

IRAK-Dependent Phosphorylation of Stat1 on Serine 727 in Response to Interleukin-1 and Effects on Gene Expression

HANNAH NGUYEN,^{1,*} MOITREYEE CHATTERJEE-KISHORE,^{2,*} ZHENGFAN JIANG,
YULAN QING, CHILAKAMARTI V. RAMANA, JOSHUA BAYES, MAIREAD COMMANE,
XIAOXIA LI, and GEORGE R. STARK

ABSTRACT

Interleukin-1 (IL-1) induces the phosphorylation of Stat1 on serine 727 but not on tyrosine 701. Analyses of mutant I1A cells, which lack the IL-1 receptor-associated kinase (IRAK), and of I1A cells reconstituted with deletion mutants of IRAK show that the IL-1-mediated phosphorylation of Stat1 on serine requires the IRAK protein but not its kinase activity and does not involve phosphatidylinositol-3'-kinase (PI3K) or the mitogen-activated protein (MAP) kinases p38 or ERK. IRAK and Stat1 interact *in vivo*, and this interaction is increased in response to IL-1, suggesting that IRAK may serve to recruit the as yet unknown IL-1-induced Stat1 serine kinase. Chemical inhibitors or dominant-negative forms of signaling components required to activate NF- κ B, ATF, or AP-1 in response to IL-1 do not affect the phosphorylation of Stat1 on serine. IL-1 and tumor necrosis factor (TNF) enhance the serine phosphorylation of Stat1 that occurs in response to interferon- γ (IFN- γ) and potentiate IFN- γ -mediated, Stat1-driven gene expression, thus contributing to the synergistic activities of these proinflammatory cytokines.

INTRODUCTION

THE PROINFLAMMATORY CYTOKINE INTERLEUKIN-1 (IL-1) mediates its effects by activating the transcription factors NF- κ B, AP-1, and ATF, which then drive the expression of IL-1-induced genes (reviewed in refs. 1-3). The signaling events following stimulation with IL-1 have been studied extensively. IL-1 causes the IL-1 receptor (IL-1R) to associate with the IL-1R accessory protein.⁽⁴⁻⁷⁾ The adapter proteins MyD88 and Tollip then bind to the receptor complex⁽⁸⁻¹⁰⁾ and recruit the IL-1R-associated kinase (IRAK). The subsequent phosphorylation of IRAK results in its association with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6)^(5,11-14) and propagates signaling pathways leading to the activation of NF- κ B, AP-1, and ATF. IRAK is ubiquitinated and degraded after activation.⁽¹⁵⁾ Downstream activation of the I κ B kinase (IKK) complex in response to IL-1 promotes the phosphorylation and degradation of the cytoplasmic inhibitor I κ B α . As a consequence, NF- κ B is liberated and translocates to the nucleus, where it binds to κ B sites in the promoters of IL-1-regulated

genes.⁽¹⁶⁻²⁶⁾ Transactivation by NF- κ B also requires its phosphorylation through a pathway that depends on phosphatidylinositol-3'-kinase (PI3K) and Akt.⁽²⁷⁾ AP-1 and ATF are phosphorylated and activated by the mitogen-activated protein kinases (MAPK) JNK,⁽²⁸⁻³⁰⁾ p38,⁽³¹⁾ and p42/p44ERK.⁽³²⁾ TNF- α , another proinflammatory cytokine, also activates NF- κ B, AP-1, and ATF as well as the expression of many genes in common with IL-1 through different but similar signaling pathways (reviewed in ref. 33). Several mutant cell lines lacking the ability to respond to IL-1 have been isolated,⁽¹⁴⁾ and the cell line I1A, which lacks IRAK, has been used to show that IRAK is essential for the activation of both NF- κ B and JNK. Moreover, I1A cells expressing various deletion mutants of IRAK have been useful in defining specific functional domains of this protein.⁽³⁴⁾

Signal transducer and activator of transcription 1 (Stat1) is the major transcription factor activated in response to interferon- γ (IFN- γ), leading to the expression of many genes and subsequent biologic effects, including regulation of cell growth, antiviral activity, and inflammation. Phosphorylation occurs

Department of Molecular Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195.

¹Present address: MethylGene, Inc., Montreal, Quebec, H4S 2A1, Canada.

²Present address: Expression Profiling Sciences/Genomics, Genetic Institute Wyeth, Cambridge, MA 02140.

*These authors contributed equally to this work.

both on tyrosine 701, which is essential for homodimerization and subsequent DNA binding of Stat1 to IFN- γ -activated sequences (GAS sites) in inducible promoters (reviewed in refs. 35, 36), and on serine 727, which lies within a proline-directed kinase or MAPK consensus motif in the transactivation domain.^(37,38) Serine phosphorylation contributes substantially to the transcriptional activity of Stat1.^(39–43) Interestingly, the phosphorylation of Stat1 and Stat3 on serine, but not tyrosine, can be stimulated by bacterial lipopolysaccharide (LPS), ultraviolet (UV) radiation, or TNF.^(40,42,44) The physiologic importance of phosphorylating Stats uniquely on serine without activating their DNA binding activity is not completely understood. However, unphosphorylated Stat1 regulates the constitutive expression of a number of genes, including caspases⁽⁴⁵⁾ and latent membrane protein-2 (LMP-2),⁽⁴⁶⁾ suggesting that increasing the activity of Stat1 through serine phosphorylation in the transactivation domain may well increase the expression of such genes. It is difficult to assess the role of serine-phosphorylated Stat1 in driving the expression of such genes as LMP-2 in response to these stress-dependent stimuli in isolation because many of these promoters include κ B sites, enabling the genes to respond strongly to active NF- κ B. We now find that IL-1 induces the phosphorylation of Stat1 on serine and have investigated the mechanism of this process as well as the role of this phosphorylated form of Stat1 in enhancing gene expression when IL-1 and IFN- γ are present together.

MATERIALS AND METHODS

Biologic reagents and cell culture

The following reagents were used: recombinant human IFN- γ (rHuIFN- γ) (Roche Applied Science, Indianapolis, IN), 1000 IU/ml; rHuIL-1 β (National Cancer Institute), 10 ng/ml; recombinant mouse IL-1 β (rMuIL-1 β) (Peprotech, Rocky Hill, NJ), 10 ng/ml; rHuTNF- α (Becton Dickinson, Franklin Lakes, NJ), 20 ng/ml; LY294,002 (Sigma, St. Louis, MO), 50 μ M; PD98059 (Cell Signaling, Beverly, MA), 50 μ M. Cells were incubated with LY for 30 min or with PD for 1 h at 37°C before cytokine treatment. T98G human glioblastoma cells, human fibrosarcoma 2fTGH cells, and the derivative U3A line⁽⁴⁷⁾ were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μ g/ml penicillin G, and 100 μ g/ml streptomycin. The cloned human embryonic kidney fibroblast 293 cell line C6 and the derivative IIA line,⁽¹⁴⁾ T98G-derived cell lines expressing dominant-negative or constitutively active forms of the IL-1 signaling components NF- κ B-inducing kinase (NIK), IKK α , IKK β , I κ B α , MAPK kinase (MKK)4, JNK, or p38, and the U3A-derived S727A cell line⁽³⁹⁾ were maintained in the same complete medium, plus either 400 μ g/ml active G418 or 1 μ g/ml puromycin. The K239A, dDD, dUD, dKD, dC1, dC2, and dC1 + dC2 cell lines⁽³⁴⁾ were maintained in the presence of both G418 and puromycin.

Western analyses

Following treatment, cells at 80% confluence in 100-mm dishes were washed once with phosphate-buffered saline (PBS), and the cell pellets were lysed for 30 min at 4°C in 100 μ l of 20 mM Tris-HCl, pH 8.0, 200 mM NaCl, 10% glycerol, 1%

Triton X-100, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.1% sodium dodecyl sulfate, 3 μ g/ml aprotinin, 2 μ g/ml pepstatin, and 1 μ g/ml leupeptin. Cellular debris was pelleted by centrifugation at 16,000g at 4°C for 10 min. Cell extracts were fractionated by electrophoresis in 8%–10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to nitrocellulose membranes. The following antibodies were used for Western analyses: antiphospho-S727 Stat1, antiphospho-Y701 Stat1 (both from Upstate Biotechnology, Lake Placid, NY), anti-C-terminal Stat1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-N-terminal Stat1 (BD Biosciences Pharmingen, San Diego, CA), anti-IRAK (Santa Cruz Biotechnology), antiphospho-(S473)Akt, antiphospho-JNK, antiphospho-p42/p44, and antiphospho-p38 (all from Cell Signaling). Horseradish peroxidase (HRP)-coupled goat antirabbit or goat antimouse immunoglobulin (Rockland, Gilbertsville, PA) was used for visualization, using the enhanced chemiluminescence (ECL) Western detection system (Perkin-Elmer Life Sciences, Boston, MA).

Immunoprecipitations

After treatment, T98G cells at 80% confluence in 150-mm dishes were washed once with PBS, and the cell pellets were lysed in for 30 min at 4°C in 200 μ l of 50 mM HEPES, pH 7.5, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM Na₄PO₇, 100 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethane sulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin. The extracts were incubated with 30 μ l of a mixture of protein A sepharose and Gamma-Bind G beads (Amersham Biosciences, Piscataway, NJ) and 2 μ g either anti-N-terminal Stat1 (BD Biosciences Pharmingen) or anti-IRAK (Santa Cruz Biotechnology), with rotation for 4 h at 4°C. The immunoprecipitates were washed three times with lysis buffer and subject to Western analysis as described.

Northern analyses

Cells were stimulated for 4–6 h. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA (20 μ g) was denatured, separated by electrophoresis in a formaldehyde-1.2% agarose gel, and transferred to Hybond-N nylon membrane (Amersham). Tryptophanyl-³H-RNA synthetase/IFP-53 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNAs were detected using specific cDNA fragments, which were labeled with α -³²P-dCTP (Amersham) by nick-translation, using the DNA megaprime labeling system (Amersham), and visualized by autoradiography. Expression levels were quantified using a PhosphorImager (Amersham).

Transfection and reporter assays

For stable transfections, cells at 60%–70% confluence seeded the previous day in 100-mm plates were transfected by the calcium phosphate method⁽⁴⁸⁾ with 2 μ g of either pSV₂-neo or pBabe-puro and 10 μ g of one of the following: pXIN-I κ Bmut superrepressor (a kind gift of Dean Ballard, Vanderbilt University School of Medicine), IKK α (K44A) dominant-negative mutant, IKK β (K44A) dominant-negative mutant, NIK (K429A, K430A) dominant-negative mutant (kind gifts of Zhaodan Cao, Tularik, CA), Flag-JNK1-APF (T183A, Y185F)

dominant-negative mutant, p38 (T180A, Y182F) dominant-negative mutant (kind gifts of Roger Davis, University of Massachusetts), HA-JNKK2-JNK1 (a fusion product of MKK7 and JNK) constitutively active JNK mutant (a kind gift of Dennis J. Templeton, University of Virginia Medical School), and SEK1(MKK4)-AL (S220A,T224L) dominant-negative mutant (a kind gift of James Woodgett, University of Toronto, ON, Canada). After 48 h, the cells were selected with either 400 $\mu\text{g/ml}$ active G418 (for pSV₂-neo transfectants) or 1 $\mu\text{g/ml}$ puromycin (for pBabe-puro transfectants) until clones appeared. For luciferase reporter assays, cells at 60%–70% confluence seeded the previous day in 150-mm plates were transfected by the calcium phosphate method with 10 μg of 4XGBPGAS-luc (Stratagene, La Jolla, CA) and 2 μg pSV2- β -gal (to verify transfection efficiency). Eight hours after transfection, the cells were divided into four or six 100-mm plates, one for each different treatment. The cells were stimulated with IFN- γ , IL-1, or TNF, individually or in combination, for 6 h beginning 48 h after transfection. Luciferase and β -galactosidase activities were determined using the Promega luciferase assay or chemiluminescence reagents, respectively (Promega, Madison, WI). Results are shown for one of at least three independent experiments, all of which were comparable.

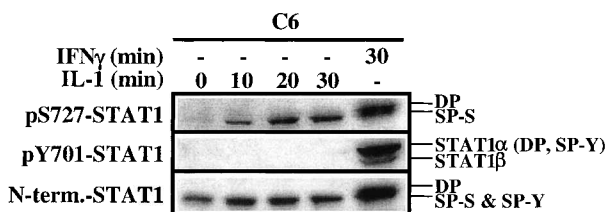
RESULTS

IL-1-mediated Stat1 serine phosphorylation requires IRAK, but not its kinase domain

Treatment of C6 or T98G cells with IL-1 increased the phosphorylation of Stat1 on serine 727 (Fig. 1). The basal levels of serine-phosphorylated Stat1 in these cells are most likely caused by the constitutive activation of kinases, for example, PI3K.⁽⁴³⁾ In contrast to IFN- γ , treatment with IL-1 did not lead to the phosphorylation of Stat1 on tyrosine 701 (Fig. 1).

A panel of mutant C6 cell lines, defective in various components of the IL-1 signaling pathway,⁽¹⁴⁾ was analyzed for the phosphorylation of Stat1 following IL-1 treatment (Fig. 2A). In contrast to parental C6 cells, in I1A cells, which lack IRAK, as well as in I2A and I3A cells, which lack as yet unidentified components upstream of IRAK, Stat1 was not phosphorylated on serine in response to IL-1. However, in I4A cells, which are defective downstream of the degradation of I κ B α , Stat1 was phosphorylated on IL-1 treatment. To investigate further the role of IRAK in the serine phosphorylation of Stat1, I1A cells reconstituted with derivatives of IRAK lacking various domains⁽³⁴⁾ were used (Fig. 2B). IRAK derivatives lacking either the death domain (dDD) or the C-terminal domain (dC1 + dC2), shown previously to be unable to restore the IL-1-mediated activation of both NF- κ B and JNK,⁽³⁴⁾ also did not restore the serine phosphorylation of Stat1 in response to IL-1 (Fig. 2C). In contrast, IRAK derivatives found previously to support the activation of both JNK and NF- κ B, lacking only one of the two C-terminal subdomains (dC1, dC2), were also able to phosphorylate Stat1 on serine in response to IL-1 (Fig. 2D), indicating that the serine phosphorylation of Stat1 requires the same domains of IRAK that are required to activate NF- κ B or JNK in response to IL-1. Expression of a point-mutant, kinase-dead derivative of IRAK (K239A) or a mutant protein lacking the kinase domain (dKD) also restored IL-1-mediated Stat1 serine

A



B

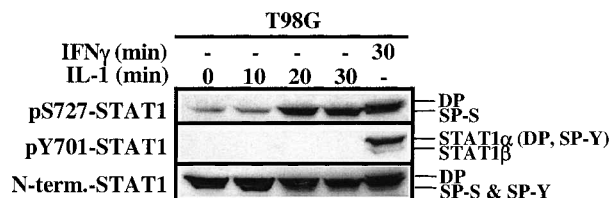


FIG. 1. Phosphorylation of Stat1 on serine but not tyrosine in response to IL-1. (A) C6 or (B) T98G cells were stimulated with IL-1 or IFN- γ for the times indicated. Cell lysates were separated by 10% SDS-PAGE, and Western analysis was carried out using antiphospho-S727-Stat1, antiphospho-Y701-Stat1, or anti-N-terminal-Stat1, sequentially. DP, Stat1 doubly phosphorylated on serine and tyrosine; SP-S, Stat1 singly phosphorylated on serine; SP-Y, Stat1 singly phosphorylated on tyrosine; Stat1 α , full-length Stat1; Stat1 β , Stat1 lacking 38 C-terminal residues.

phosphorylation, indicating that the kinase activity of IRAK is not required for the phosphorylation of Stat1 on serine and, therefore, that IRAK is not the IL-1-activated serine kinase (Fig. 2D). These latter two mutant proteins also support the IL-1-mediated activation of both NF- κ B and JNK.⁽³⁴⁾

IRAK-dependent Stat1 serine phosphorylation does not involve activation of Akt, p38, or ERK

The S727 residue of Stat1 lies in a potential MAPK site. To examine if its phosphorylation in response to IL-1 requires the activation of a MAPK pathway, the patterns of Akt, JNK, ERK, and p38 phosphorylation were analyzed in I1A cells, in which the serine is not phosphorylated in response to IL-1, and in K239A cells, which have no defect (Fig. 3A). Akt was activated constitutively in parental C6 cells, and its activation was not affected by the lack of IRAK. Similar to the pattern of Stat1 serine phosphorylation, JNK and p38 were not phosphorylated in IRAK-deficient cells but were phosphorylated in K239A cells in response to IL-1. I1A cells exhibited low constitutive levels of phosphorylated ERK, which did not increase with IL-1 treatment, and inducible activation was modestly restored in K239A cells. These results demonstrate a correlation between the activation of JNK and p38 in the serine phosphorylation of Stat1 in response to IL-1 and exclude the involvement of Akt in this process. The patterns of MAPK activation and Stat1 serine phosphorylation were also an-

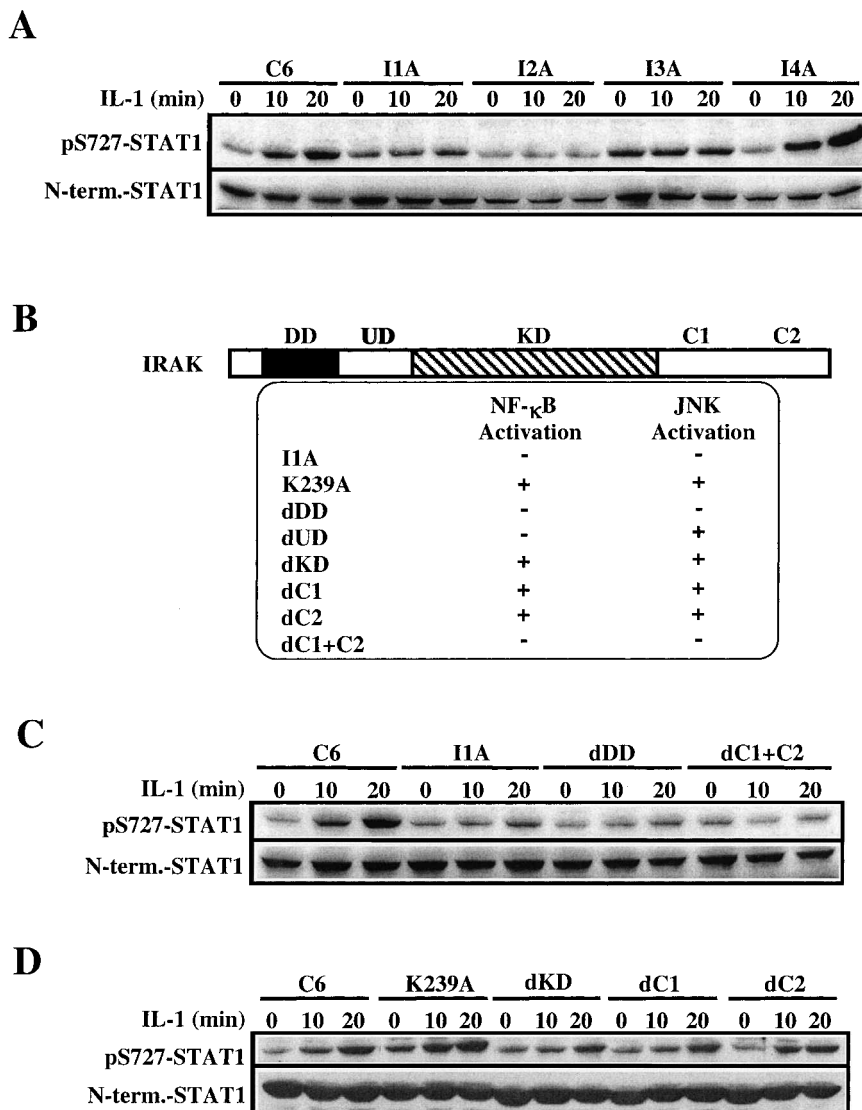


FIG. 2. Requirement for IRAK in the phosphorylation of Stat1 on serine in response to IL-1. **(A)** Analysis of Stat1 serine phosphorylation in various mutant cell lines defective in the IL-1 signaling pathway. Parental C6 and the mutant cell lines I1A, I2A, I3A, and I4A were treated with IL-1 for 10 or 20 min. Cell lysates were separated by 10% SDS-PAGE, and Western analysis was carried out with antiphospho-S727-Stat1 or anti-N-terminal-Stat1, sequentially. **(B)** Functional domains of IRAK. The IRAK protein consists of a death domain (DD), a domain of unknown function (UD), a kinase domain (KD), and C-terminal domains 1 and 2 (C1, C2). IRAK-deficient I1A cells stably expressing constructs encoding a kinase-dead point mutant of IRAK (K239A) or deletion mutants lacking one or more functional domains either restore (+) or fail to restore (-) the activation of NF- κ B or JNK or both in response to IL-1. dDD, deletion of death domain; dUD, deletion of undetermined domain; dKD, deletion of kinase domain; dC1, deletion of C-terminal domain 1; dC2, deletion of C-terminal domain 2. **(C)** Domains of IRAK required to activate NF- κ B and JNK are also required to phosphorylate Stat1 on S727 in response to IL-1. C6 and I1A cells and I1A cells stably reconstituted with the IRAK deletion mutants dDD or dC1 + dC2 were treated with IL-1 for 10 or 20 min. Cell lysates were processed as in **A**. **(D)** Domains of IRAK that are not required to activate NF- κ B and JNK are also dispensable for Stat1 S727 phosphorylation in response to IL-1. C6 cells and I1A cells stably expressing K239A, dKD, dC1, or dC2 were treated with IL-1 for 10 or 20 min. Cell lysates were processed as in **A**.

alyzed in dUD cells, in which JNK but not NF- κ B is activated⁽³⁴⁾ (Fig. 3B). In these cells, p38 was phosphorylated, JNK was partially phosphorylated, and the basal levels of phosphorylated ERK1 actually decreased with IL-1 treatment, yet there was no serine phosphorylation of Stat1 on IL-1 stimulation (Fig. 3B). These results indicate that p38 and ERK are not involved in IL-1-mediated Stat1 phosphorylation.

Several inhibitors of NF- κ B or ATF/AP1 activation do not affect IL-1-mediated Stat1 serine phosphorylation

The effects of the phosphorylation of Stat1 on serine of chemical inhibitors or of stable expression of dominant-negative or constitutively active forms of signaling components that respond to IL-1 were analyzed in T98G cells (Table 1). Inhi-

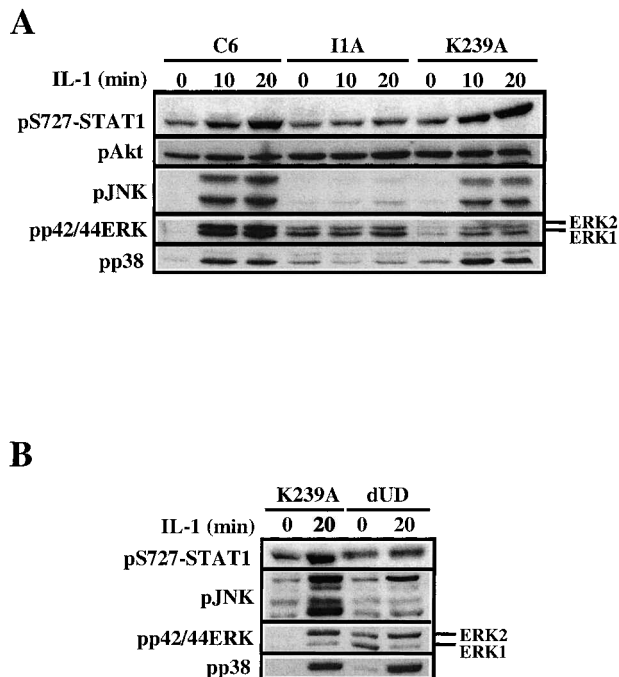


FIG. 3. Analysis of the role of MAPKs in the serine phosphorylation of Stat1 in response to IL-1. **(A)** Lack of inducible phosphorylation of JNK, ERK, or p38 but not of Akt in response to IL-1 in I1A cells. C6, I1A, or K239A cells were treated for 10 or 20 min with IL-1. Cell extracts were separated by 10% SDS-PAGE, and Western analyses were carried out with antiphospho-p38, antiphospho-p42/p44ERK, antiphospho-JNK, antiphospho-S473-Akt, and antiphospho-S727-Stat1, sequentially. **(B)** Restoration of p38, ERK, but not Stat1 phosphorylation in dUD cells in response to IL-1. K239A cells or I1A cells expressing the dUD deletion mutant of IRAK were stimulated with IL-1 for 20 min. Cell extracts were separated by 10% SDS-PAGE, and Western analyses were carried out with antiphospho-p38, antiphospho-p42/p44ERK, antiphospho-JNK, or antiphospho-S727-Stat1, sequentially.

tion of the activity of components important for the liberation of NF- κ B (NIK, IKK α , IKK β , I κ B α) or for its activation as a transcription factor (PI3K, Akt) did not affect the phosphorylation of Stat1 on serine in response to IL-1. The individual IKK subunits were also dispensable for IL-1-mediated Stat1 S727 phosphorylation (H. Nguyen, N. Sizemore, and G.R. Stark, unpublished observations) in mouse embryo fibroblasts (MEFs).^(23,49) Constitutive activators or dominant-negative inhibitors affecting the kinase activity of JNK (MKK4, JNK), p38, or ERK (mitogen-activated protein kinase kinase 1 [MEK1], chemically inhibited by PD98059) also did not affect the phosphorylation of Stat1 on serine in response to IL-1 (Table 1). These findings rule out a role for MAPKs in this process and suggest the involvement of an unknown IRAK-dependent kinase.

Stat1 and IRAK interact in vivo

To determine if IRAK is an intermediate that links Stat1 to an IL-1-activated serine kinase, immunoprecipitations followed by Western analyses were performed with untreated or IL-1-treated T98G cells (Fig. 4). Interestingly, IRAK was detected at a low level in Stat1 immunoprecipitates prior to stimulation, but the amount increased dramatically after treatment with IL-1 for 10 or 20 min. Stat1 was also detected at low levels in IRAK immunoprecipitates in the absence of treatment, and the amounts increased 10 or 20 min after IL-1 stimulation. Interestingly, serine-phosphorylated Stat1 was detected in IRAK immunoprecipitates only 20 min after IL-1 treatment, consistent with the pattern of Stat1 serine phosphorylation in T98G cells (Figs. 1 and 4B). These results show that Stat1 associates with IRAK in the absence of stimulation, that the association increases in response to IL-1, and that phosphorylation on serine follows association.

IL-1 or TNF increases transcriptional activity of Stat1 dimers through a process dependent on serine 727

IFN- γ works in concert with IL-1 or TNF to enhance inflammation through increased gene expression (reviewed in ref.

TABLE 1. EFFECTS OF DOMINANT-NEGATIVE OR CHEMICAL INHIBITION OF IL-1-DEPENDENT SIGNALING IN T98G CELLS^a

Mutant or inhibitor	Stat1 S727 phosphorylation	Positive control ^b
DN NIK	+	~50% inhibition of NF- κ B-luc activity
DN IKK α	+	~50% inhibition of NF- κ B-luc activity
DN IKK β	+	~70% inhibition of NF- κ B-luc activity
I κ B superrepressor	+	100% abrogation of NF- κ B DNA binding
50 μ M LY294,002 (PI3K inhibitor)	+	100% abrogation of Akt S473 phosphorylation
DN MKK4	+	~80% abrogation in JNK phosphorylation
CA JNK	+	~400% increase in JNK phosphorylation
DN JNK	+	~90% abrogation of JNK phosphorylation
DN p38	+	~80% abrogation of p38 phosphorylation
50 μ M PD98059 (MEK 1 inhibitor)	+	100% abrogation of ERK1/2 phosphorylation

^aCells were transfected with dominant-negative (DN) mutants or constitutively active (CA) derivatives of signaling proteins, and stable clones were analyzed for the serine phosphorylation of Stat1 by the Western method using antiphospho-S727-Stat1.

^bNF- κ B activation was assessed by EMSA to determine its binding to the κ B sequence from the promoter of the human IP-10 gene, or by reporter assays to determine NF- κ B-dependent transactivation, using a luciferase construct driven by the E-selectin promoter. The phosphorylation of Akt, JNK, ERK, and p38 was analyzed by the Western method, using phosphospecific antibodies.

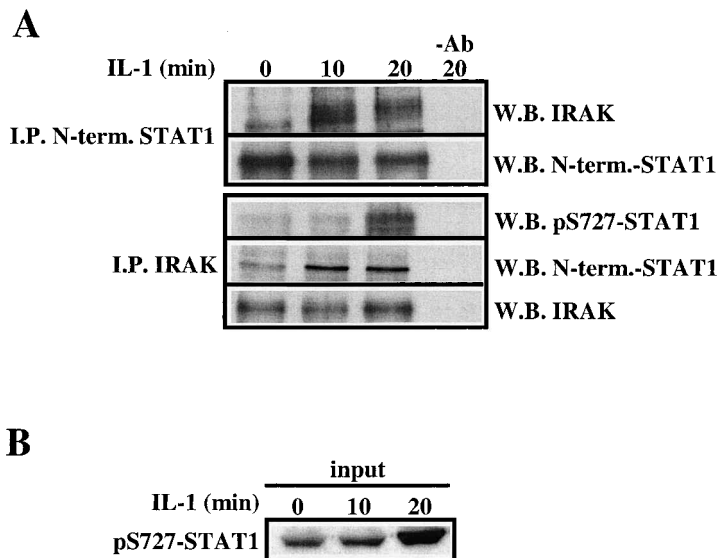


FIG. 4. *In vivo* interaction between Stat1 and IRAK. **(A)** T98G cells were treated with IL-1 for 10 or 20 min. Cellular extracts were enriched for either Stat1 or IRAK by immunoprecipitation with anti-N-terminal-Stat1 or anti-IRAK. -Ab, control immunoprecipitations performed in the absence of antibody. Immunoprecipitates were separated by 10% SDS-PAGE, and Western analyses were performed using anti-IRAK for Stat1 immunoprecipitates and sequentially by using antiphospho-S727-Stat1 or anti-N-terminal-Stat1, respectively, for IRAK immunoprecipitates. **(B)** Cellular extracts used in the immunoprecipitations (input) were also separated by 10% SDS-PAGE, and Western analyses were performed using antiphospho-S727-Stat1.

50). We examined the effects of IL-1 and TNF on Stat1 phosphorylation in combination with IFN- γ . As expected, IFN- γ treatment led to the phosphorylation of Stat1 on both serine and tyrosine, whereas IL-1 or TNF treatment caused the phosphorylation of Stat1 only on serine (Fig. 5A). Interestingly, a combination of IL-1 or TNF with IFN- γ increased the levels of Stat1 phosphorylated on serine only and of Stat1 phosphorylated on both serine and tyrosine without affecting the overall level of tyrosine phosphorylation. Similar observations were obtained in 2fTGH cells (Fig. 5B). In this case, only the effects of TNF stimulation were analyzed, as these cells do not respond to IL-1. These results reveal that IL-1 or TNF enhances the level of serine-phosphorylated Stat1 induced by IFN- γ . To determine if this enhancement affects transcriptional activation by Stat1 dimers, we examined the effects of these cytokines on the activation of a luciferase reporter construct driven by four copies of a Stat1 consensus GAS site, derived from the human guanylate-binding protein-1 (HuGBP-1) promoter (4XGBP-GAS-luc), transiently transfected into T98G cells, 2fTGH and derived Stat1-deficient U3A cells, and U3A cells expressing the S727A mutant of Stat1 (Fig. 5C). Treatment of T98G cells with IFN- γ for 6 h induced luciferase activity by \sim 15-fold, whereas treatment with IL-1 or TNF alone for 6 h did not affect reporter activation. Interestingly, treatment with IFN- γ in combination with IL-1 or TNF increased luciferase levels 30–40-fold. Similar results were observed in 2fTGH cells. As transcription of the reporter was not observed in Stat1-null U3A cells, we conclude that Stat1 dimer is the principal transcription factor involved in activating 4XGBP-GAS-luc. In U3A-S727A cells, the increase in reporter activity was \sim 4-fold in response to IFN- γ alone, and TNF did not increase this degree of activation. Similar results were obtained with pools of 2fTGH, U3A, and U3A-

S727A cells stably transfected with 4XGBP-GAS-luc (data not shown). These results reveal that IL-1 or TNF can potentiate transactivation due to the binding of Stat1 dimers to a GAS site through a process dependent on serine 727.

IL-1 or TNF increases expression of the IFN- γ -inducible IFP-53 gene through a Stat1-dependent, NF- κ B-independent process

IFN- γ plus IL-1 or TNF often increase gene expression cooperatively as a result of interactions between IFN- γ -activated Stat1 and IL-1-activated or TNF-activated NF- κ B (reviewed in ref. 50). To determine if the enhancement of Stat1 serine phosphorylation also contributes to the potentiation of endogenous gene expression, we analyzed (Fig. 6A) the expression of the IFN- γ -inducible IFP-53 gene, whose promoter is known to contain a GAS site and not an NF- κ B site,⁽⁵¹⁾ in T98G cells stably expressing a superrepressor form of the NF- κ B inhibitor I κ B α (T98G-I κ B α SR). In these cells, the DNA-binding activity of NF- κ B to its consensus site in the IP-10 promoter is completely blocked, and the IL-1-induced or TNF-induced expression of the NF- κ B-driven IL-8 gene is reduced by \sim 90%–95% in response to IL-1 or TNF (data not shown). Although IFN- γ induced the expression of IFP-53 as expected, IL-1 or TNF alone had no effect on IFP-53 mRNA levels in either the T98G-neo controls or T98G-I κ B α SR cells. Interestingly, adding IL-1 or TNF moderately but consistently enhanced the IFN- γ -mediated expression of IFP-53 mRNA not only in T98G-neo cells but also in T98G-I κ B α SR cells, indicating that the effect of IL-1 or TNF on IFN- γ -induced IFP-53 expression is independent of NF- κ B. In contrast, the potentiation of expression of the RANTES gene by IFN- γ and IL-1 or TNF, known to be me-

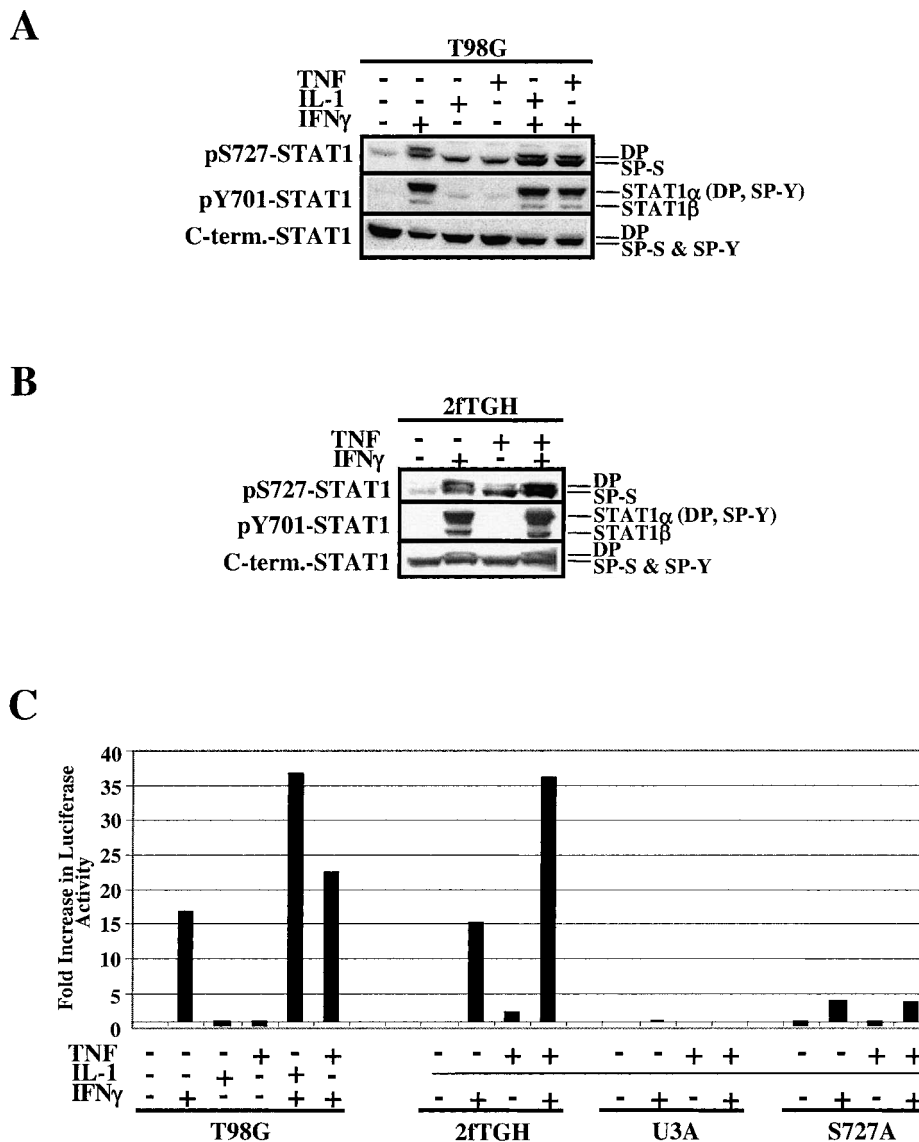


FIG. 5. Serine 727-dependent increases in IFN- γ -induced, Stat1-driven transactivation in response to IL-1 or TNF. (**A** and **B**) Increased levels of IFN- γ -induced, serine-phosphorylated Stat1 in response to IL-1 or TNF. T98G (**A**) or 2fTGH cells (**B**) were treated for 20 min with either IFN- γ , IL-1, or TNF, individually or in combination. 2fTGH cells were serum-starved for 24 h before treatment to reduce the constitutive level of Stat1 phosphorylation on S727. Cell extracts were separated by 8% SDS-PAGE, and Western analyses were carried out using antiphospho-S727-Stat1, antiphospho-Y701-Stat1 or anti-C-terminal-Stat1, sequentially. (**C**) Potentiation of IFN- γ -mediated, Stat1-driven transcription by IL-1 or TNF. T98G, 2fTGH, and derived Stat1-null U3A cells, and U3A cells reconstituted with Stat1 S727A were transfected transiently with a GBP-4XGAS-luciferase construct. Twenty-four hours after transfection, the cells were divided into four or six separate plates, one for each treatment. Forty hours after transfection, the cells were treated as indicated for 6 h. Cell extracts were assayed for luciferase activity.

diated through the cooperation of IFN- γ -activated Stat1 and IL-1-activated or TNF-activated NF- κ B,⁽⁵²⁾ was obliterated in the T98G-I κ B α SR cells compared with control T98G-neo cells (data not shown). IFN- γ -induced IFP-53 gene expression was also enhanced by TNF in 2fTGH cells (Fig. 6B). In contrast, Stat1-null U3A cells responded minimally to IFN- γ , TNF, or both, confirming that IFP-53 expression is primarily Stat1 driven (Fig. 6B). Interestingly, IFN- γ -induced IFP-53 gene expression did not increase on treating U3A-S727A cells with TNF compared with the parental 2fTGH cells (Fig. 6B), re-

vealing that the enhanced serine phosphorylation of Stat1 is important in potentiating IFN- γ -mediated IFP-53 gene expression in response to IL-1 or TNF.

DISCUSSION

Stat1 is a novel target of IL-1 signaling

We have found that IL-1 can activate the phosphorylation of Stat1 on serine in several cell lines. Therefore, in addition to

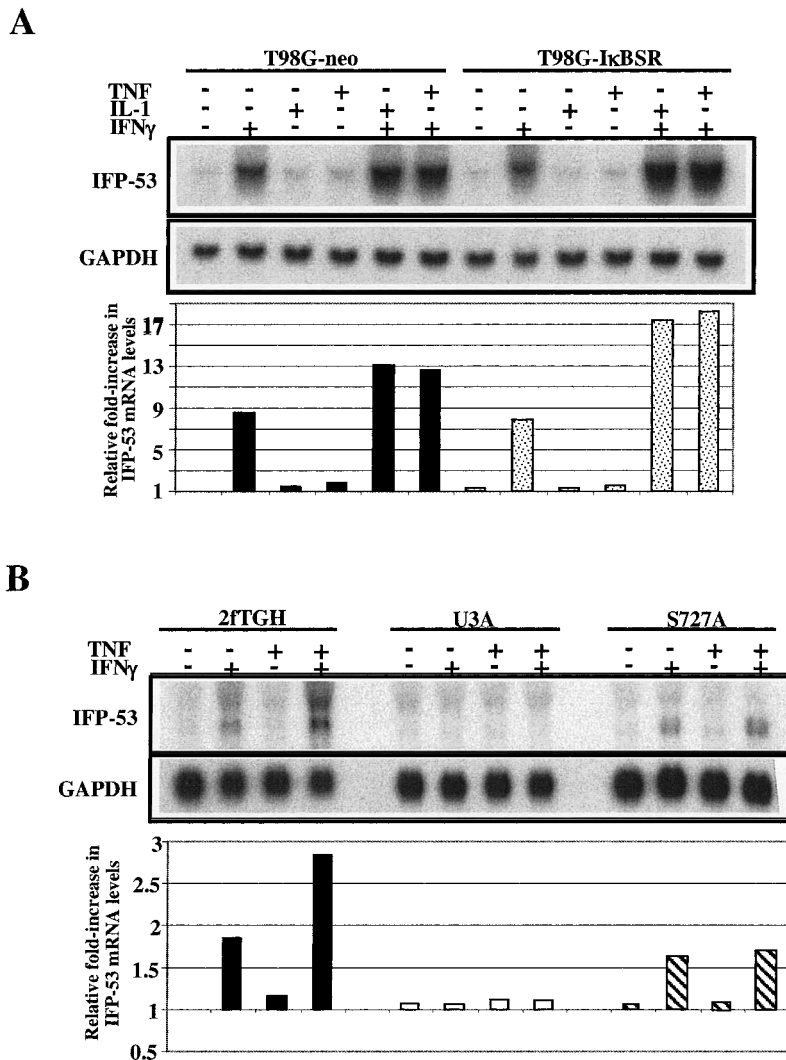


FIG. 6. Enhancement of IFN- γ -induced, Stat1-driven and NF- κ B-independent gene expression by IL-1 or TNF. **(A)** The enhancement of IFN- γ -induced IFP-53 expression by IL-1 or TNF does not involve NF- κ B. T98G cells stably transfected with a control vector containing the neomycin resistance gene (T98G-neo) or a dominant-negative superrepressor mutant of the I κ B α inhibitor of NF- κ B (T98G-I κ BSR) were stimulated as indicated for 5 h. Total RNA was analyzed by the Northern procedure, using a cDNA specific for IFN- γ -inducible IFP-53 as a probe. The transfers were stripped and reprobed with GAPDH cDNA to control for loading. Expression levels were quantified using a PhosphorImager. **(B)** The enhancement of IFN- γ -induced IFP-53 expression by IL-1 or TNF is dependent on Stat1 S727 phosphorylation. 2ftGH, U3A, and U3A-S727A cells were stimulated as indicated for 4 h. Total RNA was analyzed as in **A**.

NF- κ B and API1/ATF, Stat1 can transmit some of the biologic effects of IL-1. The results with I1A-derived 293 cells and with T98G cells expressing dominant-negative proteins suggest that IL-1-mediated Stat1 serine phosphorylation requires IRAK but not the MAPKs. TNF-mediated Stat1 serine phosphorylation depends on the activation of p38 in macrophages.⁽⁴²⁾ As IL-1 and TNF stimulate many of the same activities, one might have predicted that they would use the same mechanism for the phosphorylation of Stat1 on serine. However, IRAK, which is required for the phosphorylation of Stat1 on serine in response to IL-1, does not participate in the TNF-dependent signaling pathway, probably explaining the difference in the mechanism of

Stat1 serine phosphorylation between the two cytokines. The dispensability of JNK, p38, and ERK for the phosphorylation of Stat1 on serine in response to IL-1 is supported by a study that reveals that Stat1 is a poor substrate for all three MAPKs, tested both *in vitro* and *in vivo*.⁽⁵³⁾ Our observation that Stat1 and IRAK interact *in vivo* suggests that IRAK, while not serving as the proximal serine kinase for Stat1, may recruit the kinase to Stat1. This hypothesis is supported by the established requirement for IRAK to interact with TRAF6 in IL-1-dependent signaling pathways,^(5,11-14) as well as by its novel role as a chaperone that mediates the translocation of TAB2 and TRAF6 from the membrane to the cytosol.⁽⁵⁴⁾

Biologic role of IL-1-mediated Stat1 serine phosphorylation in enhancing IFN- γ -induced gene expression

It has been long known that IFN- γ and IL-1 or TNF, secreted after infection, together elicit inflammatory responses that are more robust than those achieved by any of these cytokines alone. We have found that combining IFN- γ with the proinflammatory cytokines increases the levels of serine-phosphorylated Stat1 as well as its transactivation function. Bacterial LPS also works in concert with IFN- γ in inflammation and has been shown previously to enhance the serine phosphorylation and transactivation potential of Stat1 in response to IFN- γ .⁽⁴⁰⁾ Furthermore, combining IFN- γ and TNF increases the DNA-binding activity and transactivation potential of NF- κ B by delaying the degradation of the I κ B β inhibitor of NF- κ B.⁽⁵⁵⁾ Although enhancement of transcriptional activity in response to combined mediators of inflammation has been demonstrated previously, IFP-53 represents the first example of a gene whose IFN- γ -mediated expression is amplified by IL-1 or TNF in a manner independent of NF- κ B and reveals an additional molecular mechanism through which inflammation is facilitated. The increases in IFP-53 gene expression observed with cytokine combinations were modest but consistent. It would be of interest to identify other IFN- γ -inducible genes whose expression is perhaps more dramatically enhanced through increased Stat1 serine phosphorylation or other cell types in which this process is more important. Probably the cooperation of distinct transcription factors and the separate enhancement of the activity of each individual transcription factor both contribute to synergistic gene expression in response to cytokine combinations.

ACKNOWLEDGMENTS

We thank Greg Manson and Huiqin Nie for technical assistance and the following investigators for expression plasmids: Dean Ballard, Zhaodan Cao, Roger Davis, Dennis J. Templeton, and James Woodgett. This work was supported by grant P01 CA 62220 from the National Institutes of Health to G.R.S. and a Cancer Research Institute Fellowship to H.N.

REFERENCES

- BARNES, P.J., and KARIN, M. (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. *N. Engl. J. Med.* **336**, 1066–1071.
- O'NEILL, L.A. (1997). Molecular mechanisms underlying the actions of the proinflammatory cytokine interleukin 1. *Biochem. Soc. Trans.* **25**, 295–302.
- O'NEILL, L.A. (1995). Towards an understanding of the signal transduction pathways for interleukin 1. *Biochim. Biophys. Acta* **1266**, 31–44.
- GREENFEDER, S.A., NUNES, P., KWEE, L., LABOW, M., CHIZZONITE, R.A., and JU, G. (1995). Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *J. Biol. Chem.* **270**, 13757–13765.
- WESCHE, H., HENZEL, W.J., SHILLINGLAW, W., LI, S., and CAO, Z. (1997). MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity* **7**, 837–847.
- HUANG, J., GAO, X., LI, S., and CAO, Z. (1997). Recruitment of IRAK to the interleukin-1 receptor complex requires interleukin-1 receptor accessory protein. *Proc. Natl. Acad. Sci. USA* **94**, 12829–12832.
- KORHERR, C., HOFMEISTER, R., WESCHE, H., and FALK, W. (1997). A critical role for interleukin-1 receptor accessory protein in interleukin-1 signaling. *Eur. J. Immunol.* **27**, 262–267.
- LORD, K.A., HOFFMAN-LIEBERMANN, B., and LIEBERMANN, D.A. (1990). Nucleotide sequence and expression of a cDNA encoding MyD88, a novel myeloid differentiation primary response gene induced by IL-6. *Oncogene* **5**, 1095–1097.
- ADACHI, O., KAWAI, T., TAKEDA, K., MATSUMOTO, M., TSUTSUI, H., SAKAGAMI, M., NAKANISHI, K., and AKIRA, S. (1998). Targeted disruption of the MyD88 gene results in loss of IL-1 and IL-18-mediated function. *Immunity* **9**, 143–150.
- BURNS, K., CLATWORTHY, J., MARTIN, L., MARTINON, F., PLUMPTON, C., MASCHERA, B., LEWIS, A., RAY, K., TSCHOPP, J., and VOLPE, F. (2000). Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. *Nat. Cell Biol.* **2**, 346–351.
- CAO, Z., HENZEL, W.J., and GAO, X. (1996). IRAK: a kinase associated with the interleukin-1 receptor. *Science* **271**, 1128–1131.
- CAO, Z., XIONG, J., TAKEUCHI, M., KURAMA, T., and GOEDDEL, D.V. (1996). TRAF6 is a signal transducer for interleukin-1. *Nature* **383**, 443–446.
- MUZIO, M., NI, J., FENG, P., and DIXIT, V.M. (1997). IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278**, 1612–1615.
- LI, X., COMMANE, M., BURNS, C., VITHALANI, K., CAO, Z., and STARK, G.R. (1999). Mutant cells that do not respond to interleukin-1 (IL-1) reveal a novel role for IL-1 receptor-associated kinase. *Mol. Cell Biol.* **19**, 4643–4652.
- YAMIN, T.T., and MILLER, D.K. (1997). The interleukin-1 receptor-associated kinase is degraded by proteasomes following its phosphorylation. *J. Biol. Chem.* **272**, 21540–21547.
- DIDONATO, J.A., HAYAKAWA, M., ROTHWART, D.M., ZANDI, E., and KARIN, M. (1997). A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature* **388**, 548–554.
- MERCURIO, F., ZHU, H., MURRAY, B.W., SHEVCHENKO, A., BENNETT, B.L., LI, J., YOUNG, D.B., BARBOSA, M., MANN, M., MANNING, A., and RAO, A. (1997). IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278**, 860–866.
- REGNIER, C.H., SONG, H.Y., GAO, X., GOEDDEL, D.V., CAO, Z., and ROTHE, M. (1997). Identification and characterization of an IkappaB kinase. *Cell* **90**, 373–383.
- ROTHWART, D.M., ZANDI, E., NATOLI, G., and KARIN, M. (1998). IKK-gamma is an essential regulatory subunit of the I kappa B kinase complex. *Nature* **395**, 297–300.
- WORONICZ, J.D., GAO, X., CAO, Z., ROTHE, M., and GOEDDEL, D.V. (1997). IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science* **278**, 866–869.
- YAMAOKA, S., COURTOIS, G., BESSIA, C., WHITESIDE, S.T., WEIL, R., AGOU, F., KIRK, H.E., KAY, R.J., and ISRAEL, A. (1998). Complementation cloning of NEMO, a component of the I kappa B kinase complex essential for NF-kappa B activation. *Cell* **93**, 1231–1240.
- ZANDI, E., ROTHWART, D.M., DELHASE, M., HAYAKAWA, M., and KARIN, M. (1997). The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell* **91**, 243–252.
- LI, Q., LU, Q., HWANG, J.Y., BUSCHER, D., LEE, K.F., IZPISUA-BELMONTE, J.C., and VERMA, I.M. (1999). IKK1-de-

- ficient mice exhibit abnormal development of skin and skeleton. *Genes Dev.* **13**, 1322–1328.
24. LI, Q., ESTEPA, G., MEMET, S., ISRAEL, A., and VERMA, I.M. (2000). Complete lack of NF-kappaB activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. *Genes Dev.* **14**, 1729–1733.
 25. HU, Y., BAUD, V., DELHASE, M., ZHANG, P., DEERINCK, T., ELLISMAN, M., JOHNSON, R., and KARIN, M. (1999). Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of I kappa B kinase. *Science* **284**, 316–320.
 26. RUDOLPH, D., YEH, W.C., WAKEHAM, A., RUDOLPH, B., NALLAINATHAN, D., POTTER, J., ELIA, A.J., and MAK, T.W. (2000). Severe liver degeneration and lack of *NF-kappaB* activation in *NEMO/IKKgamma*-deficient mice. *Genes Dev.* **14**, 854–862.
 27. SIZEMORE, N., LEUNG, S., and STARK, G.R. (1999). Activation of phosphatidylinositol-3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NFkappaB p65/RelA subunit. *Mol. Cell. Biol.* **19**, 4798–4805.
 28. KRACHT, M., TRUONG, O., TOTTY, N.F., SHIROO, M., and SAKLATAVALA, J. (1994). Interleukin 1 alpha activates two forms of p54 alpha mitogen-activated protein kinase in rabbit liver. *J. Exp. Med.* **180**, 2017–2025.
 29. XIA, Y., MAKRIKIS, C., SU, B., LI, E., YANG, J., NEMEROW, G.R., and KARIN, M. (2000). MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. *Proc. Natl. Acad. Sci. USA* **97**, 5243–5248.
 30. TOURNIER, C., DONG, C., TURNER, T.K., JONES, S.N., FLAVELL, R.A., and DAVIS, R.J. (2001). MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev.* **15**, 1419–1426.
 31. FRESHNEY, N.W., RAWLINSON, L., GUESDON, F., JONES, E., COWLEY, S., HSUAN, J., and SAKLATVALA, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of Hsp27. *Cell* **78**, 1039–1049.
 32. GUESDON, F., FRESHNEY, N., WALLER, R.J., RAWLINSON, L., and SAKLATVALA, J. (1993). Interleukin 1 and tumor necrosis factor stimulate two novel protein kinases that phosphorylate the heat shock protein hsp27 and beta-casein. *J. Biol. Chem.* **268**, 4236–4243.
 33. BAUD, V., and KARIN, M. (2001). Signal transduction by tumor necrosis factor and its relatives. *Trends Cell. Biol.* **11**, 372–377.
 34. LI, X., COMMANE, M., JIANG, Z., and STARK, G.R. (2001). IL-1-induced NFkappa B and c-Jun N-terminal kinase (JNK) activation diverge at IL-1 receptor-associated kinase (IRAK). *Proc. Natl. Acad. Sci. USA* **98**, 4461–4465.
 35. BACH, E.A., AGUET, M., and SCHREIBER, R.D. (1997). The IFN gamma receptor: a paradigm for cytokine receptor signaling. *Annu. Rev. Immunol.* **15**, 563–591.
 36. STARK, G.R., KERR, I.M., WILLIAMS, B.R., SILVERMAN, R.H., and SCHREIBER, R.D. (1998). How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264.
 37. CLARK-LEWIS, I., SANGHERA, J.S., and PELECH, S.L. (1991). Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J. Biol. Chem.* **266**, 15180–15184.
 38. GONZALEZ, F.A., RADEN, D.L., and DAVIS, R.J. (1991). Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J. Biol. Chem.* **266**, 22159–22163.
 39. WEN, Z., ZHONG, Z., and DARNELL, J.E., Jr. (1995). Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. *Cell* **82**, 241–250.
 40. KOVARIK, P., STOIBER, D., NOVY, M., and DECKER, T. (1998). Stat1 combines signals derived from IFN-gamma and LPS receptors during macrophage activation. *EMBO J.* **17**, 3660–3668.
 41. GOH, K.C., HAQUE, S.J., and WILLIAMS, B.R. (1999). p38 MAP kinase is required for Stat1 serine phosphorylation and transcriptional activation induced by interferons. *EMBO J.* **18**, 5601–5608.
 42. KOVARIK, P., STOIBER, D., EYERS, P.A., MENGHINI, R., NEININGER, A., GAESTEL, M., COHEN, P., and DECKER, T. (1999). Stress-induced phosphorylation of Stat1 at Ser727 requires p38 mitogen-activated protein kinase, whereas IFN-gamma uses a different signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 13956–13961.
 43. NGUYEN, H., RAMANA, C.V., BAYES, J., and STARK, G.R. (2001). Roles of phosphatidylinositol 3-kinase in interferon-gamma-dependent phosphorylation of Stat1 on serine 727 and activation of gene expression. *J. Biol. Chem.* **276**, 33361–33368.
 44. DECKER, T., and KOVARIK, P. (2000). Serine phosphorylation of Stats. *Oncogene* **19**, 2628–2637.
 45. KUMAR, A., COMMANE, M., FLICKINGER, T.W., HORVATH, C.M., and STARK, G.R. (1997). Defective TNF- α -induced apoptosis in Stat1-null cells due to low constitutive levels of caspases. *Science* **278**, 1630–1632.
 46. CHATTERJEE-KISHORE, M., WRIGHT, K.L., TING, J.P., and STARK, G.R. (2000). How Stat1 mediates constitutive gene expression: a complex of unphosphorylated Stat1 and IRF1 supports transcription of the LMP2 gene. *EMBO J.* **19**, 4111–4122.
 47. DARNELL, J.E., Jr., KERR, I.M., and STARK, G.R. (1994). Jak-Stat pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264**, 1415–1421.
 48. SAMBROOK, J., FRITSCH, E.F., and MANIATIS, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor University Press.
 49. LI, Q., VAN ANTWERP, D., MERCURIO, F., LEE, K.F., and VERMA, I.M. (1999). Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science* **284**, 321–325.
 50. PALUDAN, S.R. (2000). Synergistic action of pro-inflammatory agents: cellular and molecular aspects. *J. Leukocyte Biol.* **67**, 18–25.
 51. SEEGERT, D., STREHLOW, I., KLOSE, B., LEVY, D.E., SCHINDLER, C., and DECKER, T. (1994). A novel interferon-alpha-regulated, DNA-binding protein participates in the regulation of the IP53/tryptophanyl-tRNA synthetase gene. *J. Biol. Chem.* **269**, 8590–8595.
 52. OHMORI, Y., SCHREIBER, R.D., and HAMILTON, T.A. (1997). Synergy between interferon-gamma and tumor necrosis factor-alpha in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor kappaB. *J. Biol. Chem.* **272**, 14899–14907.
 53. CHUNG, J., UCHIDA, E., GRAMMER, T.C., and BLENIS, J. (1997). Stat3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation. *Mol. Cell. Biol.* **17**, 6508–6516.
 54. QIAN, Y., COMMANE, M., NINOMIYA-TSUJI, J., MATSUMOTO, K., and LI, X. (2001). IRAK-mediated translocation of TRAF6 and TAB2 in the interleukin-1-induced activation of NFkappa B. *J. Biol. Chem.* **276**, 41661–41667.
 55. CHESHIRE, J.L., WILLIAMS, B.R., and BALDWIN, A.S., Jr. (1999). Involvement of double-stranded RNA-activated protein kinase in the synergistic activation of nuclear factor-kappaB by tumor necrosis factor-alpha and gamma-interferon in preneuronal cells. *J. Biol. Chem.* **274**, 4801–4806.

Address reprint requests or correspondence to:

Dr. George R. Stark
Lerner Research Institute, NB21
The Cleveland Clinic Foundation
9500 Euclid Avenue
Cleveland, OH 44195

Tel: (216) 444-6062
Fax: (216) 444-3279
E-mail: starkg@ccf.org