Interleukin-6-induced STAT3 transactivation and Ser⁷²⁷ phosphorylation involves Vav, Rac-1 and the kinase SEK-1/MKK-4 as signal transduction components

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In the present study, signal transducer and activator of transcription 3 (STAT3) Ser⁷²⁷ phosphorylation and transactivation was investigated in relation to activation of mitogen-activated protein (MAP) kinase family members including extracellular-signal-regulated protein kinase (ERK)-1, c-Jun N-terminal kinase (JNK)-1 and p38 ('reactivating kinase') in response to interleukin (IL)-6 stimulation. Although IL-6 can activate ERK-1 in HepG2 cells, STAT3 transactivation and Ser⁷²⁷ phosphorylation were not reduced by using the MAP kinase/ERK kinase (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 for JNK1 in IL-6-induced STAT3 activation. However, SEK-1/MKK-4 [where SEK-1 stands for stress-activated protein kinase (SAPK)/ERK kinase 1, and MKK-4 stands for MAP kinase kinase 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-signalling cascade that includes Vav, Rac-1, MEKK and SEK-1/MKK-4 as signal transduction components.

Key words: cytokine signalling, gp130, serine phosphorylation.

INTRODUCTION

Interleukin (IL)-6 is a pleiotropic cytokine that mediates a variety of functions in different cells and tissues including proliferation and differentiation of haematopoietic cells, induction of the acute-phase response in liver cells and inflammation at sites of tissue injury [1–3]. IL-6 initiates its action by binding to its receptor which is composed of two subunits: an 80-kDa IL-6 binding protein and a 130-kDa transmembrane signal-transducing component (gp130) [4–6]. The gp130 receptor protein is also used by other members of the IL-6 cytokine family, including IL-11, oncostatin M, leukaemia inhibitory factor, and ciliary neurotrophic factor [7–9]. 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) [10]. Tyrosine phosphorylation of STAT3 occurs at a single residue (Tyr⁷⁰⁵) which is located in a conserved Src homology 2 (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 [10]. 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 and epidermal growth factor (EGF) [11,12]. Ser⁷²⁷ phosphorylation has been shown to enhance STAT3 transcriptional activation [12].

At present, the signalling 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. Mitogen-activated protein (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 [13]. Several mammalian MAP kinases have been identified, including extracellular-signal-regulated protein kinase (ERK) [14], stress-activated protein kinase or c-Jun N-terminal protein kinase (SAPK/JNK) [15,16], and p38 ('reactivated kinase') [17,18]. The activation of ERKs involves a signal transduction pathway that includes Ras, Raf-1 and MAP kinase/ERK kinase (MEK) downstream of receptor tyrosine kinase receptors [19–21]. JNKs are activated by 'stress stimuli' involving a signal transduction pathway that contains Ras, Rac-1, MEK kinase (MEKK) and SEK-1/MKK-4 (where SEK-1 stands for SAPK/ERK kinase 1 and MKK-4 stands for MAP kinase kinase 4) [22–28]. The signalling pathway that leads to the activation of p38 is not fully elucidated, but MKK3 and MKK6 have been identified as MKKs for p38 [29,30]. Upon activation, MAP kinases rapidly translocate to the nucleus and

Abbreviations used: STAT3, signal transducer and activator of transcription 3; MAP, mitogen-activated protein; ERK, extracellular-signal-regulated protein kinase; JNK, c-Jun N-terminal kinase; IL, interleukin; MEK, MAP kinase/ERK kinase; SAPK, stress-activated protein kinase; SEK-1, SAPK/ERK kinase 1; MKK-4, MAP kinase kinase 4; MEKK, MEK kinase; EGF, epidermal growth factor; SH, Src homology; FCS, foetal calf serum; HA, haemagglutinin; IRE, IL-6 response element; TPA, 12-O-tetradecanoylphorbol-13-acetate.

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have been shown to phosphorylate several transcription factors including Elk which is phosphorylated by ERK-1 [31,32] and c-Jun, ATF-2 and Sap-1 which are substrates for activated JNK [31,33,34].

Although signalling events downstream of the gp130 receptor have not fully been elucidated, gp130 has been shown to interact with Vav [35], a 95-kDa proto-oncogene product that is expressed in haematopoietic cells and trophoblasts [36,37]. Vav contains an array of structural motives, including zinc finger, leucine zipper, pleckstrin homology, Dbl homology and SH2 and SH3 domains [37,38]. 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 catalyse GDP/GTP exchange on Rac-1, which leads to activation of JNK [35,39,40].

In the present study, we have investigated the involvement of the ERK, JNK and p38 signalling 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 supplemented with 10% (v/v) heat-inactivated foetal 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, MA, U.S.A.), 100 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (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, U.S.A.) were used at final concentrations of 20 μ M unless stated otherwise. Antibodies against haemagglutinin (HA), STAT3, Vav, Rac-1, MKK-4, p38, JNK-1 and ERK-1 (Santa Cruz) and c-myc (9E10; Boehringer Mannheim, Corp.) were used as 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 as a 1:1000 dilution.

Expression and reporter constructs

The following plasmids were used: pIRE LUC containing two copies of the IL-6 response element (IRE) of the ICAM-1 promoter in front of the herpes simplex virus thymidine kinase promoter and the luciferase gene [41]; pSG5-STAT3 which expresses STAT3 from the SV40 promoter; pSG5-STAT3 β , which expresses a dominant-negative isoform of STAT3 lacking the 55 C-terminal amino-acid residues; pSG5-STAT3 Ser⁷²⁷ Ala, which expressed a mutant STAT3 in which the Ser⁷²⁷ is replaced by Ala; 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 [42]; pCDNA3-MEKK Δ (K432M) which expresses a dominant-negative MEKK [43]; pCDNA3-HA-JNK-1 which expresses HA-tagged JNK-1 (p46); pGEX-1-c-Jun(1-135) expressing glutathione S-transferase (GST)-c-Jun;

and the dominant-negative and constitutive-active mutants pEF-myc-Vav-C [44] and pMEX-myc-Vav-A(Δ 1-65) [39,45]. The expression vector encoding a dominant-negative mutant of Raf kinase, N Δ Raf, was provided by Dr P. Coffey (Department of Pulmonary Diseases, University Hospital Utrecht, Utrecht, The Netherlands) [46]. The pGEX-STAT3(379-770) expression vector was cloned by inserting the *Bam*HI-*Bgl*III (blunt) pSG5-STAT3 fragment into the *Bam*HI and *Sma*I sites of pGEX-4t1.

Transient transfections

HepG2 cells were seeded at 3×10^5 cells per well in 6-well plates (Costar), and 24 h later cells were transfected with 10 μ g of plasmid DNA using the calcium phosphate co-precipitation method [46]. Transfection mixtures consisted of a mixture of 3 μ g of pIRE LUC reporter, 3 μ g of pDMLacZ as a control to determine transfection efficiency, and 1-4 μ g 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 μ g of DNA. Cells were incubated with precipitate for 24 h, washed with PBS, and stimulated for an additional 24 h. Cells were collected in 200 μ l of reporter lysis buffer (Promega) and subjected to the assays for luciferase [46,47] and β -galactosidase [46,48] as described previously. 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/PAGE, Western blotting, immunoprecipitations and kinase assays

A total of 1×10^7 cells were lysed on ice in lysis buffer [20 mM Hepes, pH 7.4, 2 mM EGTA, 1 mM dithiothreitol, 1 mM Na₂VO₃ (ortho), 1% (v/v) Triton X-100, 10% (v/v) glycerol, 10 μ g/ml leupeptin, 0.4 mM PMSF]. Prior to SDS/PAGE and immunoprecipitations, protein concentrations were determined (Bio-Rad) 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 SDS/12.5% (w/v) polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (Millipore) in Tris/glycine buffer at 100 V for 1.5 h using an electroblotter (Pharmacia). Membranes were blocked with PBS buffer containing 5% (w/v) non-fat milk prior to incubation with antibodies. Binding of each antibody was detected by chemiluminescence 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 SDS/PAGE (12.5% gel). 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/HCl, pH 7.6, 0.1% Triton X-100, and 1 mM dithiothreitol), and three times with assay buffer (20 mM Mops, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM dithiothreitol, 0.1% Triton X-100). The precipitates were incubated for 30 min in 43.5 μ l of assay buffer, 20 mM MgCl₂, 25 μ M ATP, 10 μ Ci of [γ -³²P]ATP, and 5 μ g of GST-c-Jun(1-135), 5 μ g of myelin basic protein (Santa Cruz) or 5 μ g of GST-STAT3(379-770). Beads were boiled in SDS/sample buffer for 5 min and separated on an SDS/12.5% 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 [10–12]. 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 min upon IL-6 stimulation of HepG2 cells, whereas STAT3 Tyr⁷⁰⁵ phosphorylation was observed within 5 min (Figure 1A). Overexpression of STAT3 β or a STAT Ser⁷²⁷Ala mutant strongly decreased transactivation of the pIRE LUC reporter, which contained two copies of the IRE of the ICAM-1 promoter (Figure 1B). This

Figure 1 IL-6-induced STAT3 transactivation is not mediated via ERK-1 in HepG2 cells

(A) HepG2 cells (3×10^6) were grown on 92-mm-diameter Petri dishes, starved of serum overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 for various time periods. Equal amounts of whole-cell lysates were Western blotted using antibodies against STAT3 and phosphorylated STAT3(Ser⁷²⁷ and Tyr⁷⁰⁵). (B) HepG2 cells (3×10^5) were transfected with 3 μ g of pIRE LUC reporter, 3 μ g of pDM2LacZ and 4 μ g of pSG5-STAT3 β or pSG5-STAT3 Ser⁷²⁷Ala as indicated. Cells were either unstimulated (open bars) or stimulated with 25 ng/ml IL-6 (filled bars) for 24 h before harvest.

Figure 2 IL-6 does not activate JNK-1 in HepG2 cells

(A) HepG2 cells (3×10^6) were grown on 92-mm-diameter Petri dishes, starved of serum overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 or 100 ng/ml anisomycin (Ani) as indicated. Cell lysates were subjected to immunoprecipitation using anti-JNK1 antibodies and Protein A-Sepharose beads, and precipitates were used in kinase assays *in vitro* using 5 μ g of GST-c-Jun(1–135) as a substrate, as described in the Materials and methods section. 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) Kinase assays *in vitro* were as in (A); however, cells were not starved of serum but grown in 10% FCS. (C) (Upper panel) 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 antibodies. (—) indicates cells without overexpressed HA-JNK-1. (Lower panel) HepG2 cells (3×10^5) were transfected with 3 μ g of pIRE LUC reporter, 3 μ g of pDM2LacZ, and 4 μ g of pUC18 or pcDNA3-HA-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 h until harvest.

indicates that specific phosphorylation on the Ser⁷²⁷ residue of STAT3 is important for maximal STAT3 transcriptional activation. Since Ser⁷²⁷ is located in a conserved Pro-Xaa-Ser/Thr-Pro site, which has been accepted as a phosphorylation site for MAP kinases [49], the involvement of the ERK, JNK and p38 MAP kinases in STAT3 Ser⁷²⁷ phosphorylation was investi-

Figure 3 SEK-1/MKK-4 is involved in IL-6-induced STAT3 transactivation and Ser⁷²⁷ phosphorylation

(A) HepG2 cells (3×10^5) were grown on 92-mm-diameter Petri dishes, starved of serum overnight in medium containing 0.5% FCS, and stimulated with 25 ng/ml IL-6 for various time 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) (Upper panel) Lysates obtained in the transient transfection assay [4.0 μ g of 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 was detectable in untransfected cells (u), but the overexpression is clearly visible. As a control, blots were stripped and reprobed with

anti-STAT3. (Lower panel) HepG2 cells (3×10^5) were transfected with 3 μ g of pIRE LUC reporter, 3 μ g of pDM2LacZ, and 1–4 μ g of 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 h before harvest. (C) HepG2 cells (3×10^5) were grown on 92-mm-diameter Petri dishes and were transfected with 25 μ g of expression vectors for HA-STAT3 and 25 μ g of pUC18 (lanes 1 and 2) or 25 μ g of dominant-negative SEK-1/MKK-4(A-L) (lanes 3 and 4), Vav-C (lanes 5 and 6), Rac-N17 (lanes 7 and 8), or MEKKΔ(K432M) (lanes 9 and 10). After an 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 antibodies and precipitates were Western blotted using antibodies against phosphorylated STAT3(Ser⁷²⁷ and Tyr⁷⁰⁵). As a control, blots were stripped and reprobed with anti-HA antibodies.

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) HepG2 cells (10^7) were grown on 150-mm-diameter Petri dishes, starved of serum overnight in medium containing 0.5% FCS, and stimulated with 25 ng/ml IL-6 for various time periods.

ation was unaffected (Figure 3C, lanes 1–4). These results demonstrated that SEK-1/MKK-4 was activated in HepG2 in response to IL-6 and indicated that activation of SEK-1/MKK-4 was 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 [35]. Since Vav is capable of activating Rac-1 [40] and Rac-1 is a known activator of the JNK pathway [23,28,39], 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 (Figure 4A). Furthermore, transient co-transfection of the pIRE LUC reporter together with a vector expressing dominant-negative Vav-C [39] decreased IL-6-induced STAT3 reporter transactivation from 7.7 ± 0.6 -fold to 3.5 ± 0.2 -fold, while constitutive-active Vav-A ($\Delta 1-65$) [39] increased IL-6-induced STAT3 transactivation from 7.7 ± 0.6 -fold to 10.4 ± 0.1 -fold (Figure 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 analysed by Western blotting. Rac-1 transiently co-precipitates with Vav, which is maximal after 5 min and is followed by a quick release of Rac-1 that is complete after 20 min (Figure 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 -fold to 4.6 ± 0.2 -fold (Figure 4C). As expected, constitutive-active RacV12 strongly increased both basal as well as IL-6-induced STAT3-dependent activation of the pIRE LUC reporter (Figure 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 [43]. Overexpression

Cells were lysed, protein concentrations were determined, and equal amounts were used in each experiment. Vav immunoprecipitates were separated by SDS/PAGE and Western blotted with anti-Vav, anti-phosphotyrosine (PY-20) and anti-Rac-1 antibodies. (B) (Upper panel) 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 antibodies. (—) indicates lysates without overexpressed Vav-C or RacV12. (Lower panel) HepG2 cells (3×10^5) were transfected with 1 μg of pIRE LUC reporter, 1 μg of 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 h before harvest. (C) (Upper panel) Overexpression of 4.0 μg of RacV12. Lysates obtained in the transfection assay were Western blotted and the overexpressed proteins were visualized using anti-myc antibodies. Endogenous Rac-1 was detectable in untransfected cells, but the overexpression is clearly visible. (Lower panel) 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.

Figure 5 Blocking p38 increases STAT3 transactivation and Ser⁷²⁷ phosphorylation

(A) HepG2 cells (3×10^6) were grown on 92-mm-diameter Petri dishes, starved of serum 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 kinase assays were performed *in vitro* using 5 μg of myelin basic protein as a substrate as described in the Materials and methods section. As a control, the same amounts of protein used in the kinase assays were subjected to Western blotting and probed with anti-p38 antibody. (B) HepG2 cells (3×10^5) were transfected with 3 μg of pIRE LUC reporter, 3 μg of pDM2LacZ and 4 μg of pUC18 to obtain a total of 10 μg of 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 h until harvest. (C) HepG2 cells (3×10^5) were grown on 92-mm-diameter Petri dishes, starved of serum overnight in medium containing 0.1% FCS, and stimulated with 25 ng/ml IL-6 for various time periods. Where indicated, cells were preincubated for 1 h with 1 μM SB203580 prior to stimulation. Equal amounts of whole-cell lysates were Western blotted using antibodies against STAT3 and phosphorylated STAT3 (Ser⁷²⁷ and Tyr⁷⁰⁵).

of dominant-negative MEKK-1 strongly reduced IRE transactivation from 7.9 ± 0.7 -fold to 3.5 ± 0.1 -fold (Figure 4C). Furthermore, the increased IRE transactivation by overexpression of RacV12 could be blocked by overexpression of dominant-negative MEKK Δ (K432M) or 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 (Figure 4C). Also, the increased IRE transactivation in the presence of overexpressed constitutive-active Vav-A could be blocked by overexpression of dominant-negative RacN17, MEKK Δ (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 (Figure 4B).

To correlate the effects of dominant-negative Vav-C, Rac-V12 and MEKK Δ (K432M) on STAT3 transactivation at the level of STAT3 Ser⁷²⁷ phosphorylation, HA-tagged STAT3 constructs were co-transfected in HepG2 cells together with vectors expressing dominant-negative Vav-C, Rac-V12, MEKK Δ (K432M) or with pUC18 as a control. HA-STAT3 was immunoprecipitated from IL-6-stimulated cells using anti-HA antibodies and phosphorylation was analysed by Western blotting (Figure 3C, lanes 5–10). Co-expression of either dominant-negative Vav-C, dominant-negative RacN17 or dominant-negative MEKK Δ (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.

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 analysed 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 (Figure 5A). Inhibition of p38 activity had no effect on the level of IL-6 induction, which was approx. 5-fold as in control experiments (Figure 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 (Figure 5C). STAT3 Tyr⁷⁰⁵ phosphorylation was unaffected by pre-incubating HepG2 cells with SB203580 (Figure 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 [10]. 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 [10]. For STAT3, a single tyrosine residue (Tyr⁷⁰⁵) is phosphorylated in response to EGF, interferon- γ and IL-6, which involves the intrinsic- or associated tyrosine kinases of growth factor or cytokine receptors [10,11]. In addition, these

stimuli also induce STAT3 Ser⁷²⁷ phosphorylation at the extreme STAT3 C-terminus [11,12]. STAT3 Ser⁷²⁷, as well as the homologous residue in STAT1, has been shown to be required for maximal STAT-dependent transactivation [11]. 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 with the rapid STAT3 Tyr⁷⁰⁵ phosphorylation which occurs within 5 min upon receptor activation. Interestingly, in localization studies we observed that STAT3 quickly translocated to the nucleus within 5 min upon IL-6 stimulation (results not shown), which is in agreement with previous reports [12], suggesting that the Ser⁷²⁷ phosphorylation mainly occurs in the nucleus.

In HepG2 cells, we observe that IL-6 induces ERK-1 activation (results not shown), which is in agreement with previously published studies [51,52]. However, in our studies, using (i) the MEK inhibitor PD98059, (ii) overexpression 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 (J.-J. Schuringa, L. J. C. Jonk, W. H. A. Dokter, E. Vellenga and W. Kruijer, unpublished work). These results indicate that IL-6-induced STAT3 Ser⁷²⁷ phosphorylation in HepG2 cells is ERK-independent, in agreement with previously published reports [53]. In quiescent Swiss 3T3 cells, however, EGF-induced STAT3 Ser⁷²⁷ phosphorylation has been shown to be dependent on activation of ERK-1 [53]. 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 kinase assays *in vitro*. These results are further underscored by fluorescence confocal microscopy experiments, in which IL-6 did not stimulate nuclear translocation of JNK-1 (results not shown). Furthermore, no direct association between JNK-1 and STAT3 could be detected in co-immunoprecipitation studies (J. J. Schuringa, unpublished work). 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 [54]. 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 over-

expression 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 [35–37]. 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 catalyses GDP/GTP exchange on Rac-1 thereby releasing it in its active form to further propagate the signal [40]. 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 signalling 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 [23,28,39]. 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 approx. 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 is indeed capable of binding and activating p38 [29,50] and that p38 has an inhibiting effect on JNK-1-mediated Elk activation [33]. 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-signalling cascade that includes Vav, Rac-1, MEKK-1 and SEK-1/MKK-4 as signal transduction components.

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