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Induction of Jak/STAT Signaling by Activation of the Type 1 TNF Receptor

DanQun Guo,* James D. Dunbar,* Chuan He Yang,† Lawrence M. Pfeffer,† and David B. Donner²‡

Cellular responses to TNF are initiated by either of two cell surface receptors, the type 1 TNF receptor (TNFR1) and the type 2 TNF receptor (TNFR2). Although neither receptor contains an intrinsic protein tyrosine kinase, such activity has been implicated in TNF action. In this study, we show that murine TNF induces the tyrosine phosphorylation and activation of the intracellular Janus tyrosine kinases Jak1, Jak2, and Tyk2 in murine 3T3-L1 adipocytes. Activation of Jak kinases by TNF was associated with tyrosine phosphorylation of STAT1, STAT3, STAT5, and STAT6, but not STAT2 or STAT4, showing that TNF acts on a specific subset of these latent cytoplasmic transcription factors in 3T3-L1 adipocytes. Agonist antisera to TNFR1 induced Jak kinase and STAT protein phosphorylation. Phosphorylation of Jak kinases was also induced by human TNF, which selectively binds to TNFR1 on murine cells. 3²S-labeled Jak kinases were precipitated from a cell-free system and from lysates of 3T3-L1 adipocytes by a glutathione S-transferase fusion protein containing the cytoplasmic domain of TNFR1. These results suggest that the cytoplasmic domain of TNFR1 can directly interact with and form signaling complexes with Jak kinases. Jak2 was precipitated from HeLa cells by antisera to TNFR1, directly demonstrating their association in vivo. Thus, TNF activates a Jak/STAT signaling-transduction cascade by acting through TNFR1. The Journal of Immunology, 1998, 160: 2742–2750.

Tumor necrosis factor is a multifunctional cytokine that was first identified and characterized on the basis of its ability to induce the regression of tumors in animals and by the cytotoxic response that it can elicit from transformed cells (1, 2). While originally viewed as an oncolytic agent, subsequent work has shown that TNF promotes immunity, inflammation, insulin resistance, angiogenesis, and the syndrome of wasting and malnutrition known as cachexia in some chronic diseases (1, 2).

The first step in TNF action is binding to specific receptors that are expressed on virtually all nucleated cells. Two distinct receptors for TNF have been identified, the 55-kDa type 1 receptor (TNFR1)¹ and the 75-kDa type 2 receptor (TNFR2), and their cDNAs cloned (3–5). The extracellular domains of the receptors share homologies with one another and with a group of cell surface proteins that include the FAS Ag, the low affinity nerve growth factor receptor, the murine cDNA clone 4-1BB from induced tumor necrosis factor (TNF) receptors are dissimilar (3–5), and the receptors induce a distinct but overlapping repertoire of responses: TNFR1 promotes cytotoxicity, fibroblast proliferation, and antiviral responses, and plays a preeminent role in the host defense against microorganisms and pathogens (7–11); TNFR2 also plays a role in cytotoxicity, modulates hemopoiesis, and is involved in the proliferation of monocytes and T cells (12–16).

The proximal steps in the pathways through which TNF induces such responses are incompletely understood. Neither TNF receptor contains protein tyrosine kinase activity or any motif suggesting a biochemical activity (3–5). However, the induction of specific tyrosine phosphorylations is associated with cellular responses to TNF. Such phosphorylations have been related to alterations of cellular sensitivity to TNF-mediated cytotoxicity (17, 18). In addition, inhibitors of protein tyrosine kinases suppress TNF-stimulated phosphorylation of a 23-kDa nuclear protein (19), DNA fragmentation (20), activation of nuclear factor-kB, and expression of endothelial cell adhesion molecules (21, 22). We recently demonstrated that in 3T3-L1 adipocytes, TNF rapidly induces the tyrosine phosphorylation of a group of cytoplasmic proteins, including insulin receptor substrate-1. These results show that early activation of tyrosine kinase activity is a component of the cellular response to TNF (23). The priming of neutrophils by TNF is accompanied by tyrosine phosphorylation events believed to participate in the transduction of signals that direct the cells to undergo a respiratory burst (24, 25). In neutrophils, TNF, granulocyte CSF, and granulocyte-macrophage CSF induce the tyrosine phosphorylation of a common group of proteins (26, 27), which may indicate that the cytokines act, in part, through common signaling pathways.

The present study was initiated to identify protein tyrosine kinases involved in TNF signal transduction. In this regard, our attention was drawn to a family of cytoplasmic protein tyrosine kinases, Janus (Jak) kinases (28–30), which promote signaling by cytokine/hemopoietin receptors activated by ligands such as granulocyte-macrophage CSF and granulocyte CSF, and mediate the tyrosine phosphorylation of a number of intracellular targets including insulin receptor substrate-1 (31–34). We have found that...
by acting through TNFR1, TNF promotes the phosphorylation and activation of Jak protein tyrosine kinases in 3T3-L1 adipocytes. These kinases interact directly with TNFR1 in a cell-free system and in vivo. Activation of Jak kinases by TNF was accompanied by the tyrosine phosphorylation of members of the STAT family of transcription factors and the induction of STAT3 DNA-binding activity. The Jak/STAT signaling pathway represents a mechanism through which TNF transmits signals from the cell surface to the nucleus.

### Materials and Methods

#### Materials

Recombinant murine TNF (mTNF) was from R&D Systems (Minneapolis, MN). Recombinant human TNF (hTNF) and agonist Abs against mTNF were gifts from Genentech (South San Francisco, CA). Ab to hTNF was from Genzyme Corp. (Cambridge, MA). Jak1 cDNA in plasmid CMV and Jak2 cDNA in PK5 were gifts from Dr. James Ihle, St. Jude’s Children’s Research Hospital, Memphis, TN. Horseradish peroxidase-conjugated anti-phosphotyrosine mAb (RC20) was from Transduction Laboratories (Lexington, KY). Abs to Jak1, Jak2, Jak3, and Tyk2 were from Upstate Biotechnology (Lake Placid, NY). Abs to STAT1, STAT3, STAT5b, and STAT6 were from Santa Cruz Biotechnology (Santa Cruz, CA).

#### Cell culture

3T3-L1 fibroblast cells from American Type Culture Collection (Rockville, MD) were grown in 100-mm dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids (100 U/ml), and streptomycin (100 U/ml) in a humidified incubator under 5% CO2 at 37°C. Differentiation to adipocytes was conducted as described (35). Maximal adipose conversion was achieved 10 to 12 days after initiation of differentiation.

#### Immunoprecipitation and immunoblotting tyrosine-phosphorylated Jaks and STAT proteins

After treatment with TNF, cells were washed twice with ice-cold PBS and lysed by incubation in 50 mM HEPES, pH 7, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 1 mM sodium o-vanadate for 30 min at 4°C. The lysate was centrifuged (150,000 × g max, 1 h) and the infranatant was removed from the fat and passed through a 22-µm filter. Extracts (1-1.5 mg of protein) were shaken with antiserum overnight at 4°C, 40 µl of a slurry of protein G-plus/protein A-agarose was added, and the incubation was continued for 2 h at 4°C. The incubate was centrifuged briefly at 10,000 × g, and the agarose was washed with 20 mM HEPES, pH 7, 10% glycerol, 0.1% Triton X-100, 150 mM NaCl, and 1 mM sodium o-vanadate and centrifuged. This was repeated four times, after which Larenmli medium was added and the agarose was heated at 100°C for 5 min. Immunoprecipitated proteins were separated by electrophoresis and transferred to Immobilon-P (Millipore, Marlborough, MA) during an overnight transfer. Immunoblots were blocked by incubation in TBST (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% Tween-20) containing 1% BSA and then incubated with the primary Ab for 1 to 2 h at room temperature, washed three times with TBST, and, in experiments not using a primary Ab conjugated to horseradish peroxidase, incubated with anti-mouse or anti-rabbit secondary Abs for 1 h. The enhanced chemiluminescent detection system (Amersham, Arlington Heights, IL) was used for protein detection. To probe immunoblots with a second antiserum, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-ME for 30 min at 50°C. The blots were then incubated with antiserum and processed as described above.

#### Nuclear extracts and gel shift assays

Nuclei were extracted from 3T3-L1 adipocytes with buffer containing 20 mM Tris-HCl, pH 7.85, 250 mM sucrose, 0.4 M KCl, 1.1 mM MgCl2, 5 mM 2-ME, and 0.4 mM PMSF, and extracts were frozen on dry ice and stored at −80°C. For gel shift analysis, the nuclear extracts were incubated with a 32P end-labeled promoter probe for either the high affinity c-sis-inducible element (SIE) from the c-fos gene (5′-AGCTTCTAGGCTGCAAACAGCCGAAAGGAAAGCAGCCGAAACG-3′) or the IFN-stimulated response element (ISRE) from IFN-γ stimulated c-sis promoter (5′-TTCCATGTCGGCATGGAGGAAAGCAGCCGAAACG-3′) at 25°C for 30 min, and the free probe was separated from protein-DNA complexes on 5% polyacrylamide gels. For supershift assays, nuclear extracts were preincubated with a 1/50 dilution of normal rabbit serum, anti-STAT1, or anti-STAT3 at 25°C for 0.5 h. Gels were quantitated by phosphor image autoradiography.

#### In vitro binding assays

Three GST-TNFFR1IC fusion constructs were prepared by amplifying and inserting desired portions of TNFR1IC into pGEX-2T (Pharmacia Biotech, Piscataway, NJ) (36): 1) the full-length cytoplasmic domain of TNFR1IC (amino acids 205–413, GST-TNFFR1IC); 2) part of the N-terminal half of TNFR1IC (amino acids 243–315, GST-TNFFR1ICNI); and 3) the C-terminal half of TNFR1IC (amino acids 316–413, GST-TNFFR1ICOOH). After induction, cells were grown at 30°C for 3 h, suspended in lysis buffer (20 mM Tris, pH 8, 200 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 1 mM PMSF, 2 µg/ml aprotinin, 2 µg/ml leupeptin, and 0.1% 2-ME), and sonicated. The lysate was centrifuged (12,000 rpm, 30 min), and one-tenth volume of 50% GST-agarose slurry (Sigma Chemical Co., St. Louis, MO) was incubated with the supernatant for 1 h at 4°C. The beads were washed three times with lysis buffer, and the purity of the GST fusion proteins was confirmed by SDS-PAGE.

The troponin T-coupled rabbit reticulocyte lysate system (Promega Corp., Madison, WI) was used for transcription/translation of Jak1 cDNA in PR-CMV or Jak2 cDNA in PK35 (under control of the T7 or SP6 promoter, respectively), according to the instructions of the manufacturer. Rabbit reticulocyte was mixed with 1 µg of Jak cDNA along with SP6 or T7 RNA polymerase, after which an amino acid mixture without methionine plus [35S]methionine was added. After 2 h at 30°C, the purity and level of expression of each Jak were analyzed by SDS/PAGE and phosphor image analysis (Bio-Rad, Hercules, CA). Approximately 5 µg of each agarose-bound GST-TNFFR1IC construct was added into binding buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% 2-ME, 10 mM EDTA, 1 mM EGTA, 1.0 mM sodium o-vanadate, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin) and 40 µl of reticulocyte lysate that contained [35S]methionine-labeled Jak. After 1 h at 4°C, the agarose beads were washed three times with binding buffer before addition of SDS sample buffer. The samples were subjected to electrophoresis and phosphor image analysis.

GST-TNFFR1IC was also used to bind Jak kinases in lysates of control- or TNF-treated 3T3-L1 adipocytes. In this assay, 5 µg of agarose-bound GST-TNFFR1IC was added to cell lysates for 1 h at 4°C in 50 mM HEPES, pH 7, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A, and 1 mM sodium o-vanadate. The beads were washed three times in lysis buffer before bound proteins were eluted into Laemmli medium, fractionated by SDS-PAGE, and transferred to Immobilon-P (Millipore), which was probed with Abs to Jak kinases.

#### In vivo association of TNFR1 and Jak2

HeLa cells in DMEM supplemented with 10% FBS were treated with vehicle or TNF (1 nM) for 15 min at 37°C, washed twice with ice-cold PBS, and lysed by incubation in 50 mM HEPES, pH 7, 150 mM NaCl, 10% glycerol, 1.2% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A, and 1 mM sodium o-vanadate for 30 min at 4°C. Extracts were shaken with antiserum to human TNFR1 for 16 h at 4°C, after which 40 µl of a slurry of protein G-plus/protein A-agarose were added and incubation was continued for 2 h at 4°C. The incubate was centrifuged briefly at 10,000 × g, and the agarose was washed with lysis buffer and centrifuged. This process was repeated four times, after which Laemmli medium was added and the agarose was heated at 100°C for 5 min. Immunoprecipitated proteins were fractionated on 7.5% acrylamide gels and blotted onto Immobilon-P (Millipore, Marlborough, MA) during an overnight transfer. Immunoblots were blocked by incubation in PBS containing 5% fat-free milk and then incubated with the primary Ab to Jak2 for 1 to 2 h at room temperature. Blots were washed three times with TBST and then incubated with an anti-mouse secondary Ab for 1 h. The enhanced chemiluminescent detection system was used for protein detection. To reprobe immunoblots, the membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 2% SDS, and 100 mM 2-ME for 30 min at 50°C. The blots were then incubated with Ab to TNFR1 and processed as described above.

#### Results

To investigate whether mTNF stimulates phosphorylation of members of the Jak tyrosine kinase family, Jak kinases were immunoprecipitated from lysates of control- and mTNF-treated murine
3T3-L1 adipocytes. Immunoprecipitated proteins were fractionated by SDS-PAGE and immunoblotted with an Ab to phosphotyrosine. As illustrated by Figure 1, mTNF promoted the phosphorylation of Jak1 (top), Jak2 (middle), and Tyk2 (bottom). Reproducibly, Jak2 became more highly tyrosine phosphorylated than Jak1 or Tyk2 after cell stimulation with mTNF. Jak3 phosphorylation was not detected in 3T3-L1 adipocytes, as this kinase is not expressed in this cell type (data not shown).

To determine whether Jak kinase activity was stimulated by mTNF, we measured enzyme autophosphorylation in vitro (Fig. 2). To accomplish this, Jak1 or Jak2 was immunoprecipitated from control or mTNF-treated 3T3-L1 adipocytes and then reacted with ATP to permit autophosphorylation. Tyrosine phosphorylation was determined by Western blot analysis using Abs to phosphotyrosine, which showed that mTNF promotes substantial autophosphorylation of the Jak1 and Jak2 kinases. In the case of Jak1, some low level of Jak phosphorylation was detected in cells exposed to TNF alone, probably due to Jak autophosphorylation with endogenous ATP. Because of the higher level of Jak2 autophosphorylation induced by TNF in the presence of ATP, kinase activation induced by TNF alone could only be detected by grossly overexposing the autoradiograph (data not shown).

In cells responsive to ligands for cytokine/hemopoietin receptors, Jak activation leads to phosphorylation of members of the STAT family of latent transcription factors (28–30). To determine whether TNF mediates such a response, STAT proteins were immunoprecipitated from control- and mTNF-treated 3T3-L1 adipocytes, fractionated by SDS-PAGE, transferred to Immobilon-P, and probed with an Ab to phosphotyrosine. mTNF induced a time-dependent increase in the tyrosine phosphorylation of STAT1 (Fig. 3, top) and STAT3 (Fig. 3, middle), but did not affect STAT2 phosphorylation (data not shown). In addition, TNF strongly induced phosphorylation of STAT5 and weakly induced phosphorylation of STAT6 (Fig. 3, bottom), but was unable to promote phosphorylation of STAT4 (data not shown). These observations show that STAT protein phosphorylation induced by TNF is specific and selective.

The induction of DNA-binding activity attributable to STAT proteins was determined in electrophoretic mobility shift assays with oligonucleotide probes specific for the ISRE and SIE. These
probes represent conserved promoter elements present in the IFN-stimulated gene family and the c-fos gene, respectively. Figure 4 shows that TNF rapidly induced (within 15 min of addition) DNA-binding activity in nuclear lysates prepared from TNF-treated adipocytes incubated with the SIE probe. No DNA binding to the SIE was detected in the presence of excess unlabeled SIE oligonucleotide, and binding was not competed by excess ISRE oligonucleotide. Taken together, these results indicate that the binding to the probe was specific. In contrast, no DNA-binding activity could be detected in nuclear lysates prepared from TNF-treated adipocytes incubated with the ISRE probe (which detects STAT1/STAT2 heterodimers, ISGF3). To detect specific STAT proteins in the TNF-inducible DNA-protein complexes, we performed gel supershift assays with STAT-specific antisera. Anti-STAT3 supershifted the DNA-binding complex formed with the SIE probe in TNF-treated adipocytes, while neither control normal rabbit serum nor anti-STAT1 shifted any of the TNF-induced SIE DNA-protein complexes. Anti-STAT2 also failed to shift any of the TNF-induced SIE DNA-protein complexes (data not shown). Thus, TNF induced a DNA-binding activity in adipocytes attributable to STAT3.

Whereas mTNF binds TNFR1 and TNFR2 on murine cells, hTNF is selectively recognized by mTNFR1 (37). This unique species specificity, together with the availability of agonist antiserum to TNFR1 allowed us to determine whether signaling through TNFR1 induces Jak/STAT signaling, using Jak1 and Jak2, and STAT1 and STAT3 as endpoints. Treatment of murine 3T3-L1 adipocytes with hTNF resulted in phosphorylation of Jak1 and Jak2 (Fig. 5, top), which shows that TNFR1 promoted these events. Consistent with the conclusion that TNFR1 mediates phosphorylation of Jak kinases are experiments in which 3T3-L1 adipocytes were stimulated with agonist antisera specific to mTNFR1 (anti-mTNFR1). Jak1 and Jak2 were then immunoprecipitated from cell lysates and fractionated by SDS-PAGE, and Western

FIGURE 3. Stimulation of STAT phosphorylation by mTNF. 3T3-L1 adipocytes were incubated with 5 nM mTNF at 37°C for various times before STAT1 (top), STAT3 (middle), or STAT5 and STAT6 (bottom) were immunoprecipitated from cell lysates. Immune complexes were fractionated by SDS-PAGE and transferred to Immobilon-P, which was probed with an Ab to phosphotyrosine. The enhanced chemoluminescence (ECL) system was used for protein detection. To verify application of equal protein amounts, the blots were stripped and reprobed with antisera to each STAT.
blots were probed with antisera to phosphotyrosine. As shown in Figure 5 (middle), activation of TNFR1 with anti-mTNFR1 resulted in the time-dependent tyrosine phosphorylation of Jak1 and Jak2. Since Jak kinase phosphorylation promoted by TNF has not been demonstrated previously, we compared the extent of such activation with that promoted by human growth hormone, a hormone previously demonstrated to elicit such a response (38). Figure 5 (bottom) illustrates that mTNF, which activates both TNFR subtypes, and agonist antisera to TNFR1 both significantly induced phosphorylation of Jak2, although the magnitude of this response was less than that induced by human growth hormone.

Experiments similar to those described above determined whether the selective activation of TNFR1 promotes STAT protein phosphorylation. The ability of anti-mTNFR1 to replicate the effect of mTNF on STAT1 and STAT3 (Fig. 6) shows that signaling through this receptor induces STAT phosphorylation. However, our results do not preclude the possibility that signaling through TNFR2 might also activate the Jak/STAT pathway.

Since TNFR1 was demonstrated to activate Jak/STAT signaling, in vitro binding assays were used to test whether Jak kinases associate with this receptor. GST fusion proteins containing the full-length intracellular domain of TNFR1 (GST-TNFR1IC) or the N- or C-terminal halves of TNFR1IC (GST-TNFR1INH2 and GST-TNFR1COOH) were prepared and purified to homogeneity (36). GST-TNFR1IC immobilized on agarose beads bound 35S-labeled Jak1 or Jak2 (Fig. 7, top), suggesting that TNFR1 and Jak kinases interact directly, without the intermediacy of other proteins. GST fusion proteins encoding the N- and C-terminal regions of TNFR1 also bound Jak1 and Jak2, although the NH2-terminal fusion protein was more effective in this regard than the COOH-terminal fusion protein. These results suggest the presence of a primary Jak binding site in the N-terminal portion of TNFR1, which contains a proline-rich motif reminiscent of the box 1 motif with which these kinases are known to interact, as well as a secondary Jak kinase binding site in the C-terminal half of TNFR1IC. GST-TNFR1IC was also used to bind Jak1 and Jak2 in lysates of control- and mTNF-treated 3T3-L1 adipocytes (Fig. 7, bottom). Results from this experiment suggest that TNFR1 and Jak kinases interact in vivo.

This interaction was also confirmed by coimmunoprecipitation of TNFR1 and Jak2 from cell lysates. To accomplish this, TNFR1 was immunoprecipitated from vehicle- and TNF-treated HeLa cells. This human cell line was used for these experiments, as the
Among the substrates for activated Jak kinases are the STAT family of latent cytoplasmic transcription factors. Activation of cytokine receptors and their associated Jak kinases is rapidly followed by the tyrosine phosphorylation of STAT proteins, which subsequently dimerize and translocate to the nucleus. Within the nucleus, STATs bind DNA and activate the transcription of downstream genes, many of which are important to inflammatory responses (28–30). Among these are genes for acute phase reactant proteins, IFN-regulatory factor-1, and Jak2 (as summarized in Ref. 39). Prolactin and growth hormone receptors appear to function exclusively through Jak2 (39).

FIGURE 6. Stimulation of STAT1 and STAT3 phosphorylation by signaling through TNFR1. Left, 3T3-L1 adipocytes were stimulated for various times at 37°C with anti-mTNFR1, and phosphorylation of STAT1 and STAT3 was assayed, as described in the legend to Figure 5. Right, To verify application of equal amounts of protein, the blots were stripped and reprobed with antisera to STAT1 or STAT3.

Ab used for immunoprecipitation is specific to human TNFR1. Western blots of immunoprecipitates were then tested for coprecipitation of Jak2. The results illustrated in Figure 8 show that this Jak kinase constitutively associates with TNFR1.

Discussion

Many cytokine/hemopoietin receptors do not contain intrinsic protein tyrosine kinase activity, but noncovalently bind members of the Janus kinase family of cytoplasmic protein tyrosine kinases (28–30). Activation of the receptor-associated kinases is promoted by ligand-induced receptor dimerization. This process brings the receptor cytoplasmic domains into close proximity and the associated Jaks into juxtaposition, thereby allowing transphosphorylation and kinase activation. Cytokine receptors may utilize one or more members of the Jak kinase family, which is presently composed of Jaks 1, 2, and 3 and Tyk2. For example, IFN-α acts through Jak1 and Tyk2, whereas IFN-γ acts through Jak1 and Jak2 (39).

Among the substrates for activated Jak kinases are the STAT family of latent cytoplasmic transcription factors. Activation of cytokine receptors and their associated Jak kinases is rapidly followed by the tyrosine phosphorylation of STAT proteins, which subsequently dimerize and translocate to the nucleus. Within the nucleus, STATs bind DNA and activate the transcription of distinct groups of genes, many of which are important to inflammatory responses (28–30). Among these are genes for acute phase reactant proteins, IFN-regulatory factor-1, intercellular adhesion molecule 1, the β-chain of the IL-2R, Igs, and FcγRI (40–43). The alteration in gene expression plays an important role in the innate immunity of organisms (44, 45) and in the regulation of cell growth and viability (46–49).

The STAT protein family is presently composed of six distinct, but homologous members designated STAT1 through STAT6 (28–30, 39). Most STATs are widely expressed in a variety of cell types. Each STAT may be activated in response to cell stimulation by multiple ligands; however, the process is not promiscuous, in that particular ligands tend to act on a restricted array of STAT proteins. For example, IFN-γ preferentially activates STAT1, IL-6 preferentially activates STAT3, and IL-4 preferentially activates STAT6. Jak-STAT signaling is also induced in cells transformed by the human T-lymphotropic virus-1 and by the v-Src or v-Abl oncogenes, and by signaling through the G protein-coupled receptor for angiotensin II (40–43). The alteration in gene expression plays an important role in the innate immunity of organisms (44, 45) and in the regulation of cell growth and viability (46–49).

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TNF exists primarily as a trimer in solution. Consequently, its binding to the extracellular domain of TNFR1 brings the cytoplasmic domains of the receptor into proximity such that they interact and generate the signal necessary for transmission of the TNF message to downstream structures (54–57). One region in the C terminus of the cytoplasmic domain of TNFR1 plays an obligate role in signaling cytotoxicity. This death domain (58, 59) associates with itself and with cytoplasmic proteins implicated in apoptosis (60–62). Thus, as with other cytokine/hemopoietin receptors, aggregation of TNFR1 is important to TNF action. The
The present study shows that TNF induces the tyrosine phosphorylation and activation of Jak1, Jak2, and Tyk2 in 3T3-L1 adipocytes. The effect of TNF was most evident on Jak2 phosphorylation, with the effect on tyrosine phosphorylation of Jak1 and Tyk2 less pronounced. TNF also promoted the phosphorylation of Jak2 in 3T3-L1 fibroblasts and C3H10T1/2 fibroblasts (data not shown). The ability of mTNF to promote the tyrosine phosphorylation of Jak2 in 3T3-L1 adipocytes was recapitulated by an agonist antisera that specifically activates TNFR1, showing that this TNFR subtype can activate this cellular response. Consistent with this conclusion are experiments demonstrating that hTNF, which specifically binds TNFR1 on murine cells (37), also promotes Jak kinase phosphorylation. Together these observations show that Jak kinase activation is a common cellular response to TNF and that this response is mediated, at least in part, through the type 1 TNFR.

A GST fusion protein encompassing the full-length cytoplasmic domain of TNFR1 precipitated Jak2 from a cell-free translation system. This result shows that TNFR1 binds Jak2 directly and without the intermediacy of another protein. GST-TNFR1 also precipitated Jak2 from adipocyte cell lysates, showing that these proteins can interact within the intracellular milieu. Finally, TNFR1 and Jak2 were coprecipitated from cell lysates, a result that adds the Jak kinases to the list of proteins presently known to bind this TNF receptor.

In a previous study (58), we demonstrated that the C-terminal death domain in TNFR1 self-associates and mediates receptor-receptor interactions. Larger peptides that contained sequences from the N-terminal half of the cytoplasmic domain of TNFR1 interacted less strongly. These observations suggested that the conformation of the longer peptides might mask the aggregation domain, thereby diminishing receptor-receptor interactions. In the present study, we found that a fusion protein containing the full-length cytoplasmic domain of TNFR1 appears to bind Jak2 less efficiently than a protein containing only the N-terminal portion of this receptor region. This result suggests that the primary binding site to which the Jak kinases bind in the cytoplasmic domain of TNFR1 resides in its N-terminal half. Furthermore, these observations suggest that different domains in TNFR1C may positively and negatively regulate the ability of the intracellular domain to interact with cytoplasmic signaling proteins that promote cellular responses to TNF.

Our data indicate that TNF may utilize Jak kinases to mediate some of its cellular responses. Consistent with this possibility is the recent report that CD40, another member of the TNF/hormone growth factor receptor superfamily, associates with and utilizes Jak3 as a component of its signaling mechanism (63). However, further experimentation will be required to demonstrate the roles that individual Jak kinases play in the manifold actions of TNF. This is necessitated by results showing that although Tyk2, Jak1, and Jak2 are all phosphorylated in response to IL-6 (64, 65), experiments with Jak-deficient cells demonstrate that only Jak1 plays a significant role in the IL-6-dependent phosphorylation of the gp130 subunit, STAT activation, and transcriptional induction of the IFN-regulatory factor-1 gene (66).

In addition to promoting Jak kinase activity, TNF also induced the tyrosine phosphorylation of a select group of STAT proteins. In 3T3-L1 adipocytes, TNF strongly induced the tyrosine phosphorylation of STAT3 and STAT5; phosphorylation of STAT1 and STAT6 was demonstrable, but less pronounced. Tyrosine phosphorylation of STAT2 and STAT4 could not be detected. The effects of mTNF on STAT protein phosphorylation were reproduced using agonist antisera directed against TNFR1, demonstrating that it induces this cellular response.

Electromobility shift assays demonstrated that treatment of 3T3-L1 adipocytes with mTNF induced DNA-binding activity attributable to STAT3. This result, together with the ability of TNF to activate Jak kinases, shows that TNF activates the Jak/STAT signal transduction pathway. Surprisingly, we were not able to detect DNA-binding activity attributable to STAT1 in 3T3-L1 adipocytes, despite the ability of TNF to promote the tyrosine phosphorylation of this transcription factor. A recent study by Ivashkiv et al. (67) identified DNA-binding activity in activated T cells that resulted from activation of STAT1 and STAT3. Induction of CAMP signaling in these cells inhibited the DNA binding of STAT1, but not STAT3, showing that the function of these transcription factors can be regulated by cross-talk with different signaling pathways. Previous studies have also shown that serine phosphorylation may also be important for the full activation of DNA-binding activity or transcriptional activity of STAT proteins (68–71). Our results show that detection of STAT phosphorylation is insufficient in itself to demonstrate that these proteins become functional, and suggest the possibility that Jak/STAT signaling induced by TNF may be regulated by other signaling events. However, these complexities aside, our observations definitively demonstrate for the first time that TNF activates Jak/STAT signaling in cells.

References


FIGURE 8. Precipitation of TNFR1/Jak2 complexes from cells. HeLa cells were incubated in the absence or presence of 1 mM hTNF for 15 min at 37°C. TNFR1 was immunoprecipitated from these cells using an antiserum to TNFR1. After SDS-PAGE, a Western blot was probed with an Ab to Jak2. To ensure application of equal amounts of protein, the blot was stripped and reprobed with Ab to TNFR1.


