The Inhibition of Interleukin-6-dependent STAT Activation by Mitogen-activated Protein Kinases Depends on Tyrosine 759 in the Cytoplasmic Tail of Glycoprotein 130*

Lara Terstegen‡§, Petros Gatsios‡**, Johannes G. Bode‡, Fred Schaper‡, Peter C. Heinrich‡, and Lutz Graeve‡§

From the ‡Institut für Biochemie and §Interdisziplinares Zentrum für Klinische Forschung "Biomat.,” RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

Mitogen-activated protein (MAP) kinases stimulated by phorbol 13-myristate 12-acetate (PMA) have been shown to inhibit interleukin-6-induced activation of STAT3 (Sengupta, T. K., Talbot, E. S., Scherer, P. A., and Ivashkiv, L. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 11107–11112). In the present study we demonstrate that in addition to STAT3, also tyrosine phosphorylation of STAT1, signal transducer gp130, and phosphotyrosine phosphatase SHP2 underlies negative regulation by MAP kinases. Stimulation of Erks by basic fibroblast growth factor or a constitutively active mutant of Raf also led to down-regulation of STAT activity. Using chimeric receptor mutants we show that tyrosine 759 of glycoprotein 130 is crucial for the inhibitory effect of MAP kinases. Inhibition is also dependent on gene transcription and translation indicating that newly synthesized proteins are involved. Both PMA and basic fibroblast growth factor rapidly stimulate mRNA expression of the suppressor of cytokine signaling-3 (SOCS-3) and this induction is strongly reduced by an inhibitor of MAP kinase activation. Together with recent results demonstrating that SOCS-3 can bind in vitro to a phosphorylated tyrosine 759 peptide of glycoprotein 130 these data suggest SOCS-3 to be instrumental in the inhibition of the Janus kinase/STAT pathway by MAP kinases.

The Jak/STAT pathway is activated by numerous cytokines and growth factors and transduces the signal from surface receptors to respective target genes in the nucleus (1–3). In the case of interleukin-6 (IL-6), binding to its receptor (gp80) triggers the dimerization of the signal transducer gp130 resulting in the autophosphorylation/activation of noncovalently associated Jak1, Jak2, and Tyk2 (1). These tyrosine kinases phosphorylate the cytoplasmic tail of gp130 thereby creating recruitment sites for SH2 domain containing signaling components such as STAT1, STAT3, and the phosphotyrosine phosphatase SHP2 which are also subject to phosphorylation (4, 5). Activated STATs homo- or heterodimerize, translocate to the nucleus and bind to enhancer elements of target genes. SHP2 negatively regulates IL-6-induced gene transcription and was shown to act as an adaptor linking the Jak/STAT pathway to the MAP kinase pathway via Grb2 (6–8).

Recently, two families of proteins have been cloned that negatively regulate the Jak/STAT pathway, i.e. suppressor of cytokine signaling (SOCS) proteins and protein inhibitors of activated STATs (PIAS) (9–13). SOCS proteins are induced upon STAT activation and in turn inhibit the activity of Jak-PIAS bind to activated STAT dimers and prevent STAT-mediated gene activation. How PIAS are regulated is currently unknown.

A number of other mediators have been reported to down-regulate Jak/STAT activation, e.g. transforming growth factor β, granulocyte/macrophage colony stimulating factor, and angiotensin II (14–16). The protein kinase C activator phorbol 12-myristate 13-acetate (PMA) was recently shown to inhibit IL-6-induced STAT3 activation via the Erk/MAP kinases (17). The mechanism of this inhibition, however, remains to be elucidated.

Here, we demonstrate that PMA does not only inhibit tyrosine phosphorylation of STAT3 but also tyrosine phosphorylation of STAT1, gp130, and SHP2. The inhibitory effect of PMA was found to be strictly dependent on tyrosine 759, the known recruitment site for SH2 domain containing tyrosine phosphatase; PAGE, polyacrylamide gel electrophoresis; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitors of activated STATs.

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‡ Contributed equally to the results of this work.

** To whom correspondence should be addressed: Institut für Biochemie, Klinikum der RWTH Aachen, Pauwelsstrasse 30, D-52057 Aachen, Germany. Tel.: 49-241-80-88-837; Fax: 49-241-88-88-428; E-mail: gatsios@RWTH-Aachen.de.

†† Germany. Tel.: 49-241-88-88-837; Fax: 49-241-88-88-428; E-mail: gatsios@RWTH-Aachen.de.

¶ The abbreviations used are: Jak, Janus kinase; STAT, signal transducer and activator of transcription; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; Erk, extracellular signal-regulated kinase; bFGF, basic fibroblast growth factor; gp, glycoprotein; Grb2, growth factor receptor-bond protein 2; IL, interleukin; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; PMA, phorbol 13-myristate 12-acetate; PVDF, polyvinyl difluoride; SIE, Sis-inducible element; spg80, soluble interleukin-6 receptor gp80; SH2, Src homology 2; SHP2, SH2 domain containing tyrosine phosphatase; PAGE, polyacrylamide gel electrophoresis; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitors of activated STATs.

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 Experimental Procedures

Materials—PD98059 and SB202190 were from Calbiochem (Bad Soden, Germany). Anisomycin, actinomycin D, and cycloheximide were from BIOMOL (Hamburg, Germany). PMA was purchased from Sigma (Deisenhofen, Germany). Dulbecco’s modified Eagle’s medium was from

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mM Na₃VO₄, and 15% glycerol). The samples were boiled in gel electrophoresis sample buffer and the precipitated proteins were separated on 7.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE (20 mM Tris, 20 mM boric acid, 0.5 mM EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 1 h, dried, and in- stained with Coomassie Brilliant Blue R-250. Blots were washed three times with wash buffer (0.05% Nonidet P-40, 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 1 mM sodium fluoride, 1 mM EDTA, 1 mM Na₃VO₄, 0.25 mM phenylmethylsulfonyl fluoride, 5 mM m-galactosidase activity.

RESULTS

Time Dependence of Erk Activation by PMA—The electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared as described (22). EMSAs were performed using a double-stranded 32P-labeled mutated m67SIE-oligonucleotide from the c-fos promoter (m67SIE: 5'-GATCCGGAGGAGTTACCGGAATAATGCTG-3') (23, 24). The protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.55-fold TBE (20 mA, 20 mA boric acid, 0.5 mA EDTA) at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid, and 80% water for 1 h, dried, and autoradiographed. Immunoprecipitation—Cells were washed twice with phosphate-buffered saline and solubilized in 1 ml of lysis buffer (0.5% Nonidet P-40, 50 mA Tris/HCl, pH 7.4, 150 mA NaCl, 1 mA sodium fluoride, 1 mA EDTA, 1 mA Na₃VO₄, 0.25 mA phenylmethylsulfonyl fluoride, 5 mA m-galactosidase activity) for 30 min at 4 °C. Insoluble material was removed by centrifugation, and the cell lysates were incubated with specific antibodies at 4 °C for a minimum of 2 h. The immune complexes were bound to protein A-Sepharose (5 mg/ml in lysis buffer) for 1 h at 4 °C. When monoclonal antibody 15-1-2 anti-internal IgG was bound to the protein A-Sepharose beads. After centrifugation, the Sepharose beads were washed three times with wash buffer (0.05% Nonidet P-40, 50 mA Tris/HCl, pH 7.4, 100 mA NaCl, 1 mA sodium fluoride, 1 mA EDTA, 1 mA Na₃VO₄, and 15% glycerol). The samples were boiled with 15% of total DNA by using standard calcium phosphate precipitates. Cells were incubated with precipitate for 18 h, washed three times with phosphate-buffered saline, and cultured in fresh medium for at least 8 h, and stimulated with IL-6 (100 units/ml) for an additional 18 h. Cell lysates were prepared and luciferase and β-galactosidase activity was measured according to manufacturers instructions (Promega). Luciferase activities were normalized to β-galactosidase activities.

Inhibition of IL-6-induced STAT1 and STAT3 Activation by PMA—The inhibition by PMA of the IL-6-induced STAT activation was studied in human hepatoma cells (HepG2). After pretreatment with 0.1 µM PMA for 45 min IL-6 (100 units/ml) was added for 15 min. Activation of STAT1 and STAT3 requires phosphorylation of specific tyrosine residues, i.e. Tyr-701 in STAT1 and Tyr-705 in STAT3. Thus, we performed Western blot analyses of nuclear extracts using specific antibodies against the tyrosine-phosphorylated forms of STAT1 and STAT3 (Fig. 1A). Tyrosine phosphorylation of both, STAT1 and STAT3, was induced by IL-6 and clearly decreased upon PMA pretreatment (Fig. 1A). PMA incubation alone did not result in STAT activation. These results confirm recent findings with respect to STAT3 (17) and show that also STAT1 activation is affected by PMA. EMSA revealed that the reduced STAT phosphorylation results in a strongly diminished binding of STAT1 and STAT3 to the STAT1/3-specific SIE probe (Fig. 1B). Inhibition of STAT activation by PMA was partially reversed by the specific MEK inhibitor PD98059. This suggests that the inhibitory effect of PMA requires the activation of MAP kinases as recently proposed (17). Inhibition was not reversed by the specific p38 kinase inhibitor SB202190 (data not shown).

We have shown that after PMA treatment the IL-6 receptor is rapidly released from the cell surface of transfected COS-7 cells and primary human monocytes by shedding, i.e. limited proteolysis of the membrane-bound receptor form (28). This raises the question whether the observed inhibition of the IL-6-induced STAT activation by PMA is due to a loss of IL-6-binding sites at the cell surface. In order to exclude this possibility, COS-7 cells, which endogenously express only gp130 but not the IL-6 receptor, were pretreated with PMA for 45 min and then stimulated with a combination of IL-6 and a recombinant soluble IL-6 receptor. The soluble IL-6 receptor has been shown to act agonistically on cells that express only gp130 (29). As shown in Fig. 1C, the inhibitory effect of PMA on the IL-6 response was also observed in COS-7 cells which suggests that shedding of the IL-6 receptor is not involved. Similar results were obtained in HepG2 cells (data not shown). Furthermore, surface expression of gp130 was not affected by PMA (25).

Time Dependence of Erk Activation by PMA—In order to analyze which members of the MAP kinase family are activated

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S. Thié and L. Graeve, unpublished observation.
by PMA treatment, HepG2 (Fig. 2A) and COS-7 (Fig. 2B) cells were treated with PMA for 0–180 min upon which total cell lysates were analyzed by Western blot analyses using antibodies specific for the activated forms of MAP kinases. These antibodies only recognize MAP kinases that are phosphorylated at threonine and tyrosine residues in a characteristic Thr-X-Tyr motif. In both cell types activated Erk2 and to a minor extent Erk1 were detected after 5 min. Maximal activation was observed at 30 min (Fig. 2, left panel). In contrast, a negligible activation of JNKs and p38 was observed. Anisomycin, a known activator of all three MAP kinase family members (Fig. 2, right panel). These data demonstrate that the activation of Erk1 and Erk2 by PMA parallels the inhibitory effect on STAT activation. Interestingly, anisomycin did not inhibit STAT activation by IL-6 (data not shown). This could be explained by the fact that after anisomycin treatment activation of Erk1/2 was less pronounced (compare left and right panels in Fig. 2, A and B). Alternatively, co-activation of JNK and p38 MAP kinases might counteract the inhibitory effect of Erk1/2. Such a possibility is supported by our recent finding that p38 activation is necessary for STAT activation upon hyperosmotic shock (30).

**Inhibition of STAT Activation by Overexpression of a Constitutively Active Raf**—Erk1 and Erk2 are activated by MAP kinase kinase-1/2 which in turn become phosphorylated by an activated Raf kinase (31). Therefore, we overexpressed a constitutively active form of Raf (Raf-BxB (21)) in COS-7 cells and studied its effect on STAT activation (Fig. 3A). In this experiment, activation of STAT homo- and heterodimers are indicated by arrows. C, COS-7 cells were pretreated with PMA (10^{-7} M) for 45 min before stimulation with IL-6 (100 units/ml) plus sgp80 (0.5 μg/ml) for 15 min. EMSAs were performed as described above.
pressing Raf-BxB (Fig. 3C). Expression of the Eg chimera with an expected molecular mass of ~68 kDa was not affected by co-expression of Raf-BxB (Fig. 3B, right panel). Thus, also activation of the Erk2 kinase by constitutively active Raf results in an inhibition of the Jak/STAT pathway.

Tyrosine Phosphorylation of Gp130 and SHP2 Is Also Inhibited by PMA—The level at which PMA inhibits the Jak/STAT pathway is not clear. Sengupta et al. (17) demonstrated a small decrease in Jak1 and Jak2 tyrosine phosphorylation upon PMA pretreatment. In order to assess whether the signal transducer gp130 is also affected in its tyrosine phosphorylation we pretreated HepG2 cells with PMA for 45 min and then stimulated them with IL-6 for 15 min. Cell lysates were prepared and immunoprecipitations with anti-gp130 (A) or anti-SHP2 (B) antibodies were performed as described under “Experimental Procedures.” Precipitated proteins were separated by 10% SDS-PAGE, blotted onto a PVDF membrane, and analyzed by immunodetection with a specific anti-phosphotyrosine antibody (4G10) (upper panel). Blots were stripped and reprobed with anti-gp130 or anti-SHP2 antibodies (lower panel) for verification of equal loading. A, the resulting autoradiograph was scanned and quantitated using the PhosphorImager (Storm 840, Molecular Dynamics, CA).

Similar experiments were performed to investigate the tyrosine phosphorylation status of the phosphatase SHP2 which is known to contribute to the IL-6-induced signaling although its specific function remains to be elucidated (7, 32). SHP2 was proposed to be a gp130-associated adaptor connecting the signal transducer to Grb2 thereby linking the Jak/STAT to the MAP kinase pathway (8). We have recently shown that tyrosine phosphorylation of SHP2 is increased in response to IL-6 in a Jak1-dependent manner (7). PMA treatment of HepG2 cells resulted in a time-dependent decrease (maximal inhibition after 60 min) of the IL-6-induced SHP2 tyrosine phosphorylation (Fig. 4B). Together with the data of Sengupta et al. (17) these findings show that tyrosine phosphorylation of all components
of the Jak/STAT cascade (gp130, Jaks, SHP2, and STATs) is affected by PMA.

Role of the Cytoplasmic Part of Gp130—Activation of STATs and SHP2 requires prior phosphorylation of tyrosine residues within the cytoplasmic domain of gp130 which then recruit these proteins via their intrinsic SH2 domains (4, 5). To analyze the importance of specific tyrosine residues of the cytoplasmic part of gp130 for the PMA effect, we again made use of Eg chimeric receptor mutants (5) (Fig. 5A). Stimulation of COS-7 cells transfected with the wild-type Eg-chimera led to the activation of STAT1 which is diminished upon preincubation with PMA (Fig. 5B, first panel). Activation of STAT3 is consistently not observed in these cells unless overexpressed (5). A chimeric construct EgY440 containing only the Jak-binding sites (box 1 and box 2) of gp130 and the tyrosine module YDKPH of the interferon γ receptor crucial for the activation of STAT1 via interferon γ, however, resulted in a STAT1 activation that was resistant to pretreatment with PMA (Fig. 5B, fourth panel). This suggests that the activity of Jak kinases is not directly affected by PMA treatment and that sequences downstream of box 1 and box 2 of the cytoplasmic tail of gp130 are necessary for the inhibitory effect of PMA. To further analyze the role of specific tyrosine residues within the cytoplasmic part of gp130 we took advantage of the EgY759F chimera, in which phenylalanine is substituted for Tyr-759 of gp130 (7). Tyr-759 is required for the recruitment and activation of SHP2 (4). Epo-induced STAT1 activation was not inhibited by PMA in cells expressing EgY759F (Fig. 5B, second panel). In contrast, substitution of phenylalanine for Tyr-683 (EgY683F) did not affect the inhibitory effect induced by PMA (Fig. 5B, right panel). This finding suggests that Tyr-759 within the cytoplasmic part of gp130 plays an essential role in the inhibition of STAT activation by PMA.

Inhibition of IL-6-induced STAT Activation by bFGF—The FGF receptor as a receptor tyrosine kinase is known to activate the MAP kinase cascade upon FGF binding (18). To examine also whether this physiological stimulus leads to an inhibitory effect on STAT signaling, we studied NIH-3T3 cells which endogenously express FGF receptors. Upon stimulation with bFGF we detected Erk2 activation after 15 min which was transient and diminished after 30 min (Fig. 6A). Activation of JNKs and p38 was not observed (data not shown). Incubation of NIH-3T3 cells with bFGF or PMA prior to stimulation with IL-6/sgp80 resulted in an inhibition of IL-6-induced STAT activation (Fig. 6B). Also in the case of bFGF, the observed inhibition was sensitive to the MEK inhibitor PD98059 (Fig. 6C).

mRNA and Protein Synthesis Are Crucial for STAT Inhibition by PMA and bFGF—In order to determine whether the inhibitory effect on STAT activation of PMA or bFGF necessitates de novo mRNA and protein synthesis we preincubated HepG2 and NIH-3T3 cells with actinomycin D or cycloheximide, inhibitors of mRNA or protein synthesis, respectively. Cells pretreated with actinomycin D (Fig. 7A) or cycloheximide (Fig. 7B) showed a partial reversal of the inhibitory PMA/bFGF effect indicating that this inhibitory mechanism is, at least in part, mediated by the expression of newly induced genes.

PMA and bFGF Induce SOCS-3 Gene Expression—Recently, it was reported that IL-6 stimulation results in the induction of SOCS-3 gene expression, a feedback inhibitor of the Jak/STAT pathway (11). In order to study whether SOCS-3 might play a role in the inhibition of IL-6 signaling by PMA and bFGF, Northern blot analyses were performed with RNA from NIH-3T3 cells stimulated with IL-6/sgp80, PMA, or bFGF for 45 min. As shown in Fig. 8A, not only IL-6/sgp80 but also PMA and bFGF induce the expression of SOCS-3 mRNA. No increase of SOCS-1 mRNA could be detected (data not shown). To analyze whether the SOCS-3 induction by PMA and bFGF is sensitive to inhibition of Erk activation we again made use of the MEK inhibitor PD98059. NIH-3T3 cells were pretreated with PD98059 for 45 min before stimulation with PMA or bFGF for an additional 45 min and Northern blot analyses were performed. The induction of SOCS-3 mRNA by PMA and bFGF was largely reversed upon pretreatment with the MEK inhibitor (Fig. 8B).

DISCUSSION

This study presents a number of novel findings with respect to the molecular mechanism of PMA-induced and Erk-dependent down-regulation of the Jak/STAT pathway. First, a physio-

FIG. 5. Role of the cytoplasmic part of gp130. A, COS-7 cells were transiently transfected as described under “Experimental Procedures” with expression vectors for the chimeric receptors depicted. B, cells were preincubated with PMA (10−7 M) for 45 min followed by stimulation with Epo (7 units/ml) for 15 min. Nuclear extracts were prepared and EMSAs performed as described in the legend to Fig. 1.
ological activator of MAP kinases, namely bFGF, also results in a down-modulation of STAT activity. Second, transcription and translation are at least in part essential for the inhibition of the IL-6-induced STAT activation by PMA/bFGF. Third, both PMA and bFGF rapidly induce SOCS-3 mRNA expression in a MEK-dependent manner. And finally, the PMA-induced inhibition is dependent on Tyr-759 of the cytoplasmic part of the IL-6 signal transducer gp130.

Our findings strongly suggest that prestimulation of cells with activators of MAP kinases such as PMA or bFGF inhibits a subsequent STAT activation by IL-6 via the rapid induction of the negative feedback inhibitor SOCS-3. Such a model is consistent with the dependence of the inhibitory effect on de novo transcription and protein synthesis. It is, furthermore, supported by the fact that both, the inhibition of STAT activation and the induction of SOCS-3, are largely reversed by the action of the MEK1 inhibitor PD98059.

Recently, we could demonstrate in monocytic cells that activators of the MAP kinase p38, namely lipopolysaccharide and tumor necrosis factor-α, inhibit a subsequent STAT activation by IL-6 (33). This inhibition was also paralleled by a rapid SOCS-3 induction which in this case was sensitive to inhibition of p38. Interestingly, in rat hepatocytes and HepG2 cells tumor necrosis factor-α neither down-modulate STAT activation nor induce SOCS-3. Thus, in all experimental systems in which activation of MAP kinases resulted in an inhibition of the Jak/STAT pathway, SOCS-3 induction was observed. Although this is only a correlation, it strongly argues for a crucial role of SOCS-3 as a mediator of MAP kinase-dependent Jak/STAT inhibition. Final proof of our model would require the specific inhibition of SOCS-3 induction by antisense technology or gene knock-out. Unfortunately, SOCS-3 knock-out mice die at days 12–16 of embryonic development (34).

The murine SOCS-3 promoter has recently been cloned and a STAT1/3-binding site was identified and shown to be necessary for the induction of SOCS-3 by leukemia inhibitory factor (35). Our data (this study and Ref. 33) as well as recent results from other groups (36, 37) suggest also that binding sites for MAP kinase-activated transcription factors such as TCF and Elk-1 (38) must exist within the SOCS-3 regulatory region.
NIH-3T3 cells were preincubated with PD98059 (10 μM) and dephospho GAPDH (GAPDH) as a loading control.

AbFGF treatment.

pition of the IL-6-induced STAT activation.

which do not activate STATs (Fig. 8A).

IL-6 is a more potent inducer of SOCS-3 than PMA or AbFGF.

kinase-dependent transcription factors. This could explain why

due to the synergistic effect of activated STATs and MAP

imply that induction of SOCS-3 mRNA expression by IL-6 is

clear. SOCS-1 was found to bind to phosphorylated Tyr-1007 in

PMA/bFGF, exerts its inhibitory effect by binding to phospho-

X

PQ, respectively, the YSTV motif of Tyr-759 was described

to bind the SH2 domain of SOCS-3 (40). This strongly

suggests that SOCS-3, whether overexpressed or induced by

PMA/bFGF, exerts its inhibitory effect by binding to phospho-

Y

molecules. Whereas STAT3 and STAT1 are recruited to phos-

Y

photyrosine residues with the consensus sequence YYXX and

been demonstrated to act as recruitment sites for signaling

gp130? Tyr-759 is one of the five tyrosine residues which have

been demonstrated to act as recruitment sites for signaling

molecules. Whereas STAT3 and STAT1 are recruited to phos-

phosphotyrosine residues with the consensus sequence YYXX and

YXXQ, respectively, the YSTV motif of Tyr-759 was described

to bind the SH2 domain containing tyrosine phosphatase SHP2

(4, 5). However, in our study the tyrosine phosphorylation of

SHP2, which is believed to increase its catalytic activity (39), is

also inhibited by PMA making an involvement of SHP2 in our

scenario unlikely. Schmitz et al. (40) recently demonstrated

that Tyr-759 of gp130 is necessary for the inhibitory effect of

overexpressed SOCS-3 on the STAT-responsive α5-macroglobu-

lin promoter in HepG2 cells. They could further show that

SOCS-3 protein in vitro binds to a phosphorylated Tyr-759

containing peptide and that a recombinant SH2 domain of

SOCS-3 interacts with this phosphopeptide (40). This strongly

suggests that SOCS-3, whether overexpressed or induced by

PMA/bFGF, exerts its inhibitory effect by binding to phospho-

ylated Tyr-759 of gp130. How SOCS-3 blocks the activity of

Jaks and the phosphorylation of STATs remains unclear.

SOC-1 was found to bind to phosphorylated Tyr-1007 in

the activation loop of Jak2 (41). The N-terminal located kinase

inhibitory region of SOCS-1 also contributed to this binding

and was essential for Jak2 kinase inhibitory activity. A similar

mechanism was proposed for SOCS-3. However, compared with

SOC-1, the affinity of its SH2 domain for Jak2 is weaker

whereas that of its kinase inhibitory region is stronger (42).

Our model suggests that SOCS-3 can exert its inhibitory effect

not only by binding directly to Jaks but also by binding to the

receptor chain (Fig. 9). Future studies will address this ques-

tion in detail.

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Overexpression of c-Jun and c-Fos had no effect, suggesting that
transcription factors different from AP-1 are involved in this
induction. Since IL-6 does not only activate the Jak/STAT
pathway but also the MAP kinase pathway our results further imply that induction of SOCS-3 mRNA expression by IL-6 is
due to the synergistic effect of activated STATs and MAP
kinase-dependent transcription factors. This could explain why
IL-6 is a more potent inducer of SOCS-3 than PMA or AbFGF
which do not activate STATs (Fig. 8A). It should be pointed out

3 P. Gatsios, unpublished observation.
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