Molecular analysis and biological implications of STAT3 signal transduction

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Chapter 2

IL-6-induced STAT3 transactivation and ser727 phosphorylation involves Vav, Rac-1 and SEK-1/MKK-4 as signal transduction components

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Summary

In the present study, STAT3 serine 727 (ser727) phosphorylation and transactivation was investigated in relation to activation of MAP kinase family members including ERK-1, JNK-1 and p38 in response to IL-6 stimulation. Although IL-6 can activate ERK-1 in HepG2 cells, STAT3 transactivation and ser727 phosphorylation were not reduced by using the MEK inhibitor PD98059 or by overexpression of dominant-negative Raf. IL-6 did not activate JNK-1 in HepG2 cells and STAT3 was a poor substrate for JNK-1 activated by anisomycin, excluding a role of JNK1 in IL-6-induced STAT3 activation. However, SEK-1/MKK-4 was activated in response to IL-6 and overexpression of dominant-negative SEK-1/MKK-4(A-L) reduced both IL-6-induced STAT3 ser727 phosphorylation as well as STAT3 transactivation. Subsequently, the SEK-1/MKK-4 upstream components Vav, Rac-1 and MEKK were identified as components of a signal transduction cascade that leads to STAT3 transactivation in response to IL-6 stimulation. Furthermore, inhibition of p38 kinase activity with the inhibitor SB203580 did not block STAT3 ser727 phosphorylation but rather increased both basal as well as IL-6-induced STAT3 transactivation, indicating that p38 may act as a negative regulator of IL-6-induced STAT3 transactivation through a presently unknown mechanism. In conclusion, these data indicate that IL-6-induced STAT3 transactivation and ser727 phosphorylation is independent of ERK-1 or JNK-1 activity, but involves a gp130 receptor-signaling cascade that includes Vav, Rac-1, MEKK and SEK-1/MKK-4 as signal transduction components.

Introduction

Interleukin-6 (IL-6) is a pleiotropic cytokine that mediates a variety of functions in different cells and tissues including proliferation and differentiation of hematopoietic cells, induction of the acute phase response in liver cells and inflammation at sites of tissue injury [72-74]. IL-6 initiates its action by binding to its receptor that is composed of two subunits: an 80-kDa IL-6 binding protein and a 130-kDa transmembrane signal transducing component (gp130) [12,192,193].The gp130 receptor protein is also used by other members of the IL-6 cytokine family, including IL-11, Oncostatin M (OnM), Leukemia Inhibitory Factor (LIF), and Ciliary Neurotrophic Factor (CNTF) [14,194,195]. Activation of IL-6 signal transduction involves gp130 dimerization, ligand-dependent tyrosine phosphorylation of the gp130 associated protein-tyrosine kinases Jak1, Jak2, and Tyk2, as well as tyrosine phosphorylation of Signal Transducer and Activator of Transcription 3 (STAT3) [1]. Tyrosine phosphorylation of STAT3 occurs at a single residue (tyr705) which is located in a conserved SH2 domain allowing homodimerization as well as heterodimerization with other STAT family members, nuclear translocation and transcription activation following binding to specific consensus sequences of target gene promoters [1]. In addition to tyrosine phosphorylation, STAT3 is serine phosphorylated at a single residue (ser727) in response to IL-6 as well as other extracellular factors including interferon-γ (IFN-γ) and epidermal growth factor (EGF) [61,196]. Ser727 phosphorylation has been shown to enhance STAT3 transcriptional activation [196].

At present, the signaling events downstream of the gp130 receptor have not been fully explored and the kinase responsible for IL-6-induced STAT3 ser727 phosphorylation has not been identified. MAP kinases consist of a family of serine/threonine kinases, positioned at the end-point of signal transduction cascades initiated at the plasma

membrane by ligand-receptor interaction [197]. Several mammalian MAP kinases have been identified, including extracellular signal-regulated protein kinase (ERK) [198], c-Jun N-terminal protein kinase or stress activated protein kinase (SAPK/JNK) [199,200], and p38 [201,202]. The activation of ERKs involves a signal transduction pathway that includes Ras, Raf-1 and MEK downstream of receptor tyrosine kinase receptors [203-205]. JNKs are activated by "stress stimuli" involving a signal transduction pathway that contains Ras, Rac-1, MEKK and SEK-1/MKK-4 [206-212]. The signaling pathway that leads to the activation of p38 is not fully elucidated, but MKK3 and MKK6 have been identified as MAP kinase kinases (MKKs) for p38 [213,214]. Upon Activation, MAP kinases rapidly translocate to the nucleus and have been shown to phosphorylate several transcription factors including Elk that is phosphorylated by ERK-1 [215,216]and c-Jun, ATF-2 and Sap-1 that are substrates for activated JNK [216-218].

Although signaling events downstream of the gp130 receptor have not fully been elucidated, gp130 has been shown to interact with Vav [34], a 95-kDa proto-oncogene product that is expressed in hematopoietic cells and trophoblasts [219,220]. Vav contains an array of structural motives, including zinc finger, leucine zipper, pleckstrin homology, Dbl homology and SH2 and SH3 domains [219,221]. In addition, Vav has been identified as a substrate for receptor- and non-receptor tyrosine kinases while tyrosine phosphorylated Vav has recently been shown to catalyze GDP/GTP exchange on Rac-1, which leads to activation of JNK [34,222,223].

In the present study, we have investigated the involvement of the ERK, JNK and p38 signaling pathways in IL-6-induced STAT3 ser727 phosphorylation and transactivation. Our results demonstrate that STAT3 ser727 is not a direct target for phosphorylation by ERK, JNK or p38. However, IL-6-induced STAT3 ser727 phosphorylation involves activated SEK-1/MKK-4 as well as Vav, Rac and MEKK as gp130 receptor downstream components. The uncoupling between SEK-1/MKK-4 and JNK activation in response to IL-6 as well as the role of p38 in this process will be discussed.

Materials and methods

Cell culture, reagents and antibodies

The human hepatoma cell line, HepG2, was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS, Integro B.V., Zaandam, The Netherlands). Cells were stimulated with 25-ng/ml human recombinant IL-6 (generous gift from Dr. S.C. Clark, Genetics Institute, Cambridge, USA), 100 ng/ml TPA (Sigma), or 100 ng/ml Anisomycin (Sigma). The MEK inhibitor PD98059 (Santa Cruz) and the p38 inhibitor SB203580 (a gift from Dr. J.C. Lee, SmithKline Beecham Pharmaceuticals, King of Prussia, PA, USA) were used at final concentrations of 20 μM unless stated otherwise. Antibodies against hemagglutinin (HA), STAT3, Vav, Rac-1, MKK-4, p38, JNK-1 and ERK-1 (Santa Cruz) and c-myc (9E10, Boehringer Mannheim, Corp.) were used in dilutions of 1:4000, unless stated otherwise. Antibodies against phosphorylated SEK-1/MKK-4(Thr223), STAT3(Tyr705) and STAT3(Ser727) were obtained from New England Biolabs and used in a 1:1000 dilution.

Expression and reporter constructs

The following plasmids were used: pIRE LUC containing two copies of the IL-6 response element (pIRE) of the ICAM-1 promoter in front of the Herpes simplex virus thymidine

kinase promoter and the Luciferase gene [224]; pSG5-STAT3 which expresses STAT3 from the SV40 promoter; pSG5-STAT3β, which expresses a dominant negative isoform of STAT3 lacking the 55 C-terminal amino-acid residues; pSG5-STAT3 ser727ala, which expressed a mutant STAT3 in which the ser727 is replaced by alanine; pCS2+-RacN17 and pCS2+-RacV12 expressing the dominant negative and constitutive active mutant variants of Rac; pSEK-1/MKK-4(A-L) which expresses dominant negative SEK-1/MKK-4 [225]; pCDNA3-MEKKΔ(K432M) which expresses a dominant negative MEKK [226]; pCDNA3-HA-JNK-1 which expresses hemagglutinin tagged JNK-1 (p46); pGEX-1-c-jun (1-135) expressing GST-c-Jun; and the dominant negative and constitutive active mutants pEF-myc-Vav-C [227] and pMEX-myc-Vav-A(Δ1-65) [222,228]. The expression vector encoding a dominant negative mutant of Raf kinase, NΔRaf, was provided by Dr. P. Coffer (Department of Pulmonary diseases, University Hospital Utecht, Utrecht, The Netherlands) [229]. The pGEX-STAT3 (379-770) expression vector was cloned by inserting the *Bam*HI-*Bgl*II(blunt) pSG5-STAT3 fragment into the *Bam*HI and *Sma*I sites of pGEX-4t1.

Transient transfections

HepG2 cells were seeded at $3x10^5$ cells per well in 6-well plates (Costar), and 24 hours later cells were transfected with 10 μg plasmid DNA using the calcium phosphate co-precipitation method [230]. Transfection mixtures consisted of a mixture of 3 μg pIRE LUC reporter, 3 μg pDM2-LacZ as a control to determine transfection efficiency, and 1-4 μg of expression plasmids for dominant negative or constitutive active signal transduction components as mentioned in the results section. When necessary, pUC18 was added to the transfection mixture to obtain a total of 10 μg of DNA. Cells were incubated with precipitate for 24 hours, washed with phosphate buffered saline (PBS), and stimulated for an additional 24 hours. Cells were collected in 200 μl reporter lysis buffer (Promega) and subjected to the assays for luciferase [231] and β-galactosidase [232] as previously described. The data represent two independent experiments using different batches of DNA, and in each experiment transient transfections were performed in triplicate. Standard deviations were calculated using Sigmaplot (Jandel Corp.).

SDS-polyacrylamide gel electrophoresis, western blotting, immunoprecipitations, and kinase assays

A total of $1x10^7$ cells were lysed on ice in lysis buffer (20 mM HEPES pH 7.4, 2 mM EGTA, 1 mM DTT, 1 mM Na_2VO_3 (ortho), 1% Triton X100, 10% glycerol, 10 μg/ml leupeptin, and 0.4 mM PMSF). Prior to SDS- polyacrylamide gel electrophoresis and immunoprecipitations, protein concentrations were determined (Biorad), and equal amounts were used in the experiments. Whole-cell extracts were boiled for 5 min. in the presence of Laemmli sample buffer prior to separation on 12.5% SDS-polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (Millipore) in Tris-glycine buffer at 100 Volts for 1.5 h using an electroblotter (Pharmacia). Membranes were blocked with PBS buffer containing 5% non-fat milk prior to incubation with antibodies. Binding of each antibody was detected by chemiluminesence using ECL according to the manufacturer's recommendations (Amersham Corp.). For immunoprecipitations, whole cell lysates were incubated with anti-Vav or anti-HA antibodies, precipitated with Protein-A Sepharose beads (Pharmacia), and washed two times with lysis buffer. The precipitates were boiled for 5 min in Laemmli sample buffer and subjected to 12.5% SDS-

polyacrylamide gel electrophoresis. For kinase assays, JNK-1 was precipitated with Protein-A Sepharose beads, washed three times with lysis buffer, two times with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl pH 7.6, 0.1% Triton X-100, and 1 mM DTT), and three times with Assay buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM $MgCl_2$, 1mM DTT, and 0.1% Triton X-100). The precipitates were incubated for 30 min. in 43.5 µl Assay buffer, 20 mM $MgCl_2$, 25 µM ATP, 10 µCi γ-32PATP, and 5 µg GST-c-Jun (1-135), 5 µg Myelin Basic Protein (MBP, Santa Cruz) or 5 µg GST-STAT3 (379-770). Beads were boiled in SDS-sample buffer for 5 min and separated on a 12.5% SDS-polyacrylamide gel. The gel was dried, and phosphorylation of the GST substrates was detected by autoradiography.

Results

IL-6-induced STAT3 ser727 phosphorylation is not mediated by ERK-1 or JNK-1. Previously, it has been demonstrated that activation of STAT3 in response to IL-6 is dependent on phosphorylation of both tyrosine and serine amino acid residues [1,61,196]. STAT3 serine phosphorylation involves a single residue, ser727, that is located at the extreme C-terminus. To study the kinetics of STAT3 phosphorylation, HepG2 cells were stimulated with IL-6 and tyr705 and ser727 phosphorylation was examined by Western blotting using STAT3 phospho-specific antibodies. STAT3 ser727 phosphorylation was observed within 10 minutes upon IL-6 stimulation of HepG2 cells, whereas STAT3 tyr705 phosphorylation was observed within 5 min (Fig.1A). Overexpression of STAT3β or a STAT ser727ala mutant strongly decreased transactivation of the pIRE LUC reporter, which contained two copies of the IL-6 response element (IRE) of the ICAM-1 promoter (Fig.1B). This indicates that specific phosphorylation on the ser727 residue STAT3 is important for maximal STAT3 transcriptional activation.

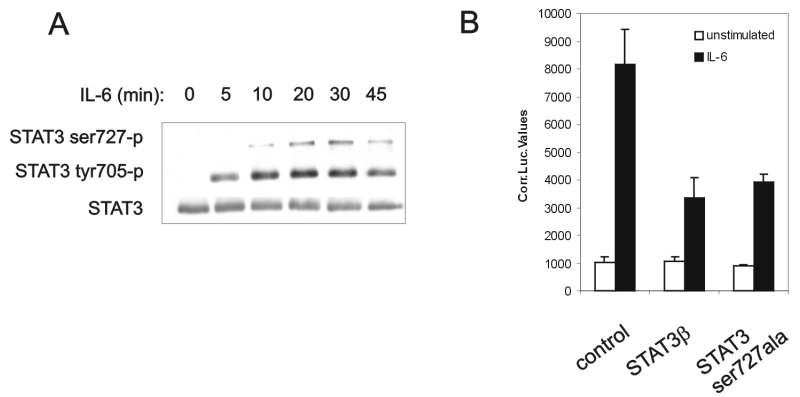

Figure 1. IL-6-induced STAT3 transactivation is dependent on ser727 phosphorylation. A, $3x10^6$ HepG2 cells were grown on 92-mm petri dishes, serum-starved overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 for varying periods. Equal amounts of whole cell lysates were Western blotted using antibodies against STAT3 and phosphorylated STAT3 ser727 and tyr705. **B,** $3x10^5$ HepG2 cells were transfected with 3 µg pIRE LUC reporter, 3 µg pDM2LacZ and 4 µg pSG5-STAT3β or pSG5-STAT3 ser727ala as indicated. Cells were either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 hrs before harvest.

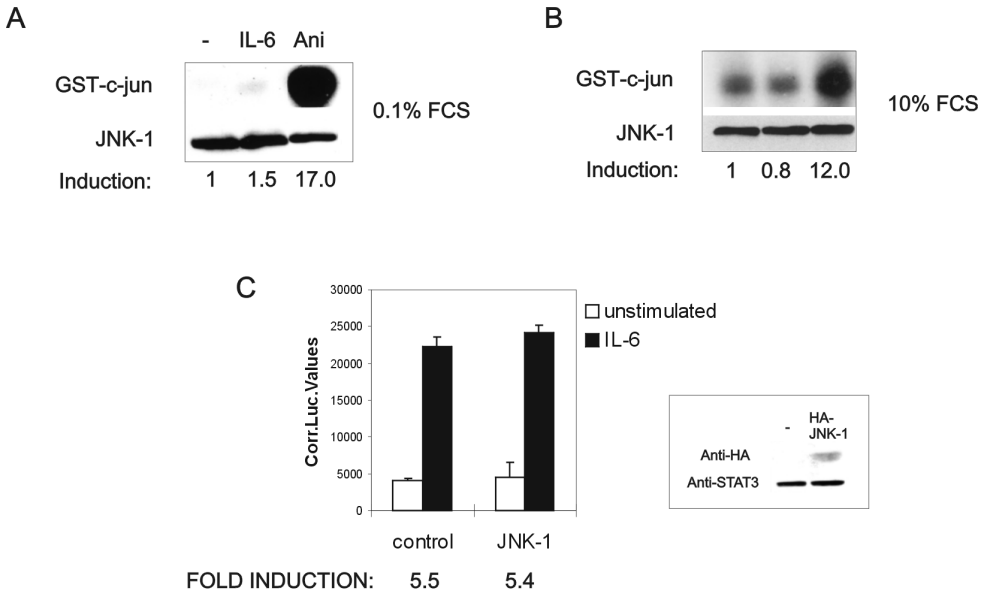

Figure 2. IL-6 does not activate JNK-1 in HepG2 cells. A, $3x10^6$ HepG2 cells were grown on 92-mm petri dishes, serum-starved overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 or 100 ng/ml Anisomycin as indicated. Cell lysates were subjected to immunoprecipitation using anti-JNK1 anti-bodies and Protein-A sepharose beads, and precipitates were used in *in vitro* kinase assays using 5 µg GST-c-Jun (1-135) as a substrate as described in Materials and Methods. As a control, the same amounts of protein used in the kinase assay were subjected to Western blotting and probed with anti-JNK-1 antibody. **B,** *In vitro* kinase assay as in **A**, but cells were not serum starved but grown on 10% FCS. **C,** $3x10^5$ HepG2 cells were transfected with 3 µg pIRE LUC reporter, 3 µg pDM2LacZ, and 4 µg pUC or pcDNA3- JNK-1 expressing the wild-type JNK-1 isoform p46. Cells were either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 hrs until harvest. *Inset:* lysates obtained in the transient transfection assay were Western blotted and overexpressed HA-JNK-1 was visualized using anti-HA antibodies. As a control, blots were stripped and reprobed with anti-STAT3. (-) indicates cells without overexpressed HA-JNK-1.

Since ser727 is located in a conserved Pro-X-Ser/Thr-Pro site, which has been accepted as a phosphorylation site for MAP kinases [84], the involvement of the ERK, JNK and p38 MAP kinases in STAT3 serine727 phosphorylation was investigated. Previous reports have indicated that IL-6-induced STAT3 ser727 phosphorylation is an ERK-1 independent process [82], and we have been able to confim these results in HepG2 cells. Blocking ERK activation by overexpression of dominant negative Raf, NΔRaf, or by using the MEK inhibitor PD09859 did not alter STAT3 ser727 phosphorylation or transactivation (data not shown). To determine the role of JNK-1 in IL-6-induced STAT3 ser727 phosphorylation, JNK-1 was immunoprecipitated from IL-6-induced HepG2 cells and its activity was determined by *in vitro* kinase assays using GST-c-Jun(1-135) as a substrate. JNK was activated only slightly by IL-6 in serum starved HepG2 cells, whereas anisomycin strongly increased JNK activity under the same conditions (Fig.2A). In HepG2 cells cultured in 10% FCS, IL-6 did not induce JNK activity over a high basal level activity (Fig.2B). Overexpression of the wild-type p46 isoform of JNK-1 did not alter transactivation of the pIRE LUC reporter, indicating that increased expression of JNK-1

does not result in increased levels of STAT transactivation (Fig.2C). In addition, using GST-STAT3 (379-770) as a substrate for immunoprecipitated JNK-1 or ERK-1 from IL-6 or TPA stimulated HepG2 cells, no phosphorylation of STAT3 was detected (data not shown). Taken together, these results strongly suggest that neither JNK-1 nor ERK-1 are responsible for IL-6-induced STAT3 ser727 phosphorylation and hence STAT3-dependent pIRE LUC reporter activation.

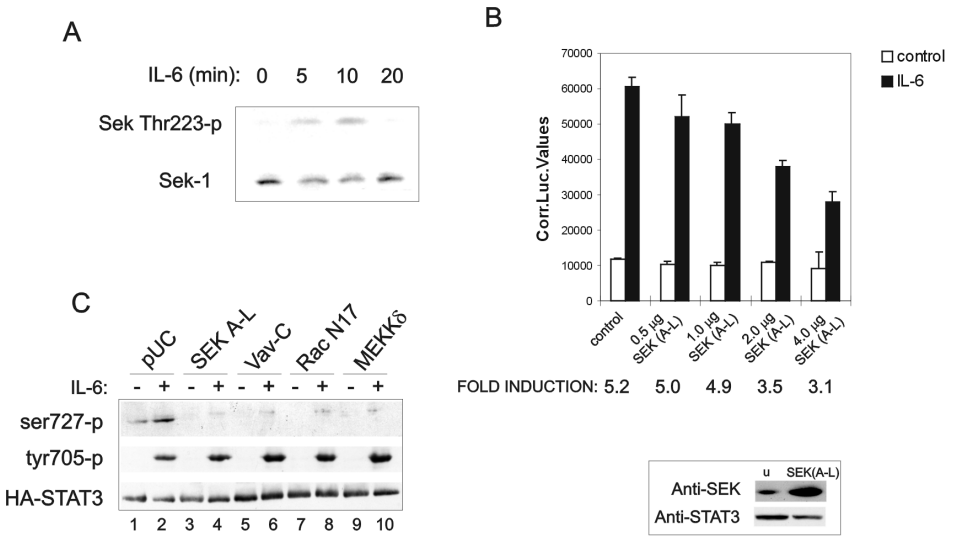

Figure 3. SEK-1/MKK-4 is involved in IL-6-induced STAT3 transactivation and ser727 phosphorylation. A, $3x10^6$ HepG2 cells were grown on 92-mm petri dishes, serum-starved overnight in medium containing 0.5% FCS, and stimulated with 25 ng/ml IL-6 for varying periods. Whole cell lysates were Western blotted using antibodies against phosphorylated SEK-1/MKK-4 (Thr223). As a control, blots were stripped and reprobed with anti-SEK-1/MKK-4 antibodies. **B,** $3x10^5$ HepG2 cells were transfected with 3 µg pIRE LUC reporter, 3 µg pDM2LacZ, and 1-4 µg dominant negative SEK-1/MKK-4 as indicated. Cells were either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 hrs before harvest. *Inset:* lysates obtained in the transient transfection assay (4.0 µg SEK-1/MKK-4(A-L)) were Western blotted and overexpressed proteins were visualized using anti-SEK-1/MKK-4 antibodies. Endogenous SEK-1/MKK-4 is detectable in untransfected cells (u), but the overexpression is clearly visible. As a control, blots were stripped and reprobed with anti-STAT3. **C,** $3x10^6$ cells were grown on 92-mm petri dishes and were transfected with 25 µg expression vectors for HA-STAT3 and 25 µg pUC18 (lanes 1,2) or 25 µg dominant negative SEK-1/MKK-4(A-L) (lanes 3,4), Vav-C (lanes 5,6), Rac-N17 (lanes 7,8), or MEKKΔ(K432M) (lanes 9,10). After overnight incubation with the DNA precipitate cells were washed and stimulated with 25 ng/ml IL-6 for 15 min. HA-STAT3 was immunoprecipitated using anti-HA antibody and precipitates were Western blotted using antibodies against phosphorylated STAT3 (ser727 and tyr705). As a control, blots were stripped and reprobed with anti-HA anti-bodies.

IL-6 activates SEK-1/MKK-4, which mediates IL-6-induced STAT3 transactivation. Although JNK-1 is not activated in response to IL-6, we further investigated the involvement of the (SAPK)/JNK pathway in IL-6-induced STAT3 transactivation. SEK-1/MKK-4 is located upstream of JNK-1 and its activation upon IL-6 stimulation was determined. IL-6-induced SEK-1/MKK-4 (Thr223) phosphorylation within 5 min as determined by Western blotting using specific SEK-1/MKK-4 phospho-threonine antibodies (Fig.3A). Maximal SEK-1/MKK-4 phosphorylation was detected at 10 min and

phosphorylation decreased to undetectable levels upon 20 min of IL-6 stimulation (Fig.3A). Since SEK-1/MKK-4 is activated in response to IL-6, the effect of over-expression of a dominant-negative mutant of SEK-1/MKK-4(A-L) [225] on the pIRE LUC reporter activity was investigated in a transient transfection assay. Overexpression of SEK-1/MKK-4(A-L) decreased STAT3 transactivation in a dose- dependent manner from 5.2 ± 0.2 to 3.1 ± 0.3 fold (Fig.3B). To study the effect of dominant-negative SEK-1/MKK-4 on IL-6-induced STAT3 ser727 phosphorylation, HA-tagged STAT3 was transiently transfected together with SEK-1/MKK-4(A-L) or pUC as a control in HepG2 cells and HA- STAT3 was immunoprecipitated from cell lysates using anti-HA antibodies and Western blotted. Co-expression of SEK-1/MKK-4(A-L) deceased both basal as well as IL-6-induced STAT3 ser727 phosphorylation while STAT3 tyr705 phosphorylation was unaffected (Fig.3C, lanes 1-4). These results demonstrate that SEK-1/MKK-4 is activated in HepG2 in response to IL-6 and indicate that activation of SEK-1/MKK-4 is important in IL-6-induced STAT3 transactivation and ser727 phosphorylation.

The SEK-1/MKK-4 upstream components Vav, Rac-1 and MEKK-1 are involved in IL-6-induced STAT3 transactivation. Previously, it has been shown that Vav associates with the gp130 receptor upon IL-6 stimulation [34]. Since Vav is capable of activating Rac-1 [223] and Rac-1 is a known activator of the JNK pathway [206,211,222], the involvement of Vav and Rac-1 in IL-6-induced STAT3 transactivation and ser727 phosphorylation was investigated. Vav tyrosine phosphorylation was increased from undetectable levels in unstimulated HepG2 cells to maximal levels after 10 min of IL-6 stimulation (Fig.4A). Furthermore, transient cotransfection of the pIRE LUC reporter together with a vector expressing dominant negative Vav-C decreased IL-6-induced STAT3 reporter transactivation from 7.7 ± 0.6 to 3.5 ± 0.2 fold, while constitutive active Vav-A (Δ1-65) increased IL-6-induced STAT3 transactivation from 7.7 ± 0.6 to 10.4 ± 0.1 fold (Fig.4B). These results indicate that Vav acts downstream of the gp130 receptor in IL-6-induced STAT3 transactivation. To investigate the association between Vav and Rac-1 upon IL-6 stimulation, Vav was immunoprecipitated from HepG2 cell lysates and Vav-associated proteins were analyzed by Western blotting. Rac-1 transiently coprecipitates with Vav, which is maximal after 5 min and is followed by a quick release of Rac-1 that is complete after 20 min (Fig.4A). Since Vav acts as a GDP/GTP exchange factor for Rac-1, by releasing it in the active GTP-bound form, we investigated the effects of constitutive-active and dominant-negative Rac-1 variants on IL-6-induced STAT3 transactivation. IL-6-induced STAT3 transactivation of the pIRE LUC reporter was reduced in a dose dependent manner by overexpression of dominant-negative RacN17 from 7.9 ± 0.7 to 4.6 ± 0.2 fold (Fig.4C). As expected, constitutive-active RacV12 strongly increased both basal as well as IL-6-induced STAT3-dependent activation of the pIRE LUC reporter (Fig.4C).
To further investigate the involvement of SEK-1/MKK-4 in IL-6-induced STAT3 transactivation, the influence of SEK-1/MKK-4 upstream kinases on STAT3 transactivation was studied. Since MEKK-1 has been shown to be an upstream activator of SEK-1/MKK-4 in many cell types, a dominant negative mutant MEKKΔ(K432M) was expressed in HepG2 cells together with the pIRE LUC reporter [226]. Overexpression of dominant-negative MEKK-1 strongly reduced IRE transactivation from 7.9 ± 0.7 to 3.5 ± 0.1 fold (Fig. 4C). Furthermore, the increased IRE transactivation by overexpression of RacV12 could be blocked by overexpression of dominant negative MEKKΔ(K432M) or

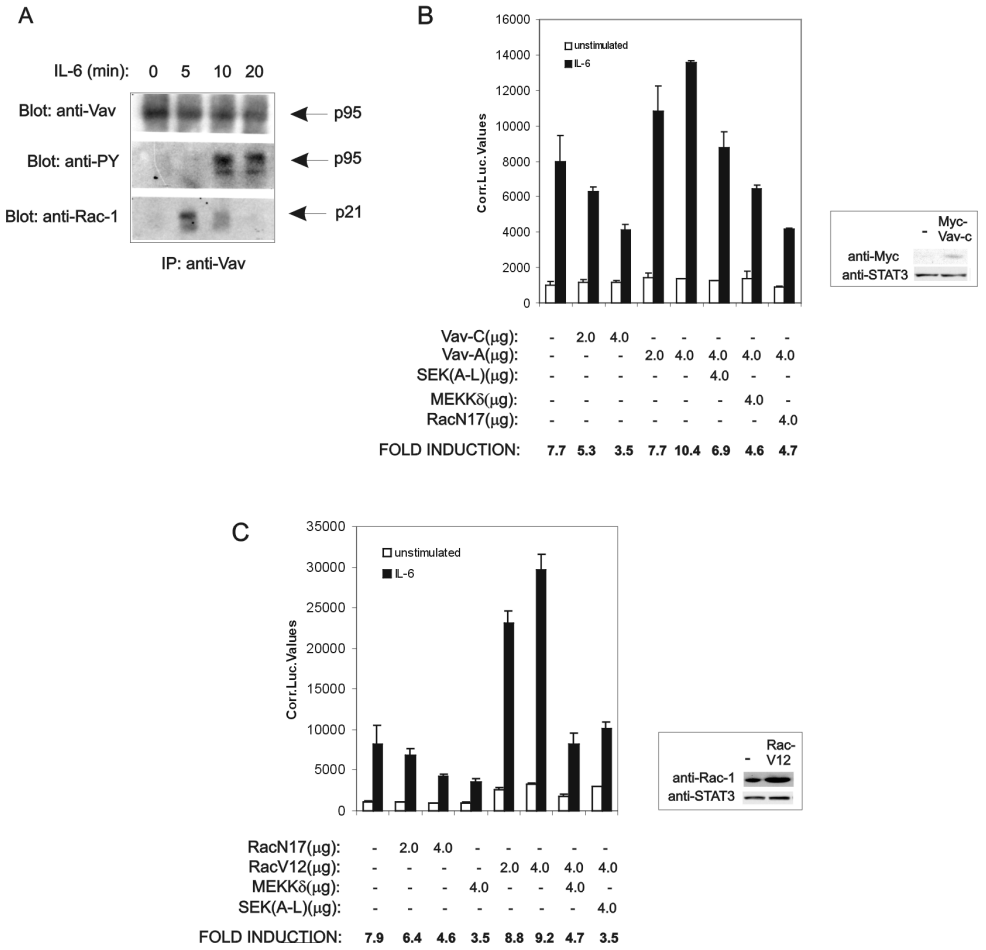

Figure 4. The SEK-1/MKK-4 upstream components Vav, Rac and MEKK are involved in IL-6-induced STAT3 transactivation and ser727 phosphorylation. A, 10^7 cells were grown on 150-mm petri dishes, serum-starved overnight in medium containing 0.5% FCS, and stimulated with 25 ng/ml IL-6 for varying periods. Cells were lysed, protein concentrations were determined, and equal amounts were used in each experiment. Vav immunoprecipitates were separated on SDS-PAGE and blotted with anti-Vav, anti-phosphotyrosine (PY-20) and anti-Rac-1 antibodies. **B,** $3x10^5$ HepG2 cells were transfected with 1 μg pIRE LUC reporter, 1 μg pDM2LacZ and increasing amounts of constitutive active or dominant negative Vav, and dominant negative SEK-1/MKK-4(A-L), MEKKΔ(K432M) or RacN17 as indicated. After overnight incubation with the DNA precipitate, cells were either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 hrs before harvest. *Inset:* overexpression of 4.0 μg myc-Vav-C. Lysates obtained in the transfection assay were Western blotted and the overexpressed proteins were visualized using anti-myc antibodies. As a control, blots were stripped and reprobed with anti-STAT3. (-) indicates lysates without overexpressed Vav-C or RacV12. **C,** Transient transfection assay as in (**B**). Cells were transfected with pIRE LUC reporter, pDM2LacZ and dominant negative RacN17, constitutive active RacV12, dominant negative MEKKΔ(K432M), or SEK-1/MKK-4(A-L) as indicated. *Inset:* overexpression of 4.0 μg RacV12. Lysates obtained in the transfection assay were Western blotted and the overexpressed proteins were visualized using anti-myc antibodies. Endogenous Rac-1 is detectable in untransfected cells, but the overexpression is clearly visible.

SEK-1/MKK-4(A-L), indicating that MEKK and SEK-1/MKK-4 act downstream of Rac in the IL-6-induced signal transduction cascade (Fig.4C). Also, the increased IRE transactivation in the presence of overexpressed constitutive active Vav-A could be blocked by overexpression of dominant negative RacN17, MEKKΔ(K432M) or SEK-1/MKK-4(A-L), indicating that Rac, MEKK-1 and SEK-1/MKK-4 act downstream of Vav in the IL-6-induced IRE transactivation (Fig. 4B).
To correlate the effects of dominant negative Vav-C, Rac-V12 and MEKKΔ(K432M) on STAT3 transactivation at the level of STAT3 ser727 phosphorylation, HA-tagged STAT3 constructs were cotransfected in HepG2 cells together with vectors expressing dominant negative Vav-C, Rac-V12, MEKKΔ(K432M) or with pUC as a control. HA-STAT3 was immunoprecipitated from IL-6 stimulated cells using anti-HA antibodies and phosphorylation was analyzed by Western blotting (Fig.3C, lanes 5-10). Co-expression of either dominant negative Vav-C, dominant-negative RacN17 or dominant negative MEKKΔ(K432M) decreased both basal as well as IL-6-induced STAT3 ser727 phosphorylation, while STAT3 tyr705 phosphorylation was unaffected.
Taken together, these experiments demonstrate that IL-6-induced STAT3 transactivation as well as STAT3 ser727 phosphorylation are both dependent on activation of SEK-1/MKK-4 and the SEK-1/MKK-4-upstream components Vav, Rac-1 and MEKK-1.

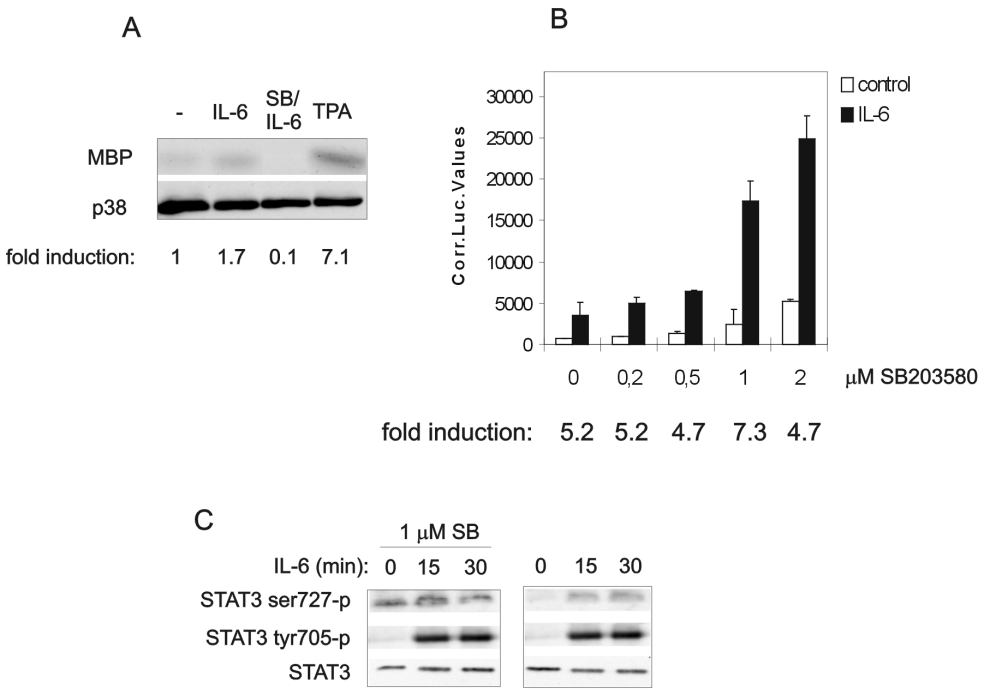

Figure 5. Blocking p38 increases STAT3 transactivation and ser727 phosphorylation. A, $3x10^6$ HepG2 cells were grown on 92-mm petri dishes, serum-starved overnight in medium containing 0.1% FCS and stimulated with 25 ng/ml IL-6, 100 ng/ml TPA, or preincubated with 1 µM SB203580 before stimulation as indicated. Cell lysates were subjected to immunoprecipitation using anti-p38 antibodies and Protein-A sepharose beads and *in vitro* kinase assays were performed using 5 µg MBP as a substrate as described in Materials and Methods. As a control, the same amounts of protein used in the kinase assay were subjected to Western blotting and probed with anti-p38 antibody. **B,** $3x10^5$

HepG2 cells were transfected with 3 μg pIRE LUC reporter, 3 μg pDM2LacZ and 4 μg of pUC18 to obtain a total of 10 μg DNA. Cells were preincubated with 0-2 μM SB203580 for 60 min, and either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 hrs until harvest. **C,** $3x10^6$ HepG2 cells were grown on 92-mm petri dishes, serum-starved overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 for varying periods. Where indicated (+SB), cells were preincubated for 1 hr with 2 μM SB203580 prior to stimulation. Equal amounts of total cell lysates were Western blotted using antibodies against STAT3 and phosphorylated STAT3 ser727 and tyr705.

The p38/mapk pathway negatively regulates STAT3 transactivation and ser727 phosphorylation. To investigate the role of p38 in STAT3-dependent transactivation, the activity of pIRE LUC was analyzed in the presence of the specific p38 inhibitor SB203580 following transient expression of this reporter in HepG2 cells. P38 activity was slightly increased in response to IL-6, but p38 kinase activity was completely blocked when cells were pre-incubated for 60 min in the presence of 1 μM SB203580 (Fig.5A). Inhibition of p38 activity had no effect on the level of IL-6 induction, which was approximately 5 fold as in control experiments (Fig.5B). These results indicate that IL-6-induced STAT3 transactivation does not require p38 activity. Interestingly however, the absolute levels of luciferase activity increased significantly in the presence of SB203580, indicating that inhibition of p38 activity has an enhancing effect on pIRE LUC transactivation. Furthermore, pre-incubation with the chemical p38 inhibitor SB203580 revealed an increased basal level of STAT3 ser727 phosphorylation (Fig.5C). STAT3 tyr705 phosphorylation was unaffected by pre-incubating HepG2 cells with SB203580 (Fig.5C). These results indicate that p38 is not directly involved in IL-6-induced STAT3 ser727 phosphorylation, but rather negatively regulates STAT3 transactivation via a presently unknown mechanism.

Discussion

STATs have been identified as a family of transcription factors that play an important role in stimulus-mediated gene expression in response to cellular stimulation by growth factors and cytokines [1]. Following ligand-receptor interaction, STATs become tyrosine phosphorylated, which allows STAT homo- or heterodimerization with other STAT family members, nuclear translocation and binding to specific consensus sequences of target gene promoters [1]. For STAT3, a single tyrosine residue (tyr705) is phosphorylated in response to EGF, INF-γ and IL-6, which involves the intrinsic- or associated tyrosine kinases of growth factor or cytokine receptors [1,61]. In addition, these stimuli also induce STAT3 ser727 phosphorylation at the extreme STAT3 C-terminus [61,196]. STAT3 ser727 as well as the homologous residue in STAT1 has been shown to be required for maximal STAT-dependent transactivation [1,61,196]. In this report, we have investigated the kinase(s) involved in STAT3 ser727 phosphorylation in response to IL-6. Stimulation of HepG2 cells with IL-6 results in STAT3 ser727 phosphorylation within 10-15 min, which occurs with decreased kinetics when compared to the rapid STAT3 tyr705 phosphorylation which occurs within 5 min upon receptor activation. Interestingly, in localization studies we observe that STAT3 quickly translocates to the nucleus within 5

min upon IL-6 stimulation (data not shown), suggesting that the ser727 phosphorylation mainly occurs in the nucleus.

In HepG2 cells, we observe that IL-6 induces ERK-1 activation (data not shown), which is in agreement with previously published studies [87,229]. However, in our studies, using (i) the MEK inhibitor PD98059, (ii) over-expression of dominant-negative Raf, NΔRaf, and (iii) co-immunoprecipitation of STAT3 with ERK-1, we failed to demonstrate a direct involvement of ERKs in STAT3 transactivation or ser727 phosphorylation. In line with these findings, the C-terminal region of STAT3 (379-770) containing ser727 was a very poor substrate for IL-6 activated ERK-1 in an *in vitro* kinase assay (unpublished observation). These results indicate that IL-6-induced STAT3 ser727 phosphorylation in HepG2 cells is ERK-independent, in agreement with previously published reports [82]. In quiescent Swiss 3T3 cells however, EGF induced STAT3 ser727 phosphorylation has been shown to be dependent on activation of ERK-1 [82]. At present, we have no explanation for the different involvement of ERKs in STAT3 ser727 phosphorylation by these different factors. However, it may be possible that the timing of ERK activation is different in response to IL-6 and EGF or involves ERK compartmentalization in a way that is not compatible with IL-6-induced STAT3 ser727 phosphorylation. Further experiments are required to resolve this issue.

Our results demonstrate that IL-6 does not induce JNK activity in HepG2 cells as determined by *in vitro* kinase assays. These results are further underscored by fluorescence confocal microscopy experiments, in which IL-6 did not stimulate nuclear translocation of JNK-1 (data not shown). Furthermore, no direct association between JNK-1 and STAT3 could be detected in co-immunoprecipitation studies (J.J. Schuringa, unpublished observation). In addition, GST-STAT3(379-770) is not phosphorylated by immunoprecipitated JNK-1 from IL-6 or anisomycin treated cells, indicating that the C-terminal part of STAT3 is a poor substrate for activated JNK-1. Taken together, these results demonstrate that JNK-1 is not mediating IL-6-induced STAT3 ser727 phosphorylation and transactivation.

In many cell types, SEK-1/MKK-4 is an upstream target for JNK-1. Surprisingly, in contrast to JNK, SEK-1/MKK-4 is quickly activated within 5-10 min upon IL-6 stimulation. Apparently, in HepG2 cells, IL-6-induced SEK-1/MKK-4 activation is not coupled to JNK-1 activation. However, SEK-1/MKK-4 activation is important in the IL-6-induced STAT3 transactivation, since overexpression of a dominant-negative mutant SEK-1/MKK-4(A-L) reduced IL-6-induced STAT3 transactivation in a dose dependent manner and completely abolished IL-6-induced STAT3 ser727 phosphorylation, indicating that SEK-1/MKK-4 is an important mediator of IL-6-induced STAT3 transactivation. Although STAT3 ser727 phosphorylation was almost completely blocked by overexpression of dominant negative SEK-1/MKK-4(A-L), the IL-6-induced STAT3 transactivation was only partially inhibited by overexpression of SEK-1/MKK-4(A-L). Possibly, there is still some residual ser727 phosphorylation which was undetectable in our Western blot analysis accounting for the residual transactivation of the pIRE LUC reporter. Clearly, overexpressed SEK-1/MKK-4(A-L) inhibited IL-6-induced STAT3 transactivation in a dose dependent manner, and the residual transactivation of the pIRE LUC reporter will depend on the balance between overexpressed dominant negative SEK-1/MKK-4(A-L) and the endogenous SEK-1/MKK-4 which is present in HepG2 cells. Whether SEK-1/MKK-4 activates a kinase which phosphorylates STAT3 on ser727 or

whether SEK-1/MKK-4 directly phosphorylates STAT3 is presently unknown and under investigation.

The observation that SEK-1/MKK-4 is involved in the IL-6-induced STAT3 ser727 phosphorylation is further underscored by experiments demonstrating the relevance of STAT3 transactivation by the SEK-1/MKK-4 upstream components Vav, Rac, and MEKK. Vav has been shown to be associated with the membrane-distal part of the gp130 transmembrane receptor in different cell types [34,219,220]. Vav is tyrosine phosphorylated within 10 min upon IL-6 stimulation in HepG2 cells and transient overexpression of dominant negative Vav decreased IL-6-induced STAT3 transactivation as well as STAT3 ser727 phosphorylation, while constitutive active Vav increased STAT3 transactivation. These results indicate that Vav can mediate IL-6-induced STAT3 transactivation. Furthermore, Vav rapidly co-immunoprecipitated with Rac-1 within 5 min of IL-6 stimulation and this association was transient. Interestingly, tyrosine phosphorylation of Vav preceded Vav-Rac-1 association, and this association was already reduced when maximal Vav tyrosine phosphorylation was detected. This finding might suggest that tyrosine phosphorylation of Vav leads to the release of Rac-1 and activation of its downstream effectors, rather than playing a role in Vav-Rac-1 association. These results are compatible with the recent observation that Vav catalyzes GDP/GTP exchange on Rac-1 thereby releasing it in its active form to further propagate the signal [223]. The involvement of Rac-1 in IL-6-induced STAT3 transactivation was demonstrated by transient overexpression of dominant negative and constitutive active mutants of Rac-1 in HepG2 cells. Dominant negative RacN17 reduced IL-6-induced STAT3 transactivation significantly, while constitutive active RacV12 significantly increased STAT3 transactivation. In line with these observations, overexpression of dominant negative RacN17 abolished IL-6-induced STAT3 ser727 phosphorylation. Rac functions downstream of Vav in this signaling cascade since the increased IRE transactivation in the presence of overexpressed constitutive active Vav could be blocked by overexpression of dominant negative RacN17. MEKK is located downstream of Rac-1 and it has been shown that MEKK becomes activated in response to activation of Rac-1 [206,211,222]. Overexpression of dominant negative MEKKΔ(K432M) strongly reduced IL-6-induced STAT3 ser727 phosphorylation and transactivation, indicating that Rac-1 might induce SEK-1/MKK-4 activity via MEKK-1 in HepG2 cells. These results are further underscored by the observations that the increased IRE transactivation in the presence of overexpressed constitutive active Vav or Rac could be blocked by dominant negative MEKKΔ(K432M) and SEK-1/MKK-4(A-L), indicating that MEKK-1 and SEK-1/MKK-4 function downstream of Vav and Rac in IL-6-induced STAT3 transactivation.

Our investigations further demonstrate that p38 is not directly involved in STAT3 ser727 phosphorylation. In contrast, blocking p38 kinase activity with the chemical inhibitor SB203580 significantly increased the basal levels as well as the IL-6-induced STAT3 transactivation, while IL-6 was still capable of inducing an additional STAT3 transactivation of approximately 5 fold. The increase in transactivation in the presence of SB203580 was also coupled to an increased basal and IL-6-induced STAT3 ser727 phosphorylation levels, while STAT3 tyr705 phosphorylation was unaffected. Possibly, p38 squelches the activity of an upstream kinase like SEK-1/MKK-4, which is blocked in the presence of SB203580. This hypothesis is supported by recent results, showing that SEK-1/MKK-4 indeed is capable of binding and activating p38 [214,233] and that p38 has an inhibiting effect on JNK-1 mediated Elk activation [218]. Whether the effects of

SB203580 on STAT3 transactivation involve cross talk between the p38 and SAPK/JNK pathways requires further investigation.
In conclusion, these data indicate that IL-6-induced STAT3 transactivation and ser727 phosphorylation involves a gp130 receptor-signaling cascade that includes Vav, Rac-1, MEKK-1 and SEK-1/MKK-4 as signal transduction components.

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