Activation of the protein tyrosine phosphatase SHP2 via the interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression

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Stimulation of the interleukin-6 (IL-6) signalling pathway occurs via the IL-6 receptor–glycoprotein 130 (IL-6R–gp130) receptor complex and results in the regulation of acute-phase protein genes in liver cells. Ligand binding to the receptor complex leads to tyrosine phosphorylation and activation of Janus kinases (Jak), phosphorylation of the signal transducing subunit gp130, followed by recruitment and phosphorylation of the signal transducer and activator of transcription factors STAT3 and STAT1 and the src homology domain (SH2)-containing protein tyrosine phosphatase (SHP2). The tyrosine phosphorylated STAT factors dissociate from the receptor, dimerize and translocate to the nucleus where they bind to enhancer sequences of IL-6 target genes. Phosphorylated SHP2 is able to bind growth factor receptor bound protein (grb2) and thus might link the Jak/STAT pathway to the ras/raf/mitogen-activated protein

kinase pathway. Here we present data on the dose-dependence, kinetics and kinase requirements for SHP2 phosphorylation after the activation of the signal transducer, gp130, of the IL-6-type family receptor complex. When human fibrosarcoma cell lines deficient in Jak1, Jak2 or tyrosine kinase 2 (Tyk2) were stimulated with IL-6–soluble IL-6R complexes it was found that only in Jak1-, but not in Jak 2- or Tyk2-deficient cells, SHP2 activation was greatly impaired. It is concluded that Jak1 is required for the tyrosine phosphorylation of SHP2. This phosphorylation depends on Tyr-759 in the cytoplasmic domain of gp130, since a Tyr-759 → Phe exchange abrogates SHP2 activation and in turn leads to elevated and prolonged STAT3 and STAT1 activation as well as enhanced acute-phase protein gene induction. Therefore, SHP2 plays an important role in acute-phase gene regulation.

INTRODUCTION

Phosphorylation and dephosphorylation of proteins are major steps in the regulation of cytokine signal transduction. The phosphorylation status of the molecules within signalling pathways is regulated by kinases and phosphatases. Binding of the specific ligand to its receptor complex leads to the activation of kinases and in turn to the phosphorylation of tyrosine motifs within the signalling receptor chain. These receptor phosphotyrosines form recruitment sites for src homology domain 2 (SH2)-containing proteins, such as signal transducer and activator of transcription (STAT) factors, phosphatases and adaptor proteins. Subsequently, these proteins are frequently also phosphorylated (for review see [1]).

Within the family of long-chain four α -helical cytokines [2], there is a group of so-called interleukin-6-type cytokines: IL-6, IL-11, ciliary neurotrophic factor, leukaemia inhibitory factor, cardiotrophin-1 and oncostatin M. Their receptor complexes share a common protein, the signal transducer glycoprotein 130 (gp130/CD130) (for review see [3]). In the case of IL-6, binding to its receptor (IL-6R/gp80) is the initial step in receptor activation. IL-6–IL-6R complexes interact with the signal transducer gp130 [4] and lead to its dimerization [5]. Tyrosine kinases of the Janus family Jak1, Jak2 and Tyk2 are constitutively

associated with the cytoplasmic part of gp130. They become activated through autophosphorylation after ligand binding and phosphorylate certain tyrosine motifs within the cytoplasmic domain of gp130 [6,7]. These phosphotyrosine motifs represent recruitment sites for the transcription factors STAT1 and STAT3 and for the SH2-containing tyrosine phosphatase (SHP2) which then also become phosphorylated [8,9]. Since in Jak1-, but not in Jak2- or in Tyk2-deficient cells, phosphorylation of gp130, STAT1 and STAT3 is greatly reduced, a major role of Jak1 is evident [10]. The kinase leading to the residual phosphorylation of gp130 in cells lacking Jak1, as well as the role of this phosphorylation in signal transduction, is still unknown. After tyrosine phosphorylation, STATs homo- or heterodimerize and subsequently translocate to the nucleus [11] where they bind to response elements in the promoters of IL-6 target genes, e.g. acute-phase protein genes, leading to their induction [12].

Although the SHP2 is a component of the IL-6R complex, its role in IL-6 signalling is presently not clear. It has been proposed to be an adaptor protein which associates with growth factor receptor bound protein (grb2) and thereby activates the mitogen-activated protein kinase (MAPK) pathway [13]. The involvement of both the Jak/STAT and MAPK pathways in IL-6-induced signal transduction has been evaluated in a few cases. Stimulation of Ba/F3 cells stably expressing gp130 leads to an IL-3-in-

Abbreviations used: CAT, chloramphenicol acetyltransferase; α_2 M, α_2 -macroglobulin; c-kit, receptor of stem cell factor; EGF, epidermal growth factor; EMSA, electrophoretic mobility-shift assay; Epo, erythropoietin; FGF, fibroblast growth factor; gp130, glycoprotein 130/CD130; grb, growth factor receptor bound protein; IL, interleukin; IL-6R, IL-6 receptor/gp80; sIL-6R, soluble IL-6R; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; SH2, src homology domain 2; SHP, SH2-containing tyrosine phosphatase; STAT, signal transducer and activator of transcription; Tyk2, tyrosine kinase 2.

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dependent, but IL-6-soluble IL-6R (sIL-6R)-dependent growth [14]. In this system STAT3 activation is required for an anti-apoptotic signal, whereas recruitment of SHP2 to the receptor is essential for the mitogenic signal [13]. In contrast, stimulation of M1 cells with IL-6 results in differentiation and growth arrest, independent of SHP2 recruitment [15].

SHP2 tyrosine phosphorylation has been described to be involved in many signalling pathways, both through receptor tyrosine kinases such as platelet-derived growth factor receptor (PDGF-R), insulin-receptor, epidermal growth factor receptor (EGF-R), fibroblast growth factor receptor (FGF-R), erythropoietin receptor (Epo-R), stem cell factor receptor (c-kit) and through non-receptor kinases such as src, Jak1 and Jak2 (for reviews see [3,16]).

Presently it is not known which kinase is responsible for the phosphorylation of SHP2 after stimulation of cells with IL-6. The residual phosphorylation of gp130 observed in Jak1-deficient cells implies that other kinases besides Jak1 might be required for SHP2 phosphorylation. Here we investigated the conditions and kinase requirements for the IL-6-dependent tyrosine phosphorylation of SHP2. We found a dose-dependent transient SHP2 phosphorylation correlating with the time-course of gp130 activation and SHP2-gp130 complex formation. This SHP2 activation requires Tyr-759 in gp130 and Jak1, but not Jak2 or Tyk2. In addition, we observed a stimulation-independent interaction of SHP2 with all three kinases. In further elucidating the role of SHP2 in gene induction, we found SHP2 to be inhibitory, i.e. inhibition of SHP2 phosphorylation led to enhanced STAT activation and transcription of an acute-phase protein gene promoter/reporter gene. The inhibitory activity is stimulation-dependent and the phosphatase activity of SHP2 is involved.

EXPERIMENTAL

Materials

Restriction enzymes were purchased from Boehringer Mannheim (Germany) and oligonucleotides were synthesized by MWG-Biotech (Germany). Vent Polymerase was obtained from New England BioLabs (Schwalbach/Ts, Germany). Recombinant Epo was a generous gift from Dr. J. Burg and Dr. K. H. Sellinger of Boehringer Mannheim (Germany). Antibodies to SHP2 were from Santa Cruz (U.S.A.), phosphotyrosine antibodies (4G10) from Upstate Laboratories (U.S.A.), antibodies to the Tyr-705-phosphorylated STAT3 from New England BioLabs and the Tyr-701-phosphorylated STAT1 from Upstate Laboratories. Antibodies to gp130 were gifts from Dr. J. Wijdenes (Besançon, France) or from Upstate Laboratories (U.S.A.). Antibodies to Jak1 and Jak2 were gifts from Dr. A. Ziemiecki (Laboratory of Clinical and Experimental Cancer Research, Bern, Switzerland). Antibodies to Tyk 2 were purchased from Transduction Laboratories (U.S.A.). Recombinant IL-6 and sIL-6R were prepared as described in [17]. The specific activity of IL-6 was 2×10^6 B-cell stimulatory factor-2 units/mg of protein.

Construction of expression vectors

Constructions were carried out by standard procedures [18]. pGL3 α_2 M-215Luc was constructed by exchange of the chloramphenicol acetyltransferase (CAT)-encoding sequence of p α_2 M-215CAT [19] by the luciferase encoding sequence of pMT7Luc [20] (α_2 M is α_2 -macroglobulin).

The expression vector pSVL-EG encoding the chimaeric Epo/gp130 receptor [8] was modified by PCR mutagenesis to code for a chimaeric Epo/gp130 receptor [pSVL-EG(YYYYYY)]

and a variant, where Tyr-759 in the cytoplasmic domain of gp130 was exchanged by Phe [pSVL-EG(YFYFYYY)]. The DNA fragments encoding the Epo/gp130 chimaeric receptors were also transferred into pRc/CMV-EG [8] and used for expression in HepG2 cells [pRc/CMV-EG(YYYYYY)] and pRc/CMV-(YFYFYYY)]. The DNA fragment coding for the transmembrane and cytoplasmic domains of the chimaeric receptors were introduced into pSVL-gp130 [17], resulting in expression vectors for wild-type gp130 [pSVL-gp130 (YYYYYY)] and for gp130 with a Tyr $\rightarrow$ Phe exchange at position 759 (pSVL-gp130-YFYFYYY). The latter constructs were used for generating stably transfected Ba/F3 cells. The cloning vector pCBC-1 has been described elsewhere [21]. pCBC-1-SHP2 was generated by introducing the DNA coding for SHP2 fused at the C-terminal end to a myc-tag into pCBC-1. pCBC-1-SHP2Cys $\rightarrow$ Ser was generated to encode a gene product that contains a Cys $\rightarrow$ Ser exchange at position 459. This SHP2Cys $\rightarrow$ Ser mutant is enzymically inactive, as demonstrated elsewhere [22,23]. The sequences of all constructs have been verified by fluorescence sequencing.

Immunoprecipitation and immunoblot analysis

For immunoprecipitation, 2×10^7 cells were lysed in 500 μ l of lysis buffer [50 mM Hepes, pH 7.5/150 mM NaCl/1 % Triton X-100 (0.1 % in co-immunoprecipitation assays)/10 % (v/v) glycerol/1 mM EGTA/1.5 mM MgCl₂/10 μ g/ml of each of aprotinin, pepstatin, leupeptin]. Equal amounts of cellular protein were incubated with the appropriate antibodies at 4 °C overnight and immunoprecipitated with 5 mg of Protein A-Sepharose (Pharmacia, Sweden). Immune complexes were separated by SDS/PAGE (7 % gel) and transferred to a PVDF membrane. Antigens were detected by incubation with the appropriate primary antibody (anti-Jak1, anti-Jak2, anti-gp130, 4G10: 1:1000; anti-SHP2, anti-Tyr705-phosphorylated STAT3, anti-Tyk2: 1:2000) and horseradish peroxidase-coupled secondary antibodies (1:2000) (Dako, Germany). The membranes were developed with an enhanced chemiluminescence kit (Amersham, Germany). To verify application of equal amounts of protein, blots were stripped and reprobed. Western blot analyses were performed at least twice under slightly modified conditions.

Transfection and reporter gene analysis

Human hepatoma HepG2 cells were grown and transient transfections by the calcium phosphate coprecipitation method were performed as described previously [19]. Cell lysis and luciferase assays were carried out using the luciferase kit (Promega, U.S.A.) according to the manufacturer's instructions. All transient-expression experiments were done at least in triplicate. Luciferase activity values were normalized to transfection efficiency monitored by the cotransfected β -galactosidase expression vector (pCR3lacZ; Pharmacia, Sweden).

Ba/F3 cells were grown and stably transfected as described previously [17]. Equal expression levels of gp130 protein were verified by FACS analysis with the B-P4 antibody specific for the extracellular domain of gp130 (results not shown).

Nuclear extract preparation and electrophoretic mobility-shift assay (EMSA)

Nuclear extracts of Ba/F3 cells were prepared, protein concentration was measured by the Bio-Rad protein assay and EMSA was performed as described in [17]. We used a STAT1- and STAT3-specific double-stranded ³²P-labelled probe: a mutated sis-inducible element (SIE) oligonucleotide of the *c-fos* promoter (m67 SIE: 5'-GAT CCG GGA GGG ATT TAC GGG

AAA TGC TG-3'). Protein-DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25 Tris/borate/EDTA buffer at 20 V/cm for 4 h. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 30 min, dried and autoradiographed.

RESULTS

The phosphorylation of SHP2 after activation of gp130 through IL-11 and IL-6 has been demonstrated in different cell types [9,24]. It was the aim of the present study to examine the characteristics of the SHP2 phosphorylation induced by stimulation of the IL-6 signalling pathway and to investigate which of the gp130-associated kinases Jak1, Jak2 and Tyk2 are required for this process.

Dose-dependent SHP2 tyrosine phosphorylation upon IL-6 stimulation of HepG2 cells

To determine the role and function of SHP2 in the induction of acute-phase protein genes, we studied the phosphorylation of SHP2 in the human hepatoma cell line HepG2. HepG2 cells were stimulated with recombinant IL-6 at increasing concentrations and immunoprecipitated SHP2 protein was analysed for tyrosine phosphorylation by Western blotting (Figure 1A). Maximal SHP2 phosphorylation was achieved at a concentration of 200

units of IL-6/ml. Larger amounts of IL-6 did not further increase the response.

The time-course of SHP2 phosphorylation corresponds to that of gp130

In order to examine the time-course of SHP2 phosphorylation we stimulated HepG2 cells with IL-6 for different periods of time. After immunoprecipitation of SHP2 and Western blotting, a transient tyrosine phosphorylation of SHP2 was observed (Figure 1B, upper panel). Phosphorylation was detected after only 5 min of stimulation; after 40 min no more phosphorylated SHP2 could be visualized. This was not due to the disappearance of SHP2 protein, as shown by reprobing with an SHP2 antibody (Figure 1B, lower panel). To compare the time-course of SHP2 phosphorylation with that of the IL-6R subunit, gp130 was immunoprecipitated with a specific antibody raised against the extracellular domain of gp130. Almost identical phosphorylation kinetics as seen for SHP2 were observed (Figure 1C, upper panel). Whereas tyrosine phosphorylation of gp130 was visible already 5 min after stimulation, no phosphorylated gp130 was detectable 40 min after stimulation. The precipitated gp130 protein could be visualized through reprobing with a specific gp130 antibody (Figure 1C, middle panel). To demonstrate complex formation between gp130 and SHP2, we reprobed the blot with specific SHP2 antibodies. As shown in the lower panel

Figure 1 SHP2 is dose-dependently and transiently phosphorylated after IL-6 stimulation

(A) HepG2 cells were stimulated for 10 min with IL-6 at the concentrations indicated (100 B-cell stimulatory factor-2 units/ml = 10 ng/ml IL-6). Cells were harvested and cellular extracts were prepared for immunoprecipitation with SHP2 antibodies. Bound proteins were separated by SDS/PAGE and immunoblotted with phosphotyrosine antibodies (upper panel). For loading controls the blot was stripped and reprobed with SHP2 antibodies (lower panel). Proteins were detected by enhanced chemiluminescence. (B) HepG2 cells were stimulated with 200 units/ml IL-6 for the times indicated. Proteins were detected as described for (A). (C) HepG2 cells were stimulated with 200 units/ml IL-6 for the times indicated and harvested. Cellular extracts were prepared for immunoprecipitation with gp130 antibodies (BT12). Bound proteins were separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine antibodies (upper panel). The blot was stripped and reprobed with either gp130 antibodies (BP4, middle panel) for the loading controls, or with SHP2 antibodies to demonstrate gp130/SHP2 association (lower panel). SHP2 protein is indicated by closed arrowheads, gp130 by open arrowheads.

Figure 2 Role of Tyr-759 in gp130 in the activation of SHP2 and STAT3 in stably transfected Ba/F3 cells stimulated with IL-6-sIL-6R complexes

(A) Wild-type Ba/F3-cells (—), stably transfected with expression vectors for gp130 [gp130(YYYYYY)] and for a gp130-mutant (Tyr-759 → Phe) [gp130(YFYYYY)] were stimulated with 200 units/ml IL-6 and 1 µg/ml sIL-6R for 15 min where indicated. Cells were harvested and cellular extracts were prepared for immunoprecipitation with antibodies raised against SHP2. The bound proteins were separated by SDS/PAGE, immunoblotted with phosphotyrosine antibodies (upper part) and for loading controls reprobed with SHP2 antibodies (lower part). Receptor expression was monitored by FACS analysis (results not shown). (B) The wild type (—) and stably transfected Ba/F3 cells [gp130(YYYYYY), gp130(YFYYYY)] described in (A) were stimulated with 200 units/ml IL-6 and 1 µg/ml sIL-6R for the times indicated. Proteins (50 µg) from nuclear extracts were separated by SDS/PAGE and immunoblotted with antibodies specific for Tyr-705-phosphorylated STAT3 (upper part) or antibodies specific for Tyr-701-phosphorylated STAT1 (middle part). The nuclear extracts were also tested for binding a STAT1- and STAT3-specific DNA probe in EMSA. DNA-protein complexes containing STAT3/STAT3 homodimers, STAT1/STAT3 heterodimers and STAT1/STAT1 homodimers are indicated on the right.

of Figure 1(C) a stimulation-dependent SHP2-gp130 complex formation was detected, corresponding to the time-courses of phosphorylation of both gp130 and SHP2.

To demonstrate that SHP2 phosphorylation is due to signalling via gp130, we stably transfected murine Ba/F3-cells, which lack endogenous gp130 [14], with wild-type gp130 or with a gp130 receptor mutant in which the SHP2 recruitment site Tyr-759 [9,24] had been exchanged by Phe. Whereas no stimulation-dependent SHP2 phosphorylation could be observed in untransfected Ba/F3 cells or Ba/F3 cells transfected with the Tyr-759 → Phe mutant receptor, the cells transfected with the wild-type gp130 gene showed tyrosine phosphorylation of SHP2 after IL-6 stimulation (Figure 2A).

To test whether impaired SHP2 activation interferes with STAT1 and STAT3 activation, we tested these Ba/F3 cells also for STAT phosphorylation after stimulation with IL-6-IL-6R

complexes. No STAT activation could be observed in nuclear extracts of untransfected Ba/F3 cells in Western blots with antibodies specific for Tyr-705-phosphorylated STAT3 or antibodies specific for Tyr-701-phosphorylated STAT1 (Figure 2B). In cells expressing the wild-type gp130 we detected transient STAT1 and STAT3 phosphorylation. Interestingly, stimulation of Ba/F3 cells expressing the Tyr-759 → Phe gp130 receptor mutant, which is not able to recruit SHP2, led to enhanced levels of STAT phosphorylation. These observations could be confirmed in EMSA experiments using a STAT1- and STAT3-specific DNA probe (Figure 2B, lower part). In Ba/F3 cells expressing the gp130 receptors we found a stimulation-dependent DNA-protein complex formation containing STAT3 homodimers, and to a lesser extent, STAT1 homodimers or STAT1/STAT3 heterodimers. The amount of STAT-DNA complexes was significantly enhanced in cells expressing the Tyr-759 → Phe gp130 receptor mutant.

Jak requirement for SHP2 phosphorylation

Jak1, Jak2 and Tyk2 have been described to be constitutively associated with gp130 and to become phosphorylated upon stimulation with IL-6 [6,7]. We tested which of the Jaks are required for SHP2 phosphorylation. For this purpose the well-established series of human fibrosarcoma cell lines, each lacking one of the three Jak kinases, and, as controls, cells where the lacking kinase had been re-transfected, were used [16]. The parental fibrosarcoma cell line 2FTGH was used to confirm the phosphorylation of SHP2 after IL-6 stimulation (Figure 3A). Owing to the lack of endogenous IL-6 α-receptors (gp80) these cells were stimulated with IL-6-sIL-6R complexes.

To examine whether Jak2 is required for the IL-6-induced phosphorylation of SHP2 we used the γ2A cells lacking Jak2 and, as a control, the stably Jak2-transfected cells (γ2A/Jak2). In both cell lines we detected a stimulation-dependent phosphorylation of SHP2 (Figure 3B). These results unambiguously demonstrate that Jak2 is not essential for IL-6-stimulated SHP2 phosphorylation. Similar experiments were performed in U1A fibrosarcoma cells which do not express Tyk2 and, for comparison, reconstituted cells (U1A/Tyk2) stably transfected with Tyk2 cDNA. Again, in both cell lines we detected a stimulation-dependent phosphorylation of SHP2, demonstrating that Tyk2 is not required for IL-6-induced phosphorylation of SHP2 (Figure 3C). Interestingly, overexpression of Jak2 or Tyk2, respectively, did not result in enhanced phosphorylation levels of SHP2.

The cell line U4A lacks endogenous Jak1, whereas U4A/Jak1 cells are stably re-transfected with the Jak1 gene. When these two cell lines were used to examine the requirement for Jak1 with regard to SHP2 phosphorylation, the U4A/Jak1 cells expressing Jak1 kinase were able to phosphorylate SHP2 after IL-6 treatment, whereas in the U4A cells lacking Jak1, a strongly impaired SHP2 tyrosine phosphorylation was observed (Figure 3D). Furthermore, we could show that a kinase-inactive Jak1 mutant (Jak1Lys → Glu) [10] is not able to restore SHP2 phosphorylation when stably transfected into U4A cells (U4A/Jak1Lys → Glu) [10] (Figure 3D).

SHP2 association with Jaks

The major role of Jak1, but not Jak2 and Tyk2, in SHP2 phosphorylation might be explained by a preferential interaction of SHP2 with Jak1 after activation of the IL-6R complex. Therefore, we tested the ability of SHP2 to interact with the respective kinases. For this purpose we precipitated SHP2 from the different stimulated and unstimulated fibrosarcoma cells (as described above) and looked for co-immunoprecipitation of the

Figure 3 Jak requirements for SHP2 activation after IL-6 stimulation

(A) 2TGH human fibrosarcoma cells were stimulated with the indicated amounts (units/ml) of IL-6 and 1 μ g/ml of sIL-6R for 15 min. Cells were harvested and cellular extracts were prepared for immunoprecipitation with antibodies raised against SHP2. The bound proteins were separated by SDS/PAGE, immunoblotted with phosphotyrosine antibodies (upper part) and reprobed with SHP2 antibodies for loading controls (lower part). (B) γ 2A fibrosarcoma cells lacking Jak2 (γ 2A) and γ 2A cells stably transfected with Jak2 (γ 2A/Jak2) were incubated without or with the 200 units/ml IL-6 and 1 μ g/ml sIL-6R for 15 min as indicated. SHP2 was detected as described in (A). (C) U1A fibrosarcoma cells lacking Tyk2 (U1A) and U1A cells stably transfected with Tyk2 (U1A/Tyk2) were stimulated and SHP2 was detected as described in (B). (D) U4A fibrosarcoma cells lacking Jak1 (U4A) and U4A cells stably transfected with Jak1 (U4A/Jak1) or with a kinase-inactive Jak1 [U4A/Jak1K $\rightarrow$ E (Lys $\rightarrow$ Glu)] were stimulated and SHP2 detected as described in (B).

various Jaks. To demonstrate the specificity of the co-immunoprecipitated kinases we compared cells lacking one of the kinases with cells reconstituted with the genes for the respective kinases. As expected, no co-immunoprecipitation of Jak1 with SHP2 was visible in the U4A cells. In contrast, in U4A cells reconstituted with the Jak1 cDNA (U4A/Jak1), co-immunoprecipitation of Jak1 with SHP2 was observed, independent of stimulation (Figure 4A, upper panel). Equal amounts of SHP2 were precipitated, as demonstrated by reblotting with anti-SHP2 antibodies (Figure 4A, lower panel). These results demonstrate that SHP2 and Jak1 form complexes before stimulation, whereas SHP2 tyrosine phosphorylation depends on stimulation with IL-6-sIL-6R complexes.

Complex formation of SHP2 with Jak2 and Tyk2 was tested similarly. γ 2A cells and γ 2A cells stably expressing Jak2 (γ 2A/Jak2) were treated as described for U4A and U4A/Jak1 cells. Again, co-immunoprecipitation of SHP2 with Jak2 occurred in the reconstituted cell line, independent of stimulation with IL-6 and sIL-6-R (Figure 4B). Corresponding results were obtained with the U1A and the U1A/Tyk2 reconstituted cells. Tyk2 co-immunoprecipitated with SHP2, independently of stimulation (Figure 4C). As a control of unspecific binding of the Jaks,

co-immunoprecipitation experiments were also performed in the absence of SHP2 antibodies (right lanes in Figure 4).

Role of SHP2 in acute-phase protein gene induction

IL-6 is the major mediator of acute-phase protein expression in the liver [25,26]. Among the various transcription factors of the STAT family, STAT3 turned out to be the most prominent one for the hepatic acute-phase protein induction [27]. To investigate the role of SHP2 in acute-phase protein gene regulation we used two methods to uncouple STAT activation from SHP2 activation. First, we stimulated the IL-6 signalling pathway in HepG2 cells via a gp130 receptor mutant lacking the SHP2 recruitment site (Tyr-759 $\rightarrow$ Phe exchange in gp130; cf. Figure 2). Secondly, we examined the influence of a dominant negative SHP2 on the induction of an acute-phase protein reporter gene.

An Epo-gp130 chimaeric receptor, containing the extracellular domain of the Epo receptor and the intracellular and transmembrane domains of gp130 wild type or the Tyr-759 $\rightarrow$ Phe mutant receptor protein, was used in the following experiments. This chimaeric receptor system allowed us to turn on the IL-6 signalling pathway independently of endogenous gp130 re-

Figure 4 SHP2 association with Jaks

(**A**) U4A fibrosarcoma cells lacking Jak1 (U4A) and U4A cells stably transfected with Jak1 (U4A/Jak1) were incubated in the absence or in the presence of 200 units/ml IL-6 and 1 μ g/ml sIL-6R for 15 min as indicated. Cells were harvested and prepared for co-immunoprecipitation with antibodies raised against SHP2. The bound proteins were separated by SDS/PAGE and probed with Jak1 antibodies (upper part) or SHP2 antibodies for loading controls (lower part). As a control of unspecific binding of the Jaks to the Protein A–Sepharose, co-immunoprecipitation experiments were also performed in the absence of SHP2 antibodies (right-most panel). (**B**) γ 2A fibrosarcoma cells lacking Jak2 (γ 2A) and γ 2A cells stably transfected with Jak2 (γ 2A/Jak2) were incubated in the presence or absence of IL-6 and sIL-6R as described for (**A**). Complex formation of SHP2 with Jak2 was analysed as described in (**A**), but with antiserum to Jak2. (**C**) U1A fibrosarcoma cells lacking Tyk2 (U1A) and U1A cells stably transfected with Tyk2 (U1A/Tyk2) were incubated with IL-6 and sIL-6R and complex formation of SHP2 with Tyk2 was analysed as described in (**A**), but with antiserum to Tyk2. The respective kinases are indicated by open arrowheads and SHP2 by a closed arrowhead.

ceptors. As a read-out system we used the activation of the promoter of the α_2 M gene linked to the luciferase reporter gene. Whereas stimulation via the intracellular wild-type gp130 receptor domain led to significant induction of the reporter gene, the exchange of Tyr-759 for Phe in the Epo–gp130 chimaeric receptor resulted in enhanced gene induction (Figure 5A).

These results suggest an inhibitory activity of phosphorylated SHP2 (or other Tyr-759-dependent activities) on the activation

of the α_2 M gene promoter. To investigate whether this inhibitory effect is due to the phosphatase activity of SHP2, we examined the influence of a dominant-negative mutant of SHP2 (SHP2Cys $\rightarrow$ Ser), lacking phosphatase activity [22,23], on the activation of the α_2 M gene promoter. We compared the stimulation-dependent reporter gene induction in HepG2 cells expressing the reporter gene construct and the Epo–gp130 chimaeric receptor with that of cells additionally cotransfected with expression vectors for wild-type SHP2 or SHP2Cys $\rightarrow$ Ser. Cotransfection of SHP2 or SHP2Cys $\rightarrow$ Ser resulted in strongly enhanced SHP2 protein levels when compared with non-cotransfected cells, as determined by Western blot analysis (Figure 5B, inset, lanes 2 and 3). Stimulation of control transfectants led to a 20-fold induction of the reporter gene construct harbouring the α_2 M gene promoter. Expression of the cotransfected wild-type SHP2 did not significantly alter the reporter gene expression. Interestingly, we observed a further enhanced reporter gene induction when the inactive phosphatase SHP2 (SHP2Cys $\rightarrow$ Ser) was coexpressed (Figure 5B). The only weak but significant increase of reporter gene induction due to SHP2Cys $\rightarrow$ Ser expression can be explained by the fact that only a small proportion of cells expresses the respective protein in the transient transfection system used, whereas the untransfected portion of the cells behaves as a control.

DISCUSSION

In contrast to its homologue SHP1, mainly present in haematopoietic cells, SHP2 is ubiquitously expressed. The recruitment to activated receptors and its subsequent tyrosine phosphorylation have been described for several signal-transduction pathways. SHP2 was found to be phosphorylated after ligand-binding to tyrosine kinase receptors (PDGF, EGF, FGF, stem cell factor, insulin) and G-protein-coupled receptors (angiotensin II) as well as cytokine receptors (IL-2, IL-3, IL-5, IL-6, IL-11, granulocyte-macrophage colony-stimulating factor, interferon α/β , Epo) (for review see [3,16]). The two N-terminal SH2 domains are both involved in receptor binding [28]. Results obtained *in vitro* have demonstrated that binding of the two SH2 domains by phosphotyrosine peptides corresponding to the SHP2 recruitment site in the PDGF-R leads to increased phosphatase activity, suggesting activation of SHP2 after receptor binding [29]. The role of the SHP2 enzymic activity, however, is still obscure. SHP2 phosphatase activity is essential for EGF, but not for PDGF, signalling [30]. Src, c-kit and PDGF-R have been shown to be substrates for SHP2 [31–33]. In some signalling pathways SHP2 acts as an adaptor protein for grb2 [32,34,35], insulin receptor substrate-1 [36,37] and/or grb7 [38]. Interestingly the nature of these protein–protein interactions depends on the signalling pathway. The grb2–SHP2 association after EGF stimulation is not phosphotyrosine-dependent but is mediated by the SH3 domain of grb2 [39]. In contrast, the PDGF-induced grb2–SHP2 interaction occurs via the SH2 domain of grb2 [40].

IL-6-dependent tyrosine phosphorylation of SHP2

The role of the activated STAT factors in the signalling of IL-6-type cytokines is well established. It is realized that SHP2 activation is important in IL-6 signalling, but the underlying mechanisms are presently not clear. However, whereas Stahl et al. [9] and Fukada et al. [13] have demonstrated the involvement of Tyr-759 for SHP2 phosphorylation by use of chimaeric receptors, we present results from experiments in which the native signal transducer gp130 was used and IL-6 stimulation performed. We confirmed the requirement of Tyr-759 in gp130

Figure 5 SHP2 counteracts IL-6-stimulated acute-phase gene induction

(A) HepG2 cells were cotransfected with 4 μ g of an α_2 M-promoter-luciferase reporter construct (pGL3 α_2 M-215Luc) and 4 μ g of a control plasmid (pRc/CMV) or a vector encoding an Epo/gp130 chimaeric receptor gene [pRc/CMV-EG(YYYYYY)] or the Epo/gp130(Y759F) mutant receptor gene [pRc/CMV-EG(YFYYYY)]. Cells were stimulated with 7 units/ml Epo and cellular protein extracts were prepared for the determination of luciferase activity. Luciferase expression was normalized to the luciferase activity in cell extracts from stimulated HepG2 cells transfected with pRc/CMV-EG(YYYYYY). (B) HepG2 cells were cotransfected with an α_2 M-promoter-luciferase reporter gene construct, the Epo-gp130 chimaeric receptor as in (A) and, either 8 μ g of control plasmid [pCBC1(control)] or expression vector for wild-type SHP2 [pCBC1-SHP2 (SHP2)] or a dominant negative mutant of SHP2 [pCBC1-SHP2Cys $\rightarrow$ Ser (SHP2C $\rightarrow$ S)]. Cells were stimulated with 7 units/ml Epo as indicated. Luciferase activity was normalized to that of stimulated control transfectants. Expression of receptor constructs as well as wild-type and dominant-negative SHP2 was monitored by Western blot analysis (insets). The amount of Epo-gp130 chimaeric receptor in untransfected and transfected HepG2 cells was monitored by Western blotting with a polyclonal antibody raised against the intracellular domain of gp130 (inset in A): lane 1, untransfected cells; lane 2, cells transfected with pRc/CMV-EG(YYYYYY); lane 3, cells transfected with pRc/CMV-EG(YFYYYY). Cell-surface expression was monitored by immunofluorescence (results not shown). The amount of SHP2 in untransfected and transfected HepG2 cells was determined by Western blotting with SHP2 antibody (inset in B): lane 1, untransfected cells; lane 2, cells transfected with wild-type SHP2; lane 3, cells transfected with SHP2Cys $\rightarrow$ Ser mutant.

for SHP2 phosphorylation in stably transfected Ba/F3 cells and demonstrated a dose-dependent SHP2 phosphorylation in HepG2 cells after IL-6 treatment. The kinetics of this phosphorylation in human hepatoma cells correlate with those of gp130 activation. We found that both SHP2 and gp130 are transiently phosphorylated, suggesting a direct link between receptor activation and SHP2 phosphorylation. STAT1 and STAT3 activation via gp130 was enhanced and prolonged by the mutation Tyr-759 $\rightarrow$ Phe in the receptor. This demonstrates that impaired SHP2 recruitment and its subsequent phosphorylation interferes with STAT activation. Our observation suggests that STAT3 and STAT1 phosphorylation are regulated by SHP2 activity.

SHP2 phosphorylation requires specific Jaks

Jak1, Jak2 and Tyk2 are constitutively associated with gp130 and become activated after stimulation [6,7]. Jak1 has been shown to play a major role in gp130, STAT1 and STAT3 activation [10]. Here we asked which Jak(s) is/are required for SHP2 activation. A set of well-established human fibrosarcoma cell lines, each lacking one of the Jaks [10], served as a powerful tool to answer this question. In U4A cells, lacking Jak1, only a residual phosphorylation of gp130 was found [10]. The kinases involved in this phosphorylation are still unknown. One explanation could be that Jak2 and Tyk2 phosphorylate gp130 predominantly at Tyr-759, which could result in a normal SHP2 activation in contrast to the impaired activation of STAT1 and

STAT3. This mechanism could not be supported by our experiments, since in the different Jak-deficient fibrosarcoma cells stimulated with IL-6-sIL-6R neither Jak2 nor Tyk2 were essential for SHP2 activation. Overexpression of Jak2 or Tyk2 in Jak2- or Tyk2-deficient cells respectively did not result in enhanced SHP2 phosphorylation. In contrast, cells lacking Jak1 showed drastically reduced SHP2 phosphorylation after stimulation. Jak1-deficient cells, which were reconstituted by stable retransfection with Jak1 cDNA, but not with the inactive Jak1Lys $\rightarrow$ Glu mutant, showed restored SHP2 phosphorylation, demonstrating that Jak1 plays a major role also in the phosphorylation of SHP2.

Our results can be explained in two ways: Jak1 may be needed for the direct phosphorylation of SHP2 or, alternatively, Jak1 is only required for gp130 activation, leading to stimulation-dependent recruitment of SHP2 to gp130 and its phosphorylation by Jak2, Tyk2 or another yet unknown kinase. The unaffected SHP2 phosphorylation in Jak2- or Tyk2-deficient cells could be explained by the assumption that each kinase can substitute for the other.

Jak-SHP2 association

Fuhrer et al. [24] have shown IL-11-induced tyrosine phosphorylation and association of SHP2 with Jak2. In a subsequent paper [41], Jak-SHP2 co-expression experiments demonstrated a constitutive phosphorylation of SHP2 (on Tyr-304 and Tyr-327) by Jak1 and Jak2, and an association of overexpressed SHP2

with Jak1 and Jak2, but not with Jak3, independent of SHP2 tyrosine phosphorylation. Interestingly, an association of endogenous SHP2 with transfected Jaks could not be detected [41]. Here we have demonstrated in fibrosarcoma cells the constitutive association of SHP2 with Jak1, Jak2 and Tyk2. Stimulation with IL-6-sIL-R complexes did not influence this protein-protein interaction.

Yin et al. [41] have shown the potential of Jak1 and Jak2 to phosphorylate SHP2 in a constitutively activated cellular system. In the present study we found that neither Jak2 nor Tyk2 is essential for stimulation-dependent SHP2 phosphorylation. Both kinases were unable to phosphorylate SHP2 in cells lacking Jak1. These results cannot be explained by the inability of both kinases to interact with SHP2, since the observed constitutive association of SHP2 with Jak1, Jak2 or Tyk2 did not lead to a constitutive SHP2 phosphorylation. The mechanism involved after ligand-binding to the receptor complex might simply be the activation of Jak1; but alternative explanations as mentioned above are possible.

Role of SHP2 in gene regulation

The role of SHP2 in acute-phase protein gene regulation mediated by IL-6 still has to be elucidated. We have demonstrated an inhibitory effect on gene induction depending on the SHP2 recruitment site in gp130. Since the exchange of Tyr-759 for Phe within this motif led to impaired SHP2 phosphorylation and enhanced inducibility of a reporter gene construct harbouring the α_2 M promoter, we conclude that SHP2 acts negatively on the IL-6 signal-transduction pathway. Whether this is due to the adaptor function of SHP2 or its enzyme activity has to be clarified. In the case of the IL-6 signalling pathway, substrates for the SHP2 enzyme are not known so far.

To investigate whether the inhibitory effect on gene induction is due to the phosphatase activity of SHP2, we overexpressed a phosphatase inactive SHP2Cys $\rightarrow$ Ser mutant to compete with endogenous wild-type SHP2. In our reporter gene assay we observed enhanced gene induction in HepG2 cells transiently transfected with the cDNA coding for this inactive SHP2Cys $\rightarrow$ Ser mutant. Therefore, our results suggest that the stimulation-dependent inhibitory effect of SHP2 is due to its phosphatase activity.

Symes et al. [42] also found Tyr-759 in gp130 to be inhibitory for gene induction and, similar to our results, these authors were able to enhance gene induction by the expression of either the N- or the N- plus C-terminal SH2 domains of SHP2. These protein fragments simply compete with SHP2 for receptor interaction and thus inhibit SHP2 recruitment and phosphorylation. Both, the N-terminal SH2 domain and the N- plus C-terminal SH2 domains lack almost two-thirds of the protein, including the phosphatase domain and potential phosphorylation sites, which may act as docking sites for adaptor proteins. Even Tyr-304 and Tyr-327, which have been described as being phosphorylated by Jak1 and Jak2, and one of them (Tyr-304) as acting as a binding site for grb2 [41], are not present. Therefore, it is not clear whether these SHP2 fragments act as dominant-negative proteins due to the lack of docking sites for adaptor proteins or due to the lack of phosphatase activity. Other C-terminal structural features lacking in these SHP2 fragments could also have been responsible for the inhibitory effect on gene induction described by Symes et al. [42].

In our experiments we focused on the role of SHP2 enzyme activity. We found that the SHP2Cys $\rightarrow$ Ser mutant lacking phosphatase activity is less able to negatively control acute-phase promoter/reporter gene induction. Since, due to a single amino

acid substitution, our dominant-negative SHP2 lacks phosphatase activity our results allow the further conclusion that SHP2 enzyme activity is involved in and acts negatively on acute-phase protein gene induction. However, the role of the adaptor function of SHP2 in gene regulation still has to be worked out.

We thank Axel Ullrich (MPI Martinsried) for providing the SHP2 cDNA, Marcel Robbertz for his help with the artwork, and Lutz Graeve for critical reading of this manuscript. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Bonn, Germany), the DAAD (ARC-program), the Fonds der Chemischen Industrie (Frankfurt, Germany) and by a grant from the British Council (ARC697) to I.M.K.

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Received 14 May 1998/28 July 1998; accepted 14 August 1998