently, both PP2 and AG490 inhibited TNF-induced expression of each target.

Our investigation of the role of c-Src and Jak2 in TNF-induced gene expression was extended to include studies with an immune cell. In THP-1 macrophages, TNF induces the expression of CCL2/MCP-1 and IL-1 β (Fig. 10 and Ref. 57). MCP-1 recruits immune cells to sites of tissue injury and infection, whereas IL-1 β is a potent mediator of the inflammatory response. Induction of MCP-1 and IL-1 β is induced largely or entirely through TNFR1 and is effected by activation of diverse signaling pathways and transcription factors (57–62). Consistent with the pleiotropic mechanisms through which CCL2/MCP-1 and IL-1 β can be induced, and the demonstration that c-Src and Jak2 are used by TNFR1 to engage diverse signaling pathways, we found that PP2 and AG490 each inhibited expression of these gene products. Our observations show that the TNFR1/kinase complex plays an important, obligatory role in the activation of gene expression in transformed cells and also in nontransformed cells that promote immune responses.

By signaling through TRADD and its associated proteins, particularly TRAF2, TNFR1 induces diverse events. TRAF2, as well as TNFR1/c-Src and TNFR1/Jak2, plays a role in the activation of JNK, p38 MAPK, and NF- κ B (63, 64). We suggest that the TNFR1-associated kinases and TNFR1/TRADD/TRAF2 act in

conjunction. Consistent with this speculation are observations showing that TRAF6 and c-Src are components of a complex used by TRANCE, a TNF family member, to activate Akt downstream of the TRANCE receptor (65). In addition, TRAF2 interacts with the T cell protein tyrosine phosphatase, which inactivates c-Src to suppress signaling through ERKs downstream of TNFR1 (66). The demonstration that c-Src associates with TNFR1 now suggests that components of the TNFR1 complex interact to regulate one another's functions. Delineation of the architecture of the TNFR1 complex, and the relationship of its associated kinases to those proteins recruited by TRADD, will lead to a more complete understanding of TNFR1 function.

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Disclosures

The authors have no financial conflict of interest.

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