Modulation of the Nuclear Factor κB Pathway by Shp-2 Tyrosine Phosphatase in Mediating the Induction of Interleukin (IL)-6 by IL-1 or Tumor Necrosis Factor

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Abstract

Shp-2, a src homology (SH)2-containing phosphotyrosine phosphatase, appears to be involved in cytoplasmic signaling downstream of a variety of cell surface receptors, although the mechanism is unclear. Here, we have determined a role of Shp-2 in the cytokine circuit for inflammatory and immune responses. Production of interleukin (IL)-6 in response to IL-1α or tumor necrosis factor (TNF)-α was nearly abolished in homozygous mutant (Shp-$2^{-/-}$) fibroblast cells. The targeted Shp-2 mutation has no significant effect on the activation of the three types of mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (Erk), c-Jun NH_2-terminal kinase (Jnk), and p38, by IL-1/TNF, indicating that Shp-2 does not work through MAP kinase pathways in mediating IL-1/TNF-induced IL-6 synthesis. In contrast, IL-1/TNF-stimulated nuclear factor (NF)-κB DNA binding activity and inhibitor of κB (IκB) phosphorylation was dramatically decreased in Shp-$2^{-/-}$ cells, while the expression and activity of NF-κB–inducing kinase (NIK), Akt, and IκB kinase (IKK) were not changed. Reintroduction of a wild-type Shp-2 protein into Shp-$2^{-/-}$ cells rescued NF-κB activation and IL-6 production in response to IL-1/TNF stimulation. Furthermore, Shp-2 tyrosine phosphatase was detected in complexes with IKK as well as with IL-1 receptor. Thus, this SH2-containing enzyme is an important cytoplasmic factor required for efficient NF-κB activation. These results elucidate a novel mechanism of Shp-2 in cytokine signaling by specifically modulating the NF-κB pathway in a MAP kinase–independent fashion.

Key words: tyrosine phosphatase • IL-1 • IL-6 • TNF • Shp-2

Introduction

TNF-α, IL-1α, IL-1β, and IL-6 are potent immunoregulatory and proinflammatory cytokines secreted by a variety of cell types. These cytokines have similar and broad ranges of physiological effects in regulating local and systemic immune responses and have also been implicated in several pathological processes (1, 2). Interestingly, both IL-1 and TNF are potent inducers of IL-6, which acts to mediate their biological effects (3). Molecular analysis of the mechanism for IL-6 induction by IL-1 and TNF has often served as a model system for dissection of cellular events of signal transduction triggered by these two cytokines.

Two types of IL-1 receptors, type I (IL-1RI) and type II (IL-1RII), have been identified, and IL-1 appears to trigger intracellular signaling cascades by binding to IL-1RI, while IL-1RII may function as a ligand sink (4). The TNF-α activity is elicited by binding to two distinct monomeric TNF receptors, TNF R-55 and TNF R-75, and most of the downstream signaling events induced by TNF binding have been associated with TNF R-55 (5). Neither IL-1 nor TNF receptor has intrinsic kinase activity and, as such, their activation signals must be transmitted into cells by accessory molecules and/or associated cytoplasmic proteins. The family of TNF receptor–associated factors are involved in information flow from both TNFR1 and TNFR2 receptors as well as IL-1 receptor (6). Furthermore, the IL-1 receptor–associated serine/threonine kinase (IRAK)[1] ap-

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[1]*Abbreviations used in this paper:* AP, activator protein; EMSA, electrophoretic mobility shift assay; Erk, extracellular signal-regulated kinase; IκB, inhibitor of κB; IKK, IκB kinase; IRAK, IL-1 receptor–associated serine/threonine kinase; Jnk, c-Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MBP, myelin basic protein; NF, nuclear factor; NIK, NF-κB–inducing kinase; SH, src homology.

pears to play a critical role in proximal events of IL-1 receptor signaling (7, 8).

Induction of IL-6 by IL-1 or TNF seems to involve multiple cytoplasmic signaling pathways. One prominent cellular event is the rapid and dramatic activation of nuclear factor (NF)-κB (9–11). NF-κB is normally inactive and sequestered in the cytoplasm as a heterodimer comprising two polypeptides of 50 kD (p50) and 65 kD (p65), which are noncovalently complexed with a cytoplasmic inhibitory protein, inhibitor of κB (IκB). Upon cytokine stimulation, IκB is rapidly phosphorylated, ubiquitinated, and then degraded, resulting in the release and subsequent nuclear translocation of active NF-κB. NF-κB is critically involved in transcriptional regulation of the *IL-6* gene, among others (12, 13). Treatment of cells with IL-1 or TNF also leads to the activation of three classes of mitogen-activated protein (MAP) kinases, p42/p44 extracellular signal-regulated kinase (Erk), p54 c-Jun NH_2-terminal kinase (Jnk), and p38 MAP kinase, in a variety of cell types (4, 14, 15). Absence of IRAK expression in targeted mutant fibroblast cells leads to a defective activation of NF-κB, Jnk, and p38 MAP kinases by IL-1 treatment, which is accompanied by a severe suppression in IL-1–induced IL-6 production (8). Accumulating biochemical data suggest that both the NF-κB and the MAP kinase pathways are required for efficient induction of IL-6 by IL-1 or TNF (1, 16). However, it is not clear whether each of these pathways is distinctly or coordinately controlled in cellular response to cytokines.

Shp-2, a widely expressed cytoplasmic tyrosine phosphatase with two src homology (SH)2 domains, has been implicated in a variety of signal transduction pathways elicited by growth factors, cytokines, hormones, antigens, and extracellular matrices (17). In previous studies, we created a Shp-2 mutant mouse model by targeting the exon 3 of the *Shp-2* gene (18). Homozygous mutant animals die at midgestation stage with severe defects in the mesodermal patterning, and all of the evidence so far suggests that this is a loss of function mutation (19–23). Using fibroblast cells derived from Shp-2 mutant embryos, we demonstrated that Shp-2 is positively involved in mitogenic stimulation of Erk activity but plays a negative role in the induction of Jnk activity by cellular stress and also in the IFN-stimulated Jak/signal transducer and activator of transcription (STAT) pathway (20, 23).

Here, we present evidence for the first time that a functional Shp-2 molecule is required for IL-6 induction by IL-1 or TNF. More importantly, we have uncovered a novel mechanism by which Shp-2 acts to promote IL-6 production via the NF-κB pathway in a MAP kinase–independent manner.

Materials and Methods

Cell Lines and Reagents. Wild-type (Shp-$2^{+/+}$) and homozygous mutant (Shp-$2^{-/-}$) embryonic fibroblast cell lines were isolated as described in detail previously (20). Reintroduction of wild-type Shp-2 protein into Shp-$2^{-/-}$ fibroblast cells was described elsewhere (21). Recombinant mouse TNF-α was purchased from R&D Systems, and recombinant mouse IL-1α was provided by Sigma-Aldrich. Antibodies specific for phospho-Erk1/2, phospho-Jnk, or phospho-p38 MAP kinase were purchased from New England Biolabs, Inc. Polyclonal anti–Shp-2, antiphosphotyrosine (pY99), anti-TNFR1, anti–IL-1RI, p50, or p65 of NF-κB antibodies were from Santa Cruz Biotechnology, Inc. Buffer A is composed of 50 mM β-glycerophosphate, pH 7.3, 2 mM EDTA, 1 mM EGTA, 5 mM β-ME, 1% Triton X-100, and 0.05 M NaCl. Buffer B contains 20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol (DTT). Both buffers were also supplemented before use with 0.2 mM Na_3VO_4, 0.4 μM microcystin, 0.1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 1 μM pepstatin A, and 1 μg/ml aprotinin.

Stimulation of Cells and Preparation of Nuclear Extracts. Fibroblast cells at ~80% confluency were starved in serum-free DMEM for 24 h before treatment with IL-1α or TNF-α. Factor stimulation was terminated by washing cells with ice-cold PBS, and cell extracts were made in the following ways. Whole cell extracts were made by homogenization of cells in Buffer A followed by high speed centrifugation. For preparation of nuclear extracts, pelleted cells were resuspended in three packed cell volumes of Buffer B, swollen for 10 min, and lysed by repeated passage through a 25-gauge needle. Nuclei were collected by centrifugation at 16,000 *g* for 20 min and then extracted in 2.5 packed cell volumes of Buffer B supplemented with 0.42 M NaCl and 20% glycerol. The supernatant, referred to as the nuclear extract, was cleared by centrifugation at 16,000 *g* for 30 min and used for electrophoretic mobility shift assay (EMSA) as described below.

EMSA. EMSAs were carried out as previously described (23). The consensus double-stranded oligodeoxynucleotide probes for NF-κB or for activator protein (AP)-1 (Santa Cruz Biotechnology, Inc.) were radioactively labeled using γ-[^{32}P] ATP and T4 polynucleotide kinase using standard procedures. Nuclear extracts containing 10 μg of total proteins were preincubated with 2 μg of polydI-dC:polydI-dC (Amersham Pharmacia Biotech) for 20 min in a 20-μl binding reaction mixture (20 mM HEPES, pH 7.9, 25 mM KCl, 4 mM $MgCl_2$, 0.5 mM DTT, 1 mM EDTA, 10% glycerol), followed by addition of 2 fmol ^{32}P-labeled DNA probe and further incubated for 30 min at room temperature. Resulting protein–DNA complexes were resolved on 5% polyacrylamide gels (acrylamide/bisacrylamide = 39:1) containing 2.5% glycerol made in 0.5× TBE (Tris/borate/EDTA) buffer. Gels were dried and exposed to X-ray films.

In Vitro Erk Kinase Assay. Erk1 kinase was precipitated from treated or nontreated cell lysates by anti-Erk antibody and protein A–Sepharose 4B beads (20). The beads were washed twice with HNTG buffer (20 mM HEPES, pH 7.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4) and once with the kinase buffer (29 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM β-ME, 1 mM Na_3VO_4). The assay was performed by mixing the beads with 1 mg/ml myelin basic protein (MBP), 80 μM ATP, and 1.5 μCi γ-[^{32}P]ATP in the kinase buffer and incubated for 10 min at 30°C. After centrifugation, supernatants were spotted on p81 Whatman paper, and the papers were washed several times in 180 mM phosphoric acid and once in 100% ethanol, air dried, and counted.

Immunoprecipitation and Immunoblot Analysis. Cell extracts were incubated with antibodies prebound to protein A–Sepharose beads overnight. The beads were washed three times with Buffer A with 0.15 M NaCl. For immunoblot analysis, samples were separated by 10% SDS-PAGE and transferred to a nitrocel-

lulose membrane. The membrane was first probed with a specific antibody and then detected using the ECL system with horseradish peroxidase–conjugated secondary antibodies (Amersham Pharmacia Biotech).

Quantitation of IL-6 Production. For detection of secreted IL-6 protein, cells were first starved in serum-free DMEM for 24 h before treatment, and then they were treated with IL-1α or TNF-α for various time periods. Supernatants were collected and analyzed for IL-6 production using a commercial ELISA kit (R&D Systems), with recombinant mouse IL-6 as a standard.

Northern Blot Analysis. Cells were starved for 24 h and treated with IL-1α (10 ng/ml) or TNF-α (20 ng/ml) for different time periods. Cells were lysed in guanidinium isothiocyanate (Kodak) in a Tris-buffered solution and isolated by CsCl density centrifugation. Total RNA (20 μg) was fractionated through 1% agarose gel containing 18% formaldehyde and transferred by capillary action to Zeta probe filter for hybridization, using a mouse IL-6 cDNA fragment (0.6 kb) as a probe (CLONTECH Laboratories, Inc.).

Results

Defective IL-6 Induction by IL-1 or TNF in Shp-2 Mutant Cells. Both IL-1 and TNF are able to induce a drastic upregulation of IL-6 gene expression, and thus assessment of IL-6 production has been used as a paradigm in dissecting intracellular signaling pathways elicited by these two cytokines. To determine the possible involvement of Shp-2 tyrosine phosphatase in this process, we first examined whether IL-6 production is altered in Shp-$2^{-/-}$ cells under treatment with IL-1 or TNF. Serum-starved cells were incubated with either IL-1α or TNF-α for various time periods, and supernatants were collected to assay for IL-6 secretion. As shown in Fig. 1 A, IL-6 production induced by IL-1α for 4, 8, and 24 h was nearly blocked in Shp-$2^{-/-}$ cells, with a maximal decrease of more than 40-fold as compared with that in wild-type cells. Similarly, a severe decrease in the levels of IL-6 secretion was observed in Shp-$2^{-/-}$ cells after TNF-α treatment (Fig. 1 B). Northern blot analysis showed much decreased amounts of *IL-6* mRNA induced by IL-1/TNF in mutant cells as compared with that in wild-type cells, pinpointing a problem at the transcription level (Fig. 1 C). These results indicate the requirement for a functional Shp-2 molecule in mediating IL-1/TNF-induced cytoplasmic signaling pathways that culminate in IL-6 induction.

Shp-2 Does Not Mediate IL-6 Induction via the Three MAP Kinase Pathways. To explore the molecular mechanism for Shp-2 involvement in mediating IL-6 induction by IL-1 or TNF, we wanted to examine the activation of three types of MAP kinases, Erk, Jnk, and p38. It has been previously reported that IL-1 and TNF activation of one or more MAP kinases in different cell types has been implicated in IL-6 induction (24, 25). We and others have also found that Shp-2 is indeed positively required for growth factor stimulation of Erk activity (20, 26–28).

Erk activity was measured in an in vitro kinase assay with MBP as a substrate. As shown in Fig. 2, A and B, activation of Erk by IL-1α or TNF-α was not significantly changed

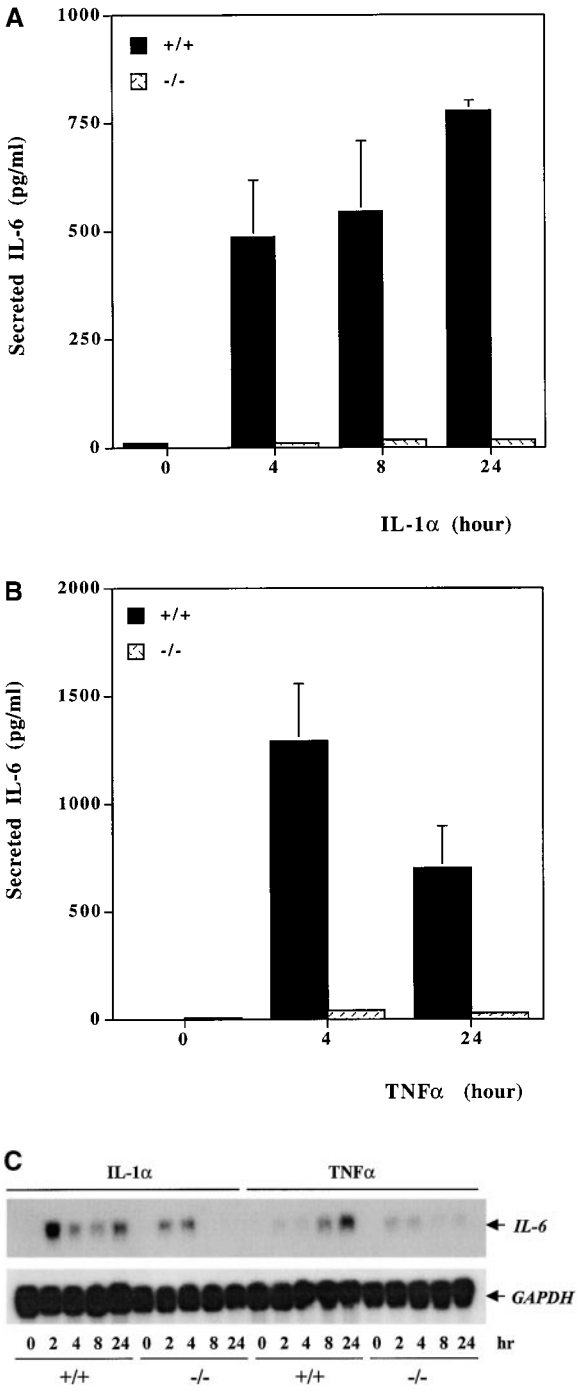

Figure 1. Suppression of IL-1α/TNF-α–induced IL-6 production in Shp-$2^{-/-}$ cells. (A and B) Measurement of IL-6 secretion. The establishment of embryonic fibroblast cell lines of wild-type and Shp-2 mutant origins was described in detail previously (20). After starvation in serum-free DMEM for 24 h, wild-type and Shp-$2^{-/-}$ cells were stimulated with 10 ng/ml IL-1α (A) or 20 ng/ml TNF-α (B) for the indicated times at 37°C. Supernatants were then collected and measured for IL-6 secretion using a commercial ELISA kit (R&D Systems), with recombinant mouse IL-6 as a standard. Data shown are the means of three independent experiments ± SD. (C). Northern blot analysis of *IL-6* transcript. Total RNA was extracted from control and factor-treated cells, and Northern blotting was performed using a specific *IL-6* cDNA probe. The membrane was stripped and reprobed with a cDNA fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control.

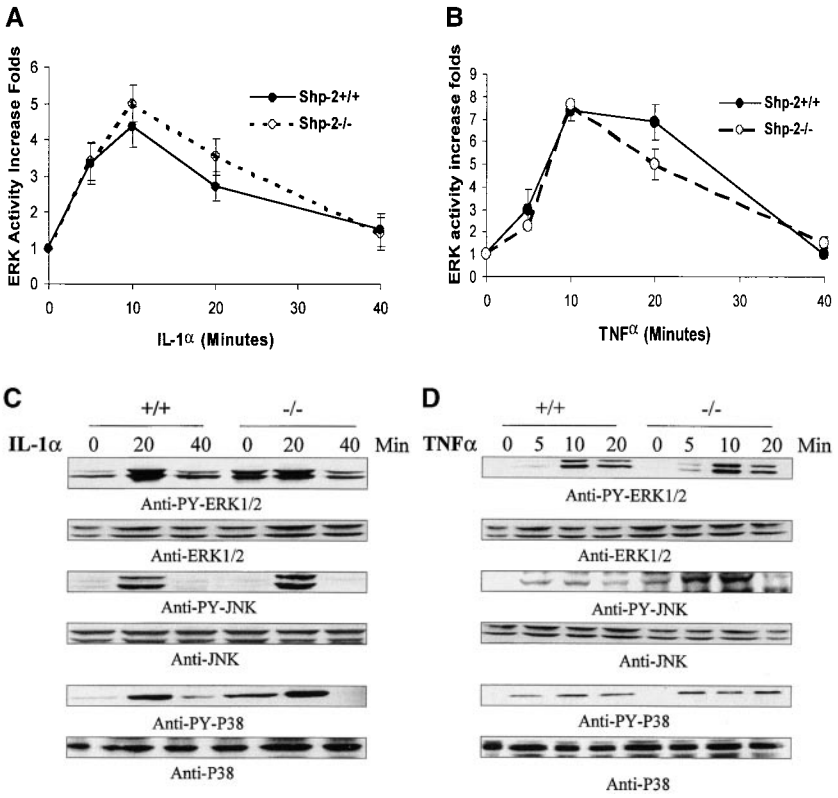

Figure 2. Normal activation of MAP kinases by IL-1α/TNF-α. (A and B) Serum-starved wild-type and Shp-$2^{-/-}$ cells were treated with 10 ng/ml IL-1α (A) or 20 ng/ml TNF-α (B) for the indicated time periods. ERK1 was immunoprecipitated for an in vitro kinase assay using MBP as a substrate as previously described (20). (C and D) Whole cell extracts were prepared from control and factor-treated cells. Equal amounts of cell lysates (40 μg of total proteins) were subjected to immunoblot analysis with anti–phospho-ERK-42/44, anti–ERK-42/44, anti–phospho-p46/54 Jnk, anti-p46/54 Jnk, anti–phospho-p38, and anti-p38 MAPK antibodies.

in Shp-$2^{-/-}$ cells, as compared with that in wild-type cells. To extend this observation, we examined extensively the activities of all three major members of the MAP kinase family, Erk, Jnk, and p38, using antibodies specific for phosphorylated forms of p44/42 Erk1/2, p54/56 Jnk, and p38 (Fig. 2, C and D). Consistent with previous observations (25), we found that stimulation of cells by IL-1α– or TNF-α–activated Jnk and p38 MAP kinases more prominently than Erk in fibroblast cells. Interestingly, in contrast to the dramatic inhibition of IL-6 induction, activation of all three types of MAP kinases was either not changed or even slightly higher in Shp-$2^{-/-}$ than in wild-type cells in response to IL-1/TNF stimulation.

Downstream of MAP kinases, AP-1 can be activated directly through phosphorylation by Jnk, and the expression of AP-1 components is induced through Jnk- and p38-dependent activation of transcription factors. To assess the effect of Shp-2 on IL-1–induced AP-1 activation, we measured AP-1 activity by EMSA assay, by incubating a radiolabeled oligonucleotide probe for the AP-1 binding site with nuclear extracts prepared from untreated and IL-1α–treated cells. Consistent with the results for the activation of MAP kinases, treatment with IL-1α resulted in a similar increase in the levels of AP-1 DNA binding in wild-type and Shp-$2^{-/-}$ cells (Fig. 3), and similar results were obtained after TNF-α stimulation (data not shown).

NF-κB Activation Is Severely Attenuated in Shp-$2^{-/-}$ Cells. The results described above indicate that the defective IL-6 induction in Shp-2 mutant cells is not due to downregulation of one or more MAP kinase activities, and it is unlikely that Shp-2 mediates cellular responses to IL-1/TNF via manipulating the MAP kinase pathways. This observation prompted us to search for other signaling routes that are influenced by Shp-2. As IL-1α and TNF-α are the two most efficient activators of NF-κB, we monitored the NF-κB activation by IL-1α or TNF-α in wild-type and Shp-$2^{-/-}$ fibroblasts by measuring DNA binding activity of NF-κB in nuclear extracts with a NF-κB–specific oligonucleotide probe. Treatment of wild-type fibroblast cells by IL-1α or TNF-α for 15, 30, and 60 min resulted in a dramatic induction of DNA binding activity of NF-κB (Fig. 4, A and B). However, compared with the induction in wild-type cells, NF-κB activation was significantly diminished in Shp-$2^{-/-}$ cells after treatment with either IL-1α or TNF-α. This is in contrast to the similar induction of AP-1 activity as well as MAP kinase activities in wild-type and

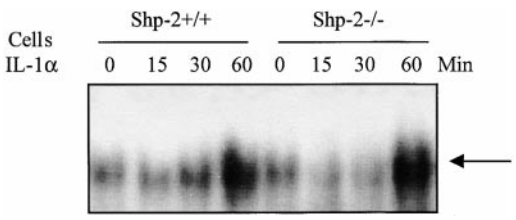

Figure 3. AP-1 activation by IL-1. Serum-starved wild-type and Shp-$2^{-/-}$ cells were treated with 10 ng/ml IL-1α for the indicated time periods. Nuclear extracts were prepared for detection of AP-1 activity using EMSA with radiolabeled probe containing the consensus AP-1 binding site (5′-CGC TTG ATG AGT CAG CCG GAA-3′; obtained from Promega). The arrow denotes the specific AP-1–DNA complexes.

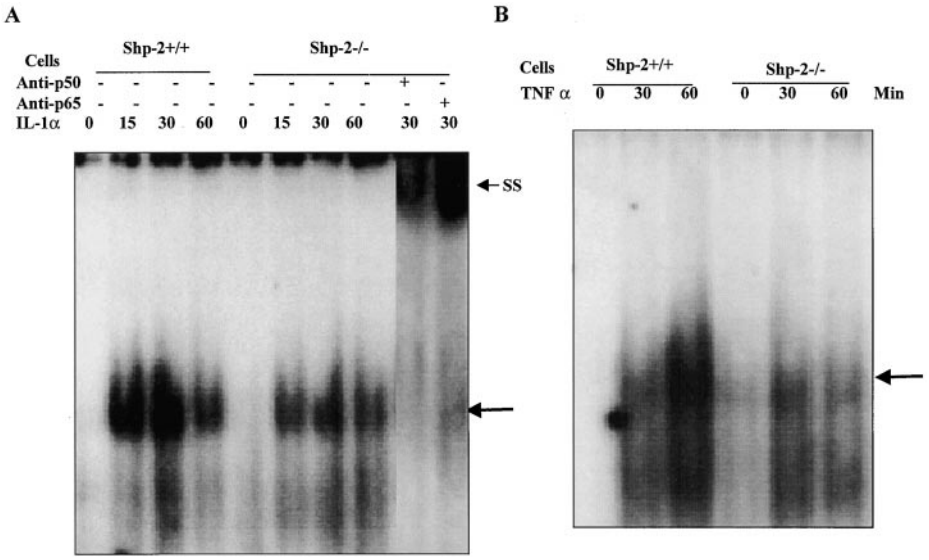

Figure 4. Reduced NF-κB activity in Shp-$2^{-/-}$ cells in response to IL-1α/TNF-α stimulation. Nuclear extracts were prepared from control and factor-stimulated cells, and NF-κB DNA binding activity was measured by EMSA with a radiolabeled probe containing the consensus NF-κB binding site (5′-AGT TGA GGG GAC TTT CCC AGG C-3′; from Santa Cruz Biotechnology, Inc.). Arrows denote the specific NF-κB–DNA complexes. In supershift assays (SS) in A and B, antibodies against the p50 or p65 subunit of NF-κB were added to the reaction mixture before incubation with the DNA probe.

Shp-$2^{-/-}$ cells as described above. Thus, Shp-2 appears to modulate IL-1α/TNF-α signaling by specifically modulating NF-κB activation rather than the MAP kinase–AP-1 pathway.

Mechanism for Shp-2 Function in the NF-κB Pathway. Our results point to a new function of Shp-2 in cytoplasmic signaling initiated by cytokines. To further dissect the mechanism for Shp-2 function in mediating NF-κB activation, we examined the phosphorylation status of IκB. The NF-κB protein is sequestered in the cytoplasm as an inactive complex with the inhibitor IκB, and cytokine-induced IκB phosphorylation is required for IκB degradation and consequent NF-κB activation. Cells were treated with either IL-1α or TNF-α for 1, 2, 3, 5, and 8 min, and cell lysates were immunoblotted with antibodies specific for phospho-IκB and IκB, respectively (Fig. 5). Stimulation with IL-1α or TNF-α induced a robust increase in the phosphorylation levels of IκB in wild-type cells. In contrast, only mild levels of IκB phosphorylation were observed in Shp-$2^{-/-}$ cells after IL-1/TNF treatment. This result confirmed that the defective NF-κB activation is caused by a severe reduction in the IκB phosphorylation in Shp-$2^{-/-}$ cells.

We then evaluated the expression and activities of several upstream kinases that are involved in IκB phosphorylation, including IκB kinase (IKK), NF-κB–inducing kinase (NIK), and Akt/protein kinase B. As detected by immunoblot analysis, the protein amounts for IKKs, NIK, and Akt were not changed in Shp-$2^{-/-}$ cells (Fig. 6). The activity of immunoprecipitated IKKα, the kinase that phosphorylates IκBα directly, was measured using purified IκBα (amino acids 1–317) as a substrate in vitro. There was no significant difference observed for IKKα activity in vitro between wild-type and Shp-$2^{-/-}$ cells after IL-1/TNF treatment (Fig. 6 A), and similar results were obtained for IKKβ (Fig. 6 B). As shown in Fig. 6 C, we detected similar levels of Akt kinase activity, and Akt was possibly involved in promoting the NF-κB pathway by activating IKK (29–31). Taken together, our results indicate that the activity of IKK, and thus the activation of upstream kinases Akt and NIK, was not altered when tested in an in vitro assay. We then searched for physical evidence for the Shp-2 involvement in the NF-κB pathway by co-immunoprecipitation

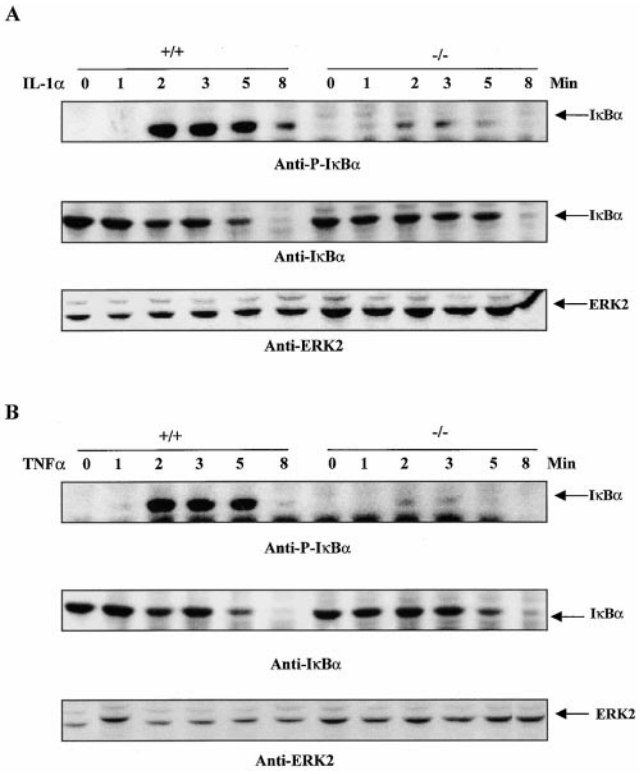

Figure 5. Diminished IκB phosphorylation in Shp-$2^{-/-}$ cells. Serum-starved cells were stimulated with either IL-1α (A) or TNF-α (B) for 1, 2, 3, 5, and 8 min, and the cell lysates were prepared for immunoblot analysis with either an anti–phospho-IκBα (anti–P-IκBα) or an anti-IκBα antibody. In parallel, the same samples were also blotted with anti-Erk antibody for loading control.

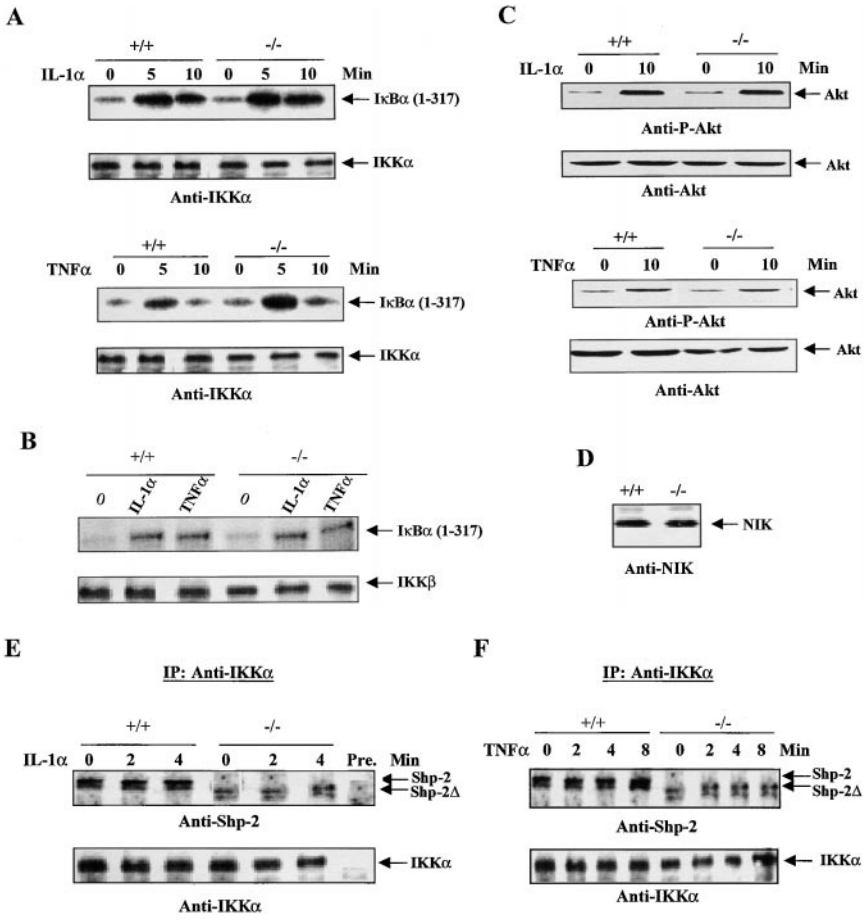

Figure 6. Expression and activity of components in the NF-κB pathway. (A and B) In vitro kinase activity assay for IKKα and IKKβ. The IKKα and -β kinases were immunoprecipitated, using their specific antibodies from control and factor-treated cell lysates and assayed for activity using purified glutathione-*S*-transferase fusion protein containing the NH_2-terminal part of IκBα (amino acids 1–317) as a substrate. The protein amount of the kinase in each sample was determined by immunoblot analysis with anti-IKKα (BD PharMingen) and anti-IKKβ (Santa Cruz Biotechnology, Inc.) antibodies, respectively. (C) Akt activation by IL-1α and TNF-α was analysed by immunoblotting with anti–P-Akt antibody (New England Biolabs, Inc.), and its protein amount was visualized by anti-Akt blotting. (D) The expression levels of NIK in wild-type and mutant cells were determined by anti-NIK immunoblotting. (E and F) Control and IL-1α/TNF-α–treated cell lysates were precipitated with anti-IKKα antibody, and the precipitates were blotted with either anti–Shp-2 or anti–IKKα antibodies. Bands for the wild-type and Shp-2^{Δ} proteins are indicated.

and found that Shp-2 was constitutively associated with IKKα, which was not influenced by IL-1/TNF treatment (Fig. 6, E and F). Therefore, Shp-2 is physically involved in a multimeric protein complex that is required for efficient phosphorylation of IκB and hence the activation of NF-κB in vivo.

To explore further the involvement of Shp-2 in IL-1 signaling, we investigated the physical interaction of Shp-2 with IL-1RI. Shp-2 was precipitated from cell lysates, and the resultant immunoprecipitates were subjected to immunoblot analysis with an anti–IL-1RI antibody. As shown in Fig. 7, Shp-2 was found in association with IL-1RI, and the complex was detected irrespective of ligand stimulation. Participation of Shp-2 in the IL-1RI complex was further confirmed in converse experiments in which the anti–IL-1RI immune complex was also found to contain Shp-2. In parallel experiments, we did not observe the physical association of Shp-2 with the TNF-α receptor (data not shown).

Rescue of the Mutant Phenotype by Reintroduction of Wild-Type Shp-2. To confirm that the defect in IL-1/TNF-induced NF-κB activation and IL-6 production were caused by absence of a functional Shp-2, we reevaluated cellular responses to the cytokine stimulation in Shp-$2^{-/-}$ cells in which a wild-type Shp-2 was reintroduced. As illustrated in Fig. 8, reintroduction of wild-type Shp-2 pro-

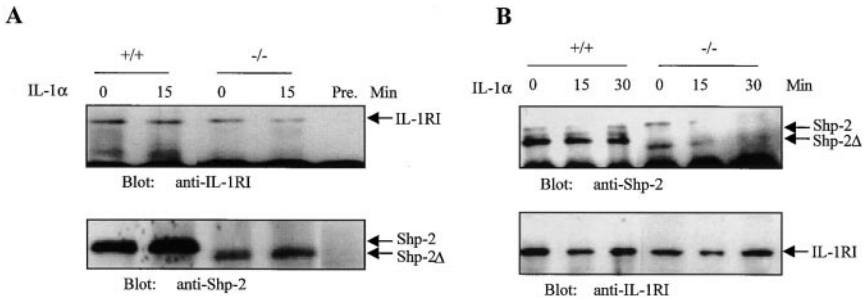

Figure 7. Interaction of Shp-2 with IL-1RI. Serum-starved wild-type and Shp-$2^{-/-}$ cells were treated with 10 ng/ml IL-1α for the indicated time periods. (A) Whole cell extracts were immunoprecipitated with anti–Shp-2 antibody or preimmune antiserum (Pre.). The resulting immunoprecipitates were resolved on SDS–polyacrylamide gel and immunoblotted with anti-IL-1RI or anti–Shp-2 antibodies as indicated. (B) Cell lysates were immunoprecipitated with anti–IL-1RI antibody and subjected to immunoblot analysis with anti–Shp-2 or anti–IL-1RI antibodies. Bands for the wild-type and Shp-2^{Δ} proteins are indicated.

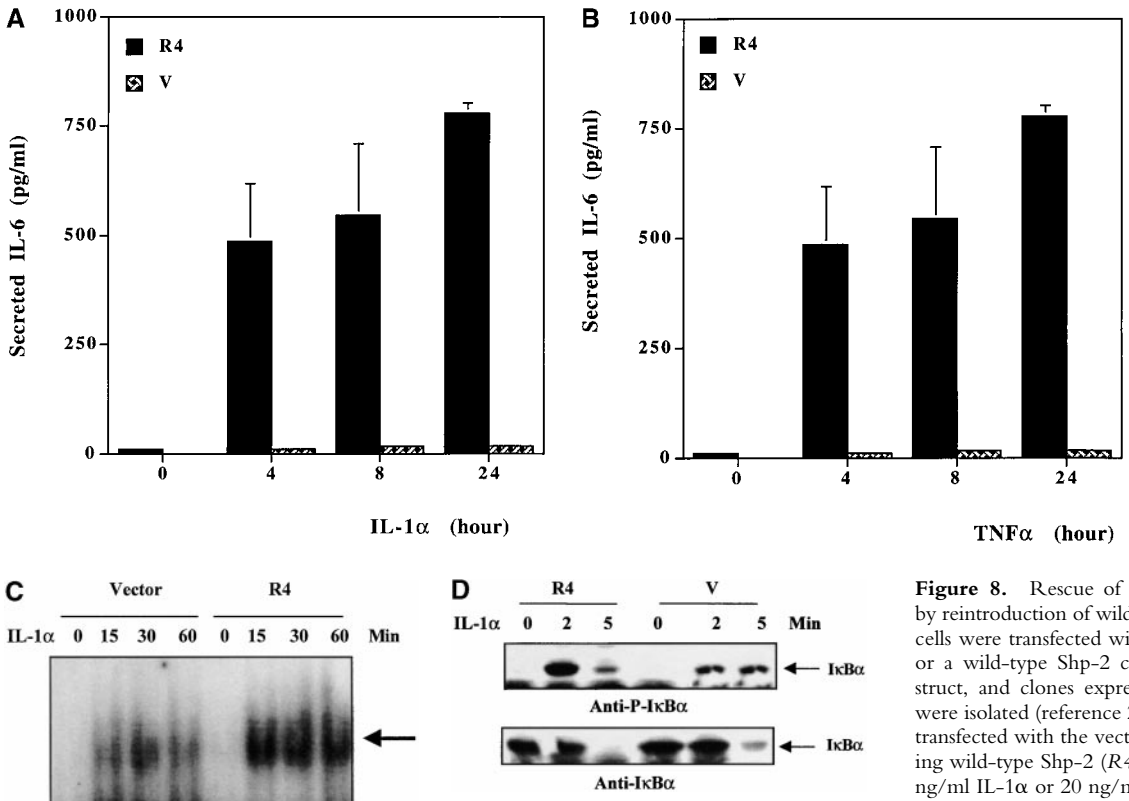

Figure 8. Rescue of the mutant phenotype by reintroduction of wild-type Shp-2. Shp-$2^{-/-}$ cells were transfected with a pcDNA3.1 vector or a wild-type Shp-2 cDNA expression construct, and clones expressing wild-type Shp-2 were isolated (reference 21). Shp-$2^{-/-}$ cell lines transfected with the vector only (V) or expressing wild-type Shp-2 (*R4*) were treated with 10 ng/ml IL-1α or 20 ng/ml TNF-α for the indicated time periods. (A and B) The amounts of secreted IL-6 in supernatants were determined by ELISA as described in Fig. 1. Data shown are the means of three independent experiments ± SD. (C) Nuclear extracts were prepared for detection of NF-κB activity as described in Fig. 4. The arrows denote the specific NF-κB–DNA complexes. (D) IkBα phosphorylation was evaluated by anti–P-IκBα antibody blotting.

tein rescued the activation of NF-κB by IL-1α or TNF-α. Furthermore, expression of wild-type Shp-2 in mutant cells resulted in a significant increase of IL-6 secretion (Fig. 8).

Collectively, these results revealed a new function for the cytoplasmic phosphotyrosine phosphatase, Shp-2, in mediating the induction of NF-κB activity and IL-6 synthesis by IL-1α and TNF-α in inflammatory and immune responses.

Discussion

In this study, we identified an important role of Shp-2 in a cytokine network by mediating the IL-6 induction by IL-1/TNF. It is well known that IL-6 acts to mediate and amplify the biological activities of IL-1 and TNF in a number of physiological and pathological processes (1). However, the molecular mechanism for the induction of IL-6 by IL-1/TNF is not fully understood. Previous studies suggest that activation of two signaling pathways, NF-κB and MAP kinases, is required for efficient induction of IL-6 by the two cytokines (4, 16). In exploring the molecular mechanism by which Shp-2 mediates the cytokine circuit, we first examined the Erk kinase activity, as our previous results indicate that Erk kinase activation is either severely diminished or even blocked in Shp-$2^{-/-}$ cells upon various growth factor stimulation (20). Surprisingly, IL-1/TNF-induced Erk activity was not significantly altered in a comparison between wild-type and Shp-$2^{-/-}$ cells. We then assessed the activities of the other two MAP kinases, Jnk and p38, upon stimulation of IL-1/TNF. Consistent with the literature (25), our results demonstrate that induction of these two types of MAP kinases are more profound than Erk in cellular responses to IL-1/TNF. However, there was no significant difference in IL-1/TNF-induced Jnk and p38 activities between wild-type and mutant cells. Thus, it is unlikely that Shp-2 acts through MAP kinase pathways to mediate the IL-6 induction by IL-1/TNF. Having obtained these results, we shifted our attention to the NF-κB pathway. Data from subsequent experiments indicate that Shp-2 indeed plays a critical role in positive regulation of IL-1/TNF-induced NF-κB activity that leads to IL-6 production, a previously unidentified mechanism for Shp-2 function in cell signaling.

NF-κB is a master transcription factor involved in the control of inflammatory response elicited by IL-1 and TNF. Previous studies suggest that stimulation of a kinase cascade by proinflammatory cytokines leads to the activation of the IKK complex that directly phosphorylates IκB (32). Now, our results indicate that Shp-2 is also an integral component of the IKK complex, and a functional Shp-2 is required for efficient phosphorylation of IκB by the IKK complex in cellular response to IL-1/TNF. This finding

might be instrumental for designing a novel therapeutic strategy for inflammatory and immune disorders by specifically manipulating Shp-2 activity in vivo. The positive effect of Shp-2 on activation of NF-κB, combined with our previously identified negative regulatory role of Shp-2 in interferon-stimulated Jak/STAT pathway (20, 23), may constitute a protective mechanism of cells against apoptosis.

We and others have described a complicated role of Shp-2 in MAP kinase cascades (20, 26–28). This phosphatase acts to promote mitogenic stimulation of Erk activity while being a negative regulator in the activation of Jnk by cellular stress (20). The most interesting part of this report is that, although all three types of MAP kinases are activated to variable extents by IL-1/TNF, Shp-2 is apparently not involved in any of the processes. In aggregate, these observations suggest that Shp-2 is engaged in multiple cytoplasmic pathways by different mechanisms, possibly due to subcellular targeting of the enzyme to different substrates. In mediating Erk induction by epidermal growth factor, Shp-2 is recruited into an oligomeric protein complex assembled on Gab1 scaffolding molecule and appears to dephosphorylate an unidentified phosphoprotein, p90, in promoting the Ras–Erk pathway (33). Results in this report show that Shp-2 participates in signaling complexes assembled around IL-1 receptor as well as IKK.

As reported previously (18–20), a mutant Shp-2 protein (Shp-2^{Δ}) lacking 65 amino acids in the SH2-N domain was expressed in the targeted mutant cells due to aberrant splicing. In epidermal growth factor (EGF)-treated cells, tyrosine-phosphorylated Gab1 selectively associates with the wild-type but not the mutant Shp-2 molecule, correlating with a defect in EGF-stimulated Erk activation (33). However, Shp-2 appears to be constitutively associated with IKK and IL-1RI, and the mutant Shp-2^{Δ} molecule was also detected in these complexes. This is similar to what we described for the interaction of Shp-2 with IFN-α and -γ receptors (23). At this moment, the molecular basis for Shp-2 activity in the NF-κB pathway is not yet fully understood. Although this is a typical tyrosine phosphatase from its molecular architecture, we have not excluded the possibility that Shp-2 may act as a dual-specificity phosphatase. It is also possible that Shp-2 functions as an adapter molecule in mediating the activation of NF-κB, and an intact SH2-N domain is possibly required for recruitment of other signaling components into the IL-1RI and IKK complexes. Shp-2^{Δ} is clearly a loss-of-function molecule, as the defects in IL-1/TNF signaling in mutant cells were rescued by reintroduction of wild-type Shp-2.

Kanakaraj et al. reported recently (8) that in IRAK-deficient fibroblast cells, IL-1–induced Jnk, p38, and NF-κB activities were all decreased, accompanied by a dramatic reduction in IL-6 secretion. At high concentrations, IL-1 induced a normal NF-κB response but not the response of Jnk and p38 in IRAK-deficient cells. But even under this condition, IL-6 induction by IL-1 was significantly lower in mutant than in wild-type cells, suggesting that efficient activation of *IL-6* gene expression requires two signals, delivered by NF-κB and MAP kinases. Consistently, another recent report by Vanden Berghe et al. (34) suggests a cooperation between NF-κB and MAP kinases for TNF-induced IL-6 gene expression in that Erk and p38 modulate the transactivation potential of NF-κB without affecting its DNA binding activity.

Defects in both the NF-κB and MAP kinase pathways in IRAK-deficient cells suggest an involvement of IRAK in a proximal common event downstream of IL-1 receptor that signals activation of both pathways. In this regard, it is interesting to note that, although Shp-2 apparently operates in signal relay proximally from IL-1 receptor complex, it has a specific effect on the NF-κB pathway only, without influencing the MAP kinase cascades. Therefore, pharmaceutical interference of this specific activity of Shp-2 in cell signaling might be clinically useful for the treatment of IL-1–mediated inflammatory diseases without side-effects.

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