The role of the inhibitors of interleukin-6 signal transduction SHP2 and SOCS3 for desensitization of interleukin-6 signalling

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The immediate early response of cells treated with IL-6 (interleukin-6) is the activation of the signal transducer and activator of transcription (STAT)3. The Src homology domain 2 (SH2)-containing protein tyrosine phosphatase SHP2 and the feedback inhibitor SOCS3 (suppressor of cytokine signalling) are potent inhibitors of IL-6 signal transduction. Impaired function of SOCS3 or SHP2 leads to enhanced and prolonged IL-6 signalling. The inhibitory function of both proteins depends on their recruitment to the tyrosine motif 759 within glycoprotein gp130. In contrast to inactivation, desensitization of signal transduction is regarded as impaired responsiveness due to pre-stimulation. Usually, after activation the sensing receptor becomes inactivated by modifications such as phosphorylation, internalization or degradation. We designed an experimental approach which allows discrimination between desensitization and inactivation of IL-6 signal transduction. We observed that pre-stimulation with IL-6 renders cells less sensitive to further stimulation with IL-6. After several hours, the cells become sensitive again. We show that not only signal transduction through previously activated receptors is affected by desensitization but signalling through receptors which were not targeted by the first stimulation was also attenuated (*trans*-desensitization). Interestingly, in contrast to inhibition, desensitization does not depend on the presence of functional SHP2. Furthermore, cells lacking SOCS3 show constitutive STAT3 activation which is not affected by pre-stimulation with IL-6. All these observations suggest that desensitization and inhibition of signalling are mechanistically distinct.

Key words: inflammation, interleukin-6, signal transducer and activator of transcription 3 (STAT3), signal transduction, Src homology domain 2-containing protein tyrosine phosphatase (SHP2), suppressor of cytokine signalling 3 (SOCS3).

INTRODUCTION

IL-6 (interleukin-6) is a cytokine with a wide spectrum of activities. It is the major regulator for the expression of acute-phase protein genes in liver cells [1–4] but it also stimulates the differentiation of B- and T-cells and the proliferation of keratinocytes, mesangial cells and plasmacytoma cells, whereas the proliferation of breast carcinoma cells and also melanoma cells is inhibited (for a review see [5]). IL-6 exerts its action by binding and activating a receptor complex composed of a specific α-receptor [IL-6Rα, gp (glycoprotein)80, CD126] and the signal-transducing subunit gp130 (CD130) [6–8]. Ligand binding to the α-receptors leads to dimerization of gp130 and activation of constitutively associated Janus kinases JAK1, JAK2 and Tyk2 [9,10]. In turn, gp130 becomes tyrosine phosphorylated at its cytoplasmic tail and recruits transcription factors of the family of STAT (signal transducers and activators of transcription), STAT1 and STAT3, to specific phosphotyrosine motifs [11,12]. Subsequently, STAT factors are also tyrosine-phosphorylated, dissociate from the receptor complexes and translocate to the nucleus where STAT homo- and/or heterodimers bind to specific DNA elements in the promoters of IL-6 target genes [13,14].

Negative regulation of signalling is as important as the activation of signalling pathways (for reviews see [15,16]). Several mechanisms are proposed to be involved in the termination of IL-6 signal transduction: (i) gp130 is internalized ligand-independently. After formation of the IL-6-ternary receptor complex, IL-6Rα co-internalizes with gp130 [17–19]. (ii) Protein tyrosine phosphatase SHP2 [SH2 (Src homology domain 2)-containing protein tyrosine phosphatase] is recruited to the phosphotyrosine 759 motif of activated gp130 [11] and counteracts receptor and STAT activation as well as IL-6-induced gene induction [20–22]. (iii) The IL-6-induced feedback-inhibitor SOCS (suppressor of cytokine signalling) 3 is recruited to the same site in gp130 and inhibits JAK activity. SOCS1 acts similarly but does not require the receptor-tyrosine motif 759 [23,24]. Recent investigations have shown SOCS3 to be a central regulator for IL-6 signalling *in vivo* and suggest that SOCS3 and SOCS1 have reciprocal functions in IL-6 and interferon-γ signal transduction [25–27]. (iv) Protein inhibitors of activated STATs (PIAS) bind specifically to activated STAT dimers and attenuate activity [28,29]. (v) Proteasomal degradation of STAT factors [30] and (vi) STAT-inactivating phosphatase [31–33] in the nucleus have been described. (vii) Finally, nuclear export appears to negatively regulate JAK/STAT-dependent gene induction [34–36].

In the past, inhibition of cytokine signalling has mainly been studied by analysing the time-dependent disappearance of activated signalling components in long-term kinetic studies. For

Abbreviations used: Epo, erythropoietin; EpoR, Epo receptor; gp, glycoprotein; IL-6, interleukin-6; IL-6Rα, IL-6 α-receptor; sIL-6R, soluble IL-6R; JAK, Janus kinase; STAT, signal transducer and activator of transcription; SH2, Src homology domain 2; SHP, SH2-containing protein tyrosine phosphatase; SOCS, suppressor of cytokine signalling; DMEM, Dulbecco's modified Eagle's medium; MEF, murine embryonal fibroblast; EMSA, electrophoretic mobility shift assay; HRP, horseradish peroxidase; OSM, oncostatin M.

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example, the mutation of the inhibitory tyrosine-759 in gp130 leads to prolonged STAT activation and enhanced gene induction of IL-6-dependent genes [20,21,37].

In contrast, desensitization of signalling is observed as an unresponsiveness to cytokine stimulation immediately after a first stimulation. In this study we present an experimental approach which allows us to discriminate desensitization and inactivation of IL-6 signal transduction. We explored the mechanisms leading to the desensitization of IL-6-signal transduction and asked (i) whether desensitization is due to receptor disappearance or inactivation after ligand binding, (ii) whether only pre-activated receptors are refractory (*cis*-desensitization) or whether pre-stimulation also affects subsequent signalling through non-pre-stimulated receptors (*trans*-desensitization). (iii) We analysed the level in the signal cascade at which desensitization occurs. (iv) Finally, we investigated whether signalling is desensitized through the IL-6 signalling inhibitors SHP2 and SOCS3.

Interestingly, in contrast to many other signalling pathways, down regulation of surface expression of receptor subunits is not responsible for desensitization. Consequently, signalling through receptors which were not pre-activated is attenuated (*trans*-desensitization). Cells lacking functional SHP2 are also desensitized after stimulation with IL-6, whereas the lack of SOCS3 leads to constitutive STAT3 activation which is not affected by pre-activation of the IL-6 signalling pathway. These observations further indicate that desensitization and inhibition of IL-6 signalling should be considered as different mechanisms counteracting signal transduction.

EXPERIMENTAL

Materials

Restriction enzymes were purchased from Roche Molecular Biochemicals (Mannheim, Germany); *Taq* polymerase was from Hybaid (Heidelberg, Germany); oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany); DMEM (Dulbecco's modified Eagle's medium) and DMEM/F12 medium were from Life Technologies (Eggstein, Germany); fetal calf serum was from Seromed (Berlin, Germany); recombinant human IL-6 and sIL-6R (soluble IL-6R) gp80 were prepared as described [38]. Recombinant Epo (erythropoietin) was a gift of Dr J. Burg and Dr K. H. Sellinger (Roche, Mannheim, Germany). Recombinant IL-5 was purchased from Cell Concepts (Umkirch, Germany). The internal control plasmid DNA pCH110 was from Amersham Bioscience (Uppsala, Sweden) and actinomycin D was from Sigma-Aldrich (Taufkirchen, Germany). Antibodies for detection of SOCS3 in Western blots were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). These antibodies were biotinylated as described by Pierce (Rockford, IL, U.S.A.). Antibodies raised against JAK1 were from Pharmingen (Heidelberg, Germany). Antibodies to the Tyr-705-phosphorylated STAT3 were obtained from New England BioLabs (Beverly, MA, U.S.A.). Antibodies to the extracellular region of gp130 (BP8) and to IL-6Rα (gp80) (B-N12) were gifts from Dr J. Wijdenes (Diaclone, Besançon, France). Phosphotyrosine-specific antibodies (4G10) and antibodies to the cytoplasmic region of gp130 were purchased from Upstate Laboratories (Lake Placid, NY, U.S.A.). The polyclonal antiserum against JAK1 was kindly supplied by Dr A. Ziemiecki (University of Bern, Switzerland).

Preparation, cultivation and stimulation of cells

Human HepG2 hepatoma cells were grown in DMEM/nut. mix F12 and MEFs (murine embryonal fibroblasts) were grown in DMEM. Both media were supplemented with 10 % (20 % for SHP2-mutated cells) fetal calf serum, streptomycin (100 mg/l), and penicillin (60 mg/l). SOCS3-deficient cells were generated by infecting MEFs from homozygote SOCS3-floxed mice with Cre-recombinase-expressing adenovirus. Infection was repeated until no SOCS3-floxed alleles were detectable by genomic PCR as described previously [25]. MEFs stably expressing chimaeric EpoR (Epo receptor)–gp130 or Epo–gp130(Y759F) fusion proteins were generated by retroviral gene transfer (pM5-EpoR/gp130-NeoGFP) and subsequent selection on G418-containing cell culture medium. Expression of the chimaeric receptors was monitored by Western blotting (results not shown). Medium was changed and adjusted to 5 ml 24 h before experiments were carried out. Cells grown in a 100 mm dish were stimulated with IL-6, Epo or IL-5 at the concentrations indicated. Nuclear extracts were prepared as described previously [39]. Protein concentration was determined using a Bio-Rad (Munich, Germany) protein assay.

EMSA (electrophoretic mobility shift assay)

EMSAs were performed as described previously [13]. The protein–DNA complexes were separated on a 4.5 % polyacrylamide gel containing 7.5 % glycerol in 0.25-fold TBE (20 mM Tris base, 20 mM boric acid and 0.5 mM EDTA, pH 8.0) at 20 V/cm for 4 h. Gels were fixed in 10 % methanol, 10 % acetic acid and 80 % water for 1 h, dried and autoradiographed. The double-stranded STAT3-specific ^{32}P-labelled oligonucleotide used was a mutated m67SIE-oligonucleotide from the c-*fos* promoter (m67SIE: 5′-GATCCGGGAGGGATTTACGGGAA-ATGCTG-3′) [40]. The results shown are representative for at least three independent similar experiments.

Plasmids

Standard cloning procedures were performed as outlined by Sambrook and Russell [41]. Vectors encoding chimaeric IL-5R–gp130 fusion proteins were described previously [37]. The chimaeric IL-5R–gp130 receptors are composed of the extracellular IL-5Rα or IL-5Rβ region and the transmembrane and intracellular parts of gp130 [IL-5Rα/gp130(YYYYYY) and IL5-Rβ/gp130(YYYYYY)) or mutants of gp130 (IL-5Rα/gp130(Y**F**YYYY) and IL5-Rβ/gp130(Y**F**YYYY)] where a phenylalanine residue (in bold) substitutes for tyrosine-759 of gp130. To allow expression in the human hepatoma cell line HepG2, these constructs were inserted into the pRcCMV expression vector (Invitrogen, Groningen, The Netherlands) to give pRcCMV-IL-5Rα/gp130(YYYYYY), pRcCMV-IL-5Rα/gp130(Y**F**YYYY), pRcCMV-IL-5Rβ/gp130(YYYYYY), and pRcCMV-IL-5Rβ/gp130(Y**F**YYYY). pRcCMV-EpoR/gp130(YYYYYY) and pRcCMV-EpoR/gp130(Y**F**YYYY) are expression vectors for EpoR–gp130 chimaeric receptor constructs containing the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130 and were described previously [42]. The retroviral vector pM5-EpoR/gp130-NeoGFP is based on the MPSV (myeloproliferative sacrome virus). The cDNAs were cloned in a bicistronic configuration into the multiple cloning site: the cDNA encoding an EpoR–gp130 chimaeric receptor is followed by the internal ribosomal entry site of the human NRF gene (negative regulatory factor gene; kindly provided by Dr H. Hauser, Braunschweig, Germany [43]) and the cDNA for an enhanced green fluorescent protein–neomycin-resistance fusion protein. Two different retroviral vectors were generated to express EpoR/gp130(YYYYYY) or EpoR/gp130(Y**F**YYYY)

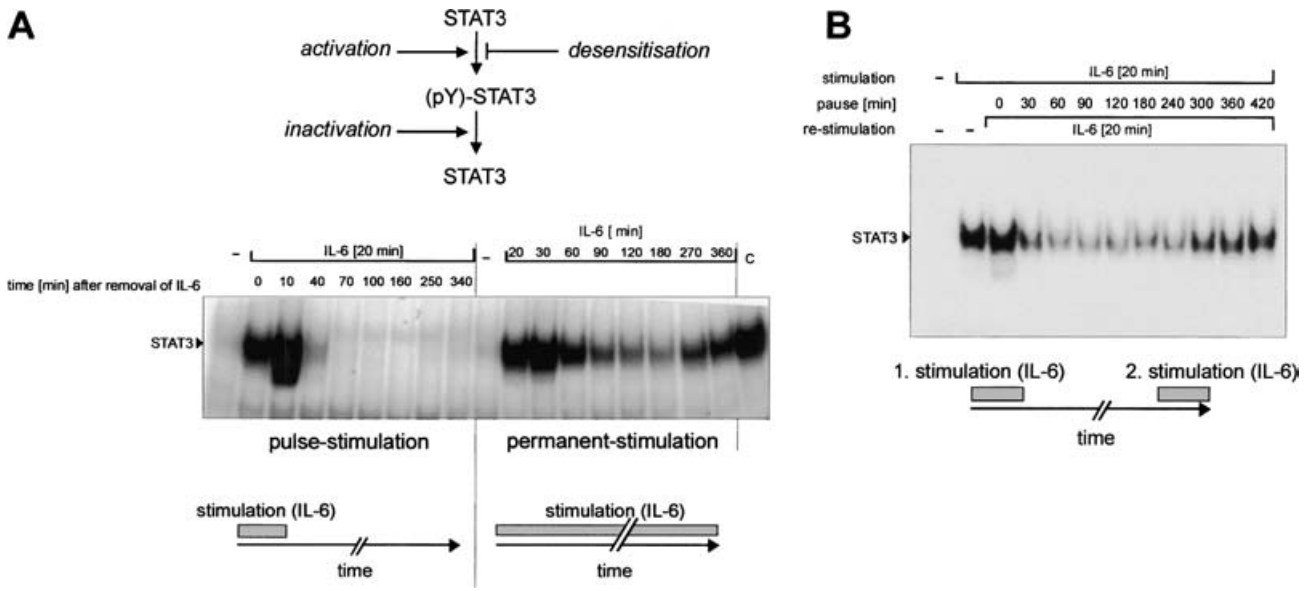

Figure 1 Kinetics of pulse stimulation, permanent stimulation and desensitization

(**A**) Kinetics of pulse stimulation and permanent stimulation of HepG2 cells with IL-6. HepG2 cells were stimulated with 100 units/ml IL-6 for the times indicated. Left-hand panel: medium was removed after stimulation and cells were incubated in fresh, IL-6-free medium for the times indicated before harvesting the cells. For determination of STAT3 activation, nuclear extracts were prepared and equal amounts of protein were analysed by an EMSA with a STAT3-specific probe (m67SIE), as described in the Experimental section. Lane C, supernatant from cells incubated for 6 h with IL-6 was transferred to untreated HepG2 cells for 20 min. The upper part of the figure illustrates schematically the difference between inhibition of signalling and desensitization. (**B**) Pre-stimulation induces desensitization of IL-6-induced STAT-activation. HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min (lane 2). For the kinetic analysis (lanes 3–12) medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Finally cells were treated a second time with 100 units/ml IL-6 for 20 min before they were harvested and STAT3 activation was determined as described for (**A**).

[42]. These chimaeric receptors contain tyrosine → phenylalanine substitutions within the cytoplasmic part of the receptor as indicated. The sequences of all constructs were verified by fluorescence sequencing.

Transfection procedure

For transfection of HepG2 cells, cells were grown on 60 mm dishes to 30 % confluency and transfected in DMEM supplemented with 10 % fetal calf serum. Calcium phosphate precipitates were adjusted with control vectors to equal amounts of DNA. Cells were incubated with the precipitate for 16 h, washed twice with PBS and left for additional 10–24 h in fresh medium.

Northern blotting analysis

Total RNA was isolated from cultured HepG2 cells by using the Qiagen total RNA kit (Qiagan, Hilden, Germany) according to manufacturer's instructions. Gel electrophoresis and Northern blot analysis were performed with 10 μg of total RNA using a ^{32}P-labelled murine SOCS3 cDNA [44].

Immunoprecipitation and immunoblot analysis

For immunoprecipitation, 2×10^7 cells were lysed in 500 μl of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 % Triton X-100, 10 % glycerol, 1 mM EGTA and 1.5 mM $MgCl_2$). Before adding antibodies protein amounts were adjusted to be equal. Buffers were supplemented with 10 μg/ml of each aprotinin, pepstatin and leupeptin. Immune complexes were separated by SDS/PAGE and transferred on to a PVDF membrane. Antigens were detected by incubation with specific primary antibodies and HRP (horseradish peroxidase)-coupled secondary antibodies (Dako, Hamburg, Germany). SOCS3 detection was performed with biotinylated SOCS3-specific primary antibodies and HRP-coupled streptavidin (1:5000; Pierce). The membranes were developed with an enhanced chemiluminescence kit (Amersham Biosciences).

FACS analyses

Cells (5×10^5) were harvested and washed with FACS buffer (PBS containing 5 % fetal calf serum and 0.1 % NaN_3). The cells were re-suspended in 100 μl of ice-cold FACS buffer and incubated with 1 μg of antibodies raised against the extracellular part of the respective receptors. After washing, mouse IgG-specific phycoerythrin-conjugated goat antibodies were added and receptor expression was monitored using a FACScalibur (Becton Dickinson, Heidelberg, Germany). IL-6 binding to the cell surface was analysed by using the IL-6-Fluorokine kit of R&D Systems (Minneapolis, MN, U.S.A.) as described by the manufacturer. Briefly, binding of biotinylated IL-6 to cell-surface receptors was monitored with avidin-conjugated fluorescein using the FACScalibur.

RESULTS

Kinetics of pulse stimulation and permanent stimulation

IL-6-induced STAT activation is known to appear transiently. STAT inactivation is currently proposed to be mediated by phosphatases as well as by degradation [30–33]. In the present study, we have compared the kinetics of STAT3 activation after short-term stimulation with those after continuous IL-6 stimulation (Figure 1). STAT3 activity was monitored by an EMSA

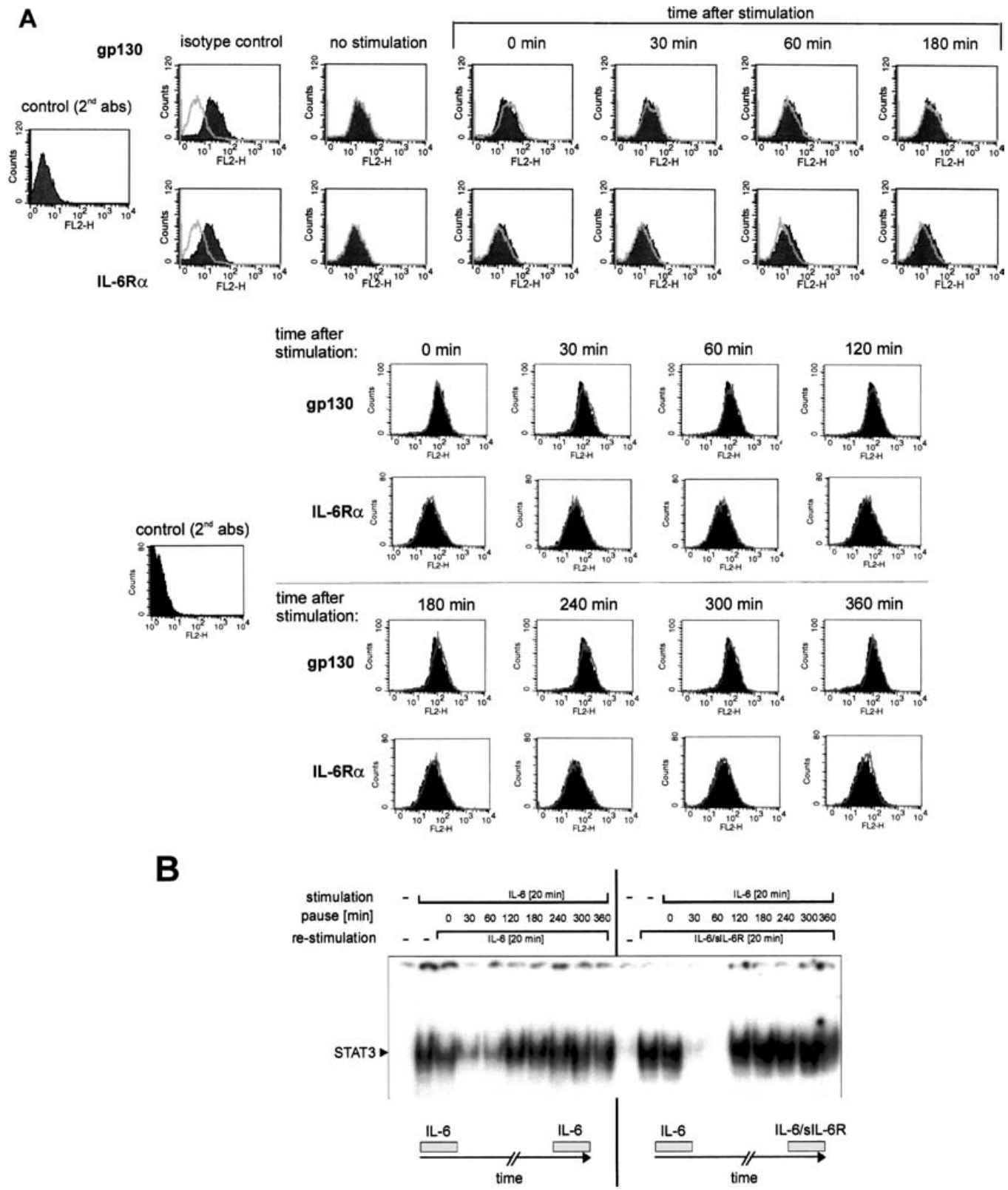

Figure 2 For legend see facing page

where binding to a specific STAT3 probe was measured. Stimulation of HepG2 cells with IL-6 for 20 min led to a transient STAT3 activation with a delayed peak 10 min after removal of the cytokine. The decrease in DNA-binding activity 40 min post-stimulation indicates that STAT3 is also rapidly inactivated (Figure 1A, left-hand panel). In contrast, the continuous presence

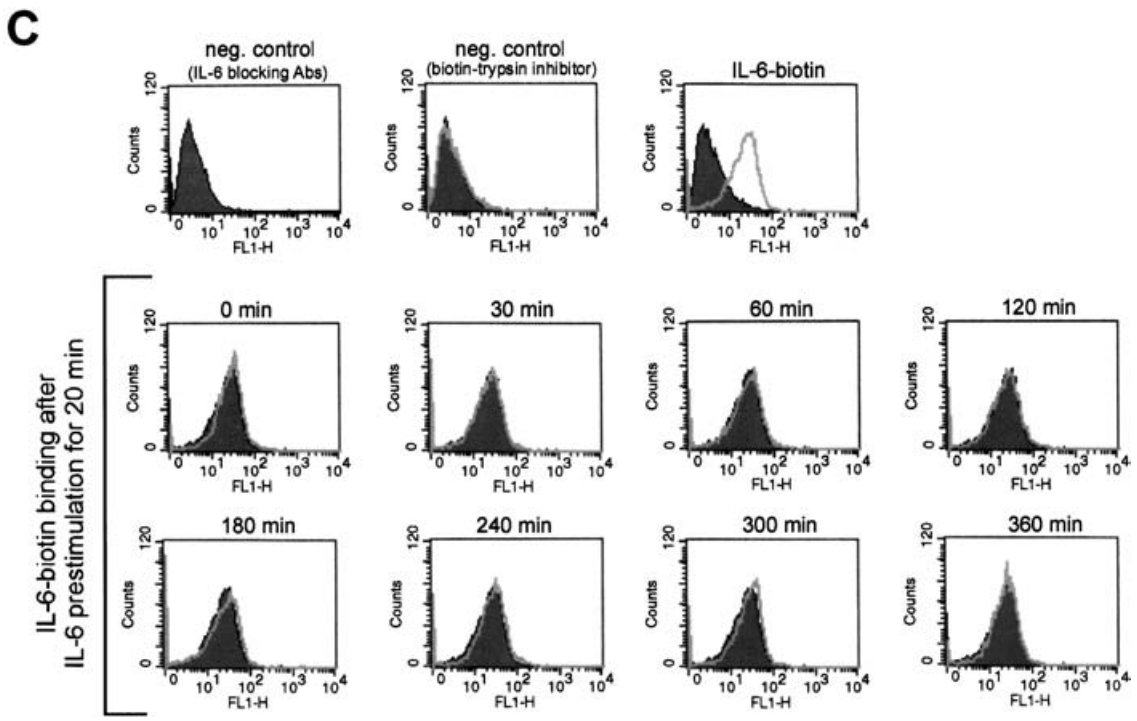

Figure 2 Surface expression of receptor components after stimulation with IL-6

(**A**) HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min. Medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Surface expression of the gp130 and the IL-6Rα (gp80) was monitored by FACS analyses, using antibodies raised against the extracellular parts of these receptor subunits (open histograms). Filled histograms show FACS controls before stimulation with IL-6. A FACS control with a secondary antibody only is shown on the left (control 2nd abs). The isotype control was performed with antibodies against STAT3. FL2-H, fluorescence intensity. The lower part represents more detailed kinetics and was performed essentially as in the upper part. (**B**) Surplus of sIL-6Rα does not overcome desensitization. HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min (lanes 2 of both parts of the Figure). For the kinetic analyses (lanes 3–10, both parts) medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Finally cells were treated a second time with 100 units/ml IL-6 plus 0.4 μg/ml sIL-6Rα for 20 min before they were harvested and STAT3 activation was determined as described in the legend to Figure 1(**A**). (**C**) HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min. Medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Afterwards binding of biotinylated IL-6 to the cell surface was monitored by FACS analyses using phycoerythrin-conjugated streptavidin (open histograms). Filled histograms show binding of biotinylated IL-6 without pre-stimulation. For controlling specificity an IL-6 blocking antibody was used to block binding of biotin-IL-6 to the cell surface (left-hand negative control). Additionally biotin-labelled soya bean trypsin inhibitor was used instead of biotinylated IL-6 (open histogram, right-hand negative control). For comparison, biotin–IL-6 binding was analysed without pre-stimulation (IL-6-biotin).

of IL-6 did not lead to persistence of STAT3 activation (Figure 1A, right-hand panel), i.e. STAT–DNA-binding decreased after 1 h and remaind lower for the following 3 h followed by restored sensitivity towards IL-6. We tested whether the decrease in STAT3–DNA-binding might be due to a loss of IL-6 activity in the culture medium during long-term stimulation. Therefore the conditioned medium of HepG2 cells incubated with IL-6 for 6 h was transferred to untreated HepG2 cells for 20 min and STAT3 activity monitored by EMSA (Figure 1A, lane C). Since STAT3 activation was as prominent as with fresh IL-6 (compare with 20 min stimulation in Figure 1A, lane 2) the reduced STAT activity after 1 h of stimulation was not due to reduced IL-6 activity in the culture medium. Thus, the kinetics of STAT3 activation in case of continuous stimulation depends on STAT inactivation as well as desensitization of signal transduction (see the scheme in Figure 1A).

IL-6 stimulation induces desensitization for a subsequent IL-6-induced STAT activation

To determine the kinetics of desensitization, the effect of pausing between first and second stimulation on STAT activation measured by STAT3–DNA-binding activity in an EMSA was analysed (Figure 1B). We observed that immediately after a first stimulation with IL-6 for 20 min (Figure 1B, lane 2, reading from the left) human hepatoma cells (HepG2) are refractory to a second IL-6 stimulus (Figure 1B, lanes 4–12). After stimulation of HepG2 cells STAT3 remains active for at least 30 min. Therefore the shortest break between pre-stimulation and stimulation had to last at least 30 min. After a pause of 30 min, there is probably some weak STAT activity remaining from the first stimulation (Figure 1B, lane 4). Nevertheless, Figure 1(B) shows that IL-6 signalling is refractory for at least 4 h after the first stimulation.

Altered surface expression of receptor components is not responsible for desensitization

The simplest explanation for desensitization would be the disappearance of the specific receptor subunits. To monitor cell surface expression of gp130 and IL-6Rα during the different times of pausing, FACS analyses were performed with antibodies specific for the extracellular parts of gp130 and IL-6Rα. Figure 2(A) shows that no significant disappearance of either transmembrane receptors occurred during the period analysed (360 min, Figure 2A).

The presence of soluble agonistic IL-6Rα allows signalling independent of IL-6Rα surface expression. To confirm the

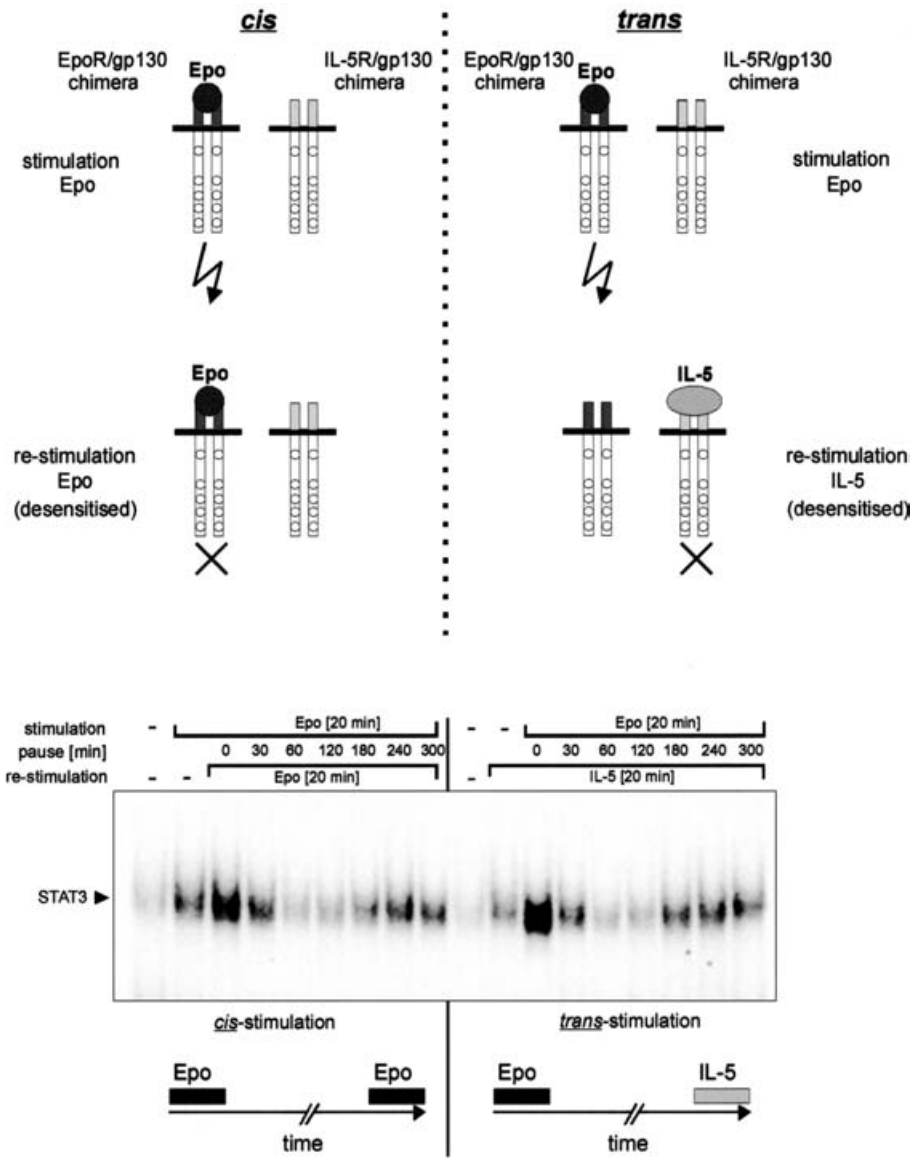

Figure 3 Desensitization of non-pre-activated receptors

HepG2 cells where transfected with vectors encoding the chimaeric receptors EpoR–gp130 and IL-5R–gp130. The first stimulation was performed with 7 units/ml of Epo for 20 min. After pausing for the times indicated, a second stimulation was carried out with 7 units/ml Epo (left part) or 80 ng/ml IL-5 (right part) for 20 min. STAT3 activation was measured as described in the legend to Figure 1. The scheme illustrates the order of activating the two different chimaeric receptors in *cis* (left) or in *trans* (right).

previous results we also analysed desensitization in presence of a surplus of agonistic sIL-6Rα to overcome potential depletion of IL-6Rα by internalization. As shown in Figure 2(B) the addition of sIL-6Rα into medium enhances the amount of activated STAT3 but does not overcome desensitization, indicating that desensitization is not due to depletion of IL-6Rα. The more efficient desensitization in cells stimulated with IL-6/sIL-6R may be due to the enhanced STAT3 activation, assuming that STAT3 activation is crucial for desensitization.

Finally, we monitored whether pre-stimulation of HepG2 cells with IL-6 affects subsequent binding of IL-6 to the cell surface. We analysed whether binding of biotinylated IL-6 to the cells alters in response to pre-stimulation. The FACS analyses in Figure 2(C) show that through the whole time period of the experiment binding of biotinylated IL-6 (open histograms in the kinetics) is not affected by previous stimulation with the cytokine (filled histograms in the kinetics). All these results further corroborate the data in Figure 2(A), indicating that desensitization is not mediated by receptor internalization and are in line with previous work showing that internalization of gp130 is not dependent on signalling [17–19].

Desensitization is mediated through *trans*-acting factors

We asked whether desensitization is due to a specific modification of the activated receptor complexes resulting in insensitive receptor complexes (*cis*-desensitization) or whether signalling through receptors which have not been activated during pre-stimulation is also affected (*trans*-desensitization). The latter mechanism requires the presence of *trans*-acting factors which

counteract signalling through pre-activated receptors as well as through non pre-activated receptors. To answer this question, we had to achieve stimulation of two populations of receptors independently. Therefore, two sets of different chimaeric receptors known to mimic signalling through gp130 were used (Figure 3, scheme). Chimaeric receptors composed of the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130 as well as chimaeric receptors containing the extracellular parts of IL-5Rα or IL-5Rβ and the transmembrane and cytoplasmic parts of gp130 were expressed in HepG2 cells. Pre-stimulation was performed by addition of Epo into the culture medium and incubation for 20 min. The effect of pre-stimulation on a subsequent stimulation of the same EpoR–gp130 chimaeric receptors (*cis*-desensitization) as well as through non-pre-activated IL-5R–gp130 chimaeras (*trans*-desensitization) was analysed. Figure 3 clearly shows that pre-stimulation with Epo leads to desensitization of both chimaeric receptor complexes. The kinetics of refraction are almost identical for *cis*- (left-hand panel) and *trans*-stimulation (right-hand panel). From these results we conclude that desensitization of IL-6 signalling does not function through a specific receptor inactivation but through *trans*-active inhibitory factors. Also these results corroborate the data presented in Figure 2 which indicate that desensitization is not mediated by receptor internalization, since stimulation-dependent internalization would not lead to *trans*-desensitization.

STAT3 and JAK1 activation are affected by desensitization

To elaborate at which level of the IL-6 signal-transduction cascade activation of signalling components is blocked, we monitored STAT, gp130 and JAK1 tyrosine phosphorylation by Western blotting using the same time course for stimulation as in the experiments described above. Figure 4 shows that, after stimulation with IL-6, STAT and gp130 tyrosine phosphorylation as well as that of JAK1 are decreased due to pre-treatment of the cells with IL-6. Thus desensitization most probably affects STAT3 activity through impaired JAK1 activation.

The inhibitory tyrosine-759 of gp130 is crucial for desensitization

The tyrosine-759 of gp130 mediates inhibitory functions of SHP2 and SOCS3 on gp130-dependent signal transduction [22,23]. Therefore, we analysed whether desensitization occurs through a tyrosine-759-dependent inhibitory mechanism. Desensitization of signalling through EpoR–gp130 chimaeric receptors or EpoR–gp130 chimaeras containing a tyrosine-759-to-phenylalanine substitution within the gp130 cytoplasmic part was analysed in cells stably expressing the corresponding receptors. Cells were pre-stimulated with IL-6/sIL-6R for 20 min. Subsequently, the effect of pre-stimulation on a following signal transduction through the wild-type EpoR–gp130 chimaera or the corresponding Y759F mutant was analysed. Figure 5 shows that the strong Epo-mediated STAT activation (lanes 3 and 13, reading from the left) is affected by IL-6 pre-stimulation only in cells expressing the wild-type EpoR/gp130(YYYYYY) but not the mutated EpoR/gp130(Y**F**YYYY) receptor (lanes 4–10 or 14–20, respectively). Thus the inhibitory tyrosine-759 within gp130 makes the receptor sensitive towards *trans*-desensitization, indicating that tyrosine-759 of gp130 is crucial for desensitization.

SHP2 is not involved in desensitization

JAK/STAT and gp130 activation could be counteracted via tyrosine-759 in gp130 by the protein tyrosine phosphatase SHP2

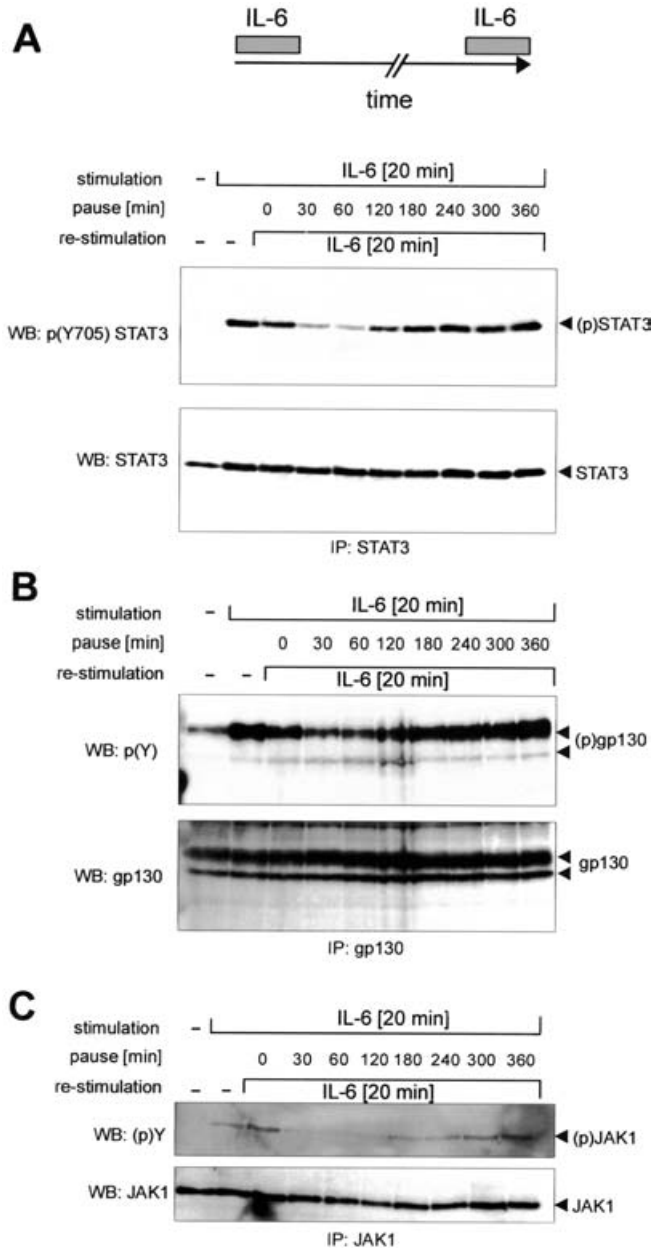

Figure 4 STAT3, gp130 and JAK tyrosine phosphorylation during desensitization

HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min. Medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Finally, cells were treated a second time with 100 μg/ml IL-6 for 20 min and cellular extracts were prepared. Lysates were incubated with antibodies against STAT3 (**A**), gp130 (**B**) or JAK1 (**C**). Protein–antibody complexes were separated by SDS/PAGE and analysed by Western blotting (WB) with the antibodies specific for activated STAT3 [p(Y705)STAT3] (**A**) or phosphotyrosine (pY; **B** and **C**; upper panels). For monitoring loading of the gel, blots were re-probed with antibodies against STAT3, gp130 or JAK1, respectively (lower panels).

or by SOCS inhibitors. To analyse whether the protein tyrosine phosphatase SHP2 is involved in desensitization, studies in MEFs lacking functional wild-type SHP2 [45] were performed. These cells express an N-terminally truncated SHP2 lacking the amino acids 46–110 within the N-terminal SH2 domain. Impaired recruitment of SHP2 to gp130 led to enhanced IL-6 signalling in these cells [22]. In both wild-type cells and in cells expressing

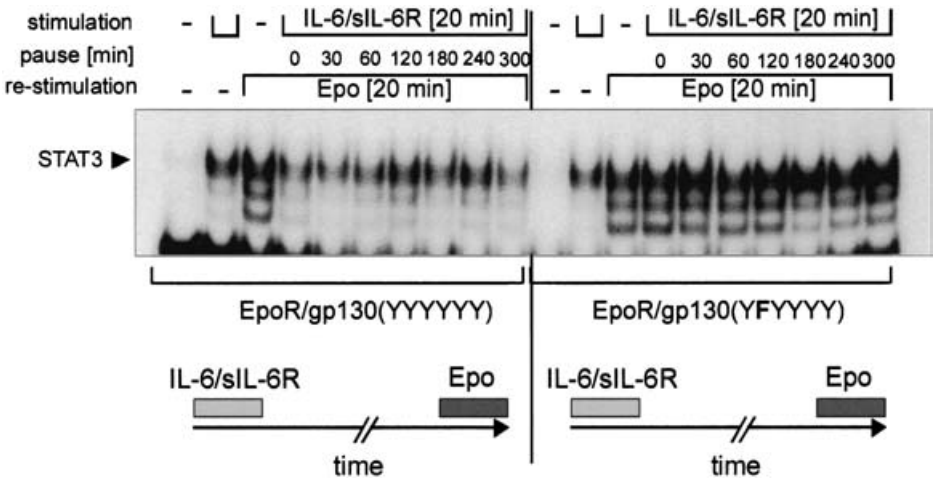

Figure 5 Involvement of the inhibitory tyrosine-759 within gp130 in desensitization

Murine embryonal fibroblasts transduced with retroviral vectors encoding the chimaeras EpoR/gp130(YYYYYY) (right-hand panel) or EpoR/gp130(Y**F**YYYY) (left-hand panel). Cells were stimulated with Epo (lanes 3 or 13, reading from the left). Desensitization of EpoR–gp130-mediated signalling was analysed after pre-stimulation with 100 units/ml IL-6 and 1 μg/ml sIL-6R (lanes 4–10 or 14–20). After pausing for the times indicated, the second stimulation was done with 7 units/ml Epo for 20 min. Stimulation with 100 units/ml IL-6 and 1 μg/ml sIL-6R alone is shown in lane 2 or 12. STAT3 activation was determined as described in the legend to Figure 1.

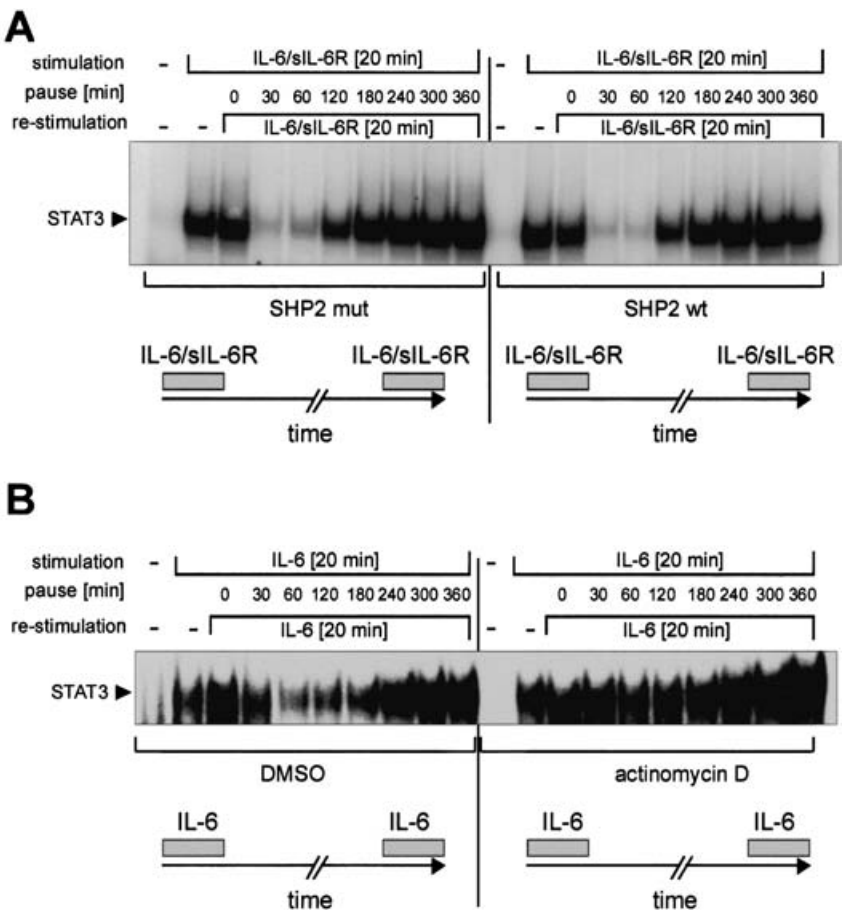

Figure 6 Desensitization does not depend on SHP2 but on transcriptional activity

(**A**) Murine embryonal 3T3 fibroblasts SHP2-exon 3 (SHP2 mut)-deficient mice or corresponding wild-type cells (SHP2 wt) were treated with 100 units/ml IL-6 and 1 μg/ml sIL-6R for 20 min. Medium was removed after stimulation and cell were left in fresh, IL-6-free medium for the times indicated. Finally cells were treated a second time before cells were harvested. STAT3 activation was determined as described in the legend to Figure 1. (**B**) To block transcriptional activities HepG2 cells were pre-incubated with 5 μg/ml actinomycin D for 30 min (right-hand panel) and as a control with the same amount DMSO (left-hand panel). Subsequently, the cells were stimulated with 100 units/ml IL-6 for 20 min, medium was removed and cells were left in fresh, IL-6-free medium for the times indicated. Finally, cells were treated a second time with 100 units/ml IL-6 for 20 min before the cells were harvested. During the whole time course of the experiment actinomycin D or DMSO was present in the media. STAT3 activation was determined as described in the legend to Figure 1.

the truncated SHP2 desensitization was clearly detectable (Figure 6A), suggesting that the inhibitory function of SHP2 cannot be responsible for signal attenuation after IL-6 pre-stimulation. The differences observed with respect to the kinetics of desensitization of HepG2 cells and MEFs are likely to be due to the different nature of hepatocytes and fibroblasts.

Desensitization of IL-6 signalling occurs through a transcription-dependent mechanism

SOCS1 and SOCS3 are inhibitors of IL-6 signal transduction. Both proteins are induced by IL-6, act as feedback inhibitors and may thus be responsible for the refraction of signal transduction. To test whether induction of a new protein is required for desensitization we investigated whether a block of transcription by actinomycin D affects the inhibition of STAT activation after IL-6 pre-stimulation. Indeed, as shown in Figure 6(B) treatment of HepG2 cells with actinomycin D compensated the inhibition of STAT activation induced by pre-treatment with IL-6.

Kinetics of SOCS1 and SOCS3 induction resemble that of IL-6 desensitization

We asked whether the kinetics of SOCS1 and SOCS3 induction after pre-stimulation correlate with the kinetics of desensitization of IL-6 signalling. Therefore HepG2 cells were stimulated for 20 min with IL-6 and, after pausing, the amounts of SOCS3 mRNA and protein were measured by Northern or Western blotting respectively (Figures 7A and 7B). SOCS3 mRNA and protein were detectable already very early in the time course. The disappearance of the SOCS3 protein correlated with the reappearance of STAT3 activation shown in the previous experiments. SOCS1 mRNA appeared with similar kinetics (Figure 7C). Due to the lack of suitable antibodies we were not able to measure SOCS1 protein levels. Our results demonstrate clearly that both SOCS1 and SOCS3 are IL-6-induced immediate-early genes. Furthermore, since SOCS mRNAs were only transiently detectable, SOCS1 and SOCS3 are possibly responsible for desensitization of IL-6 signal transduction.

Desensitization in SOCS3-deficient cells

Because of the central role of SOCS3 as a regulator for IL-6 signalling *in vivo* [25–27] we analysed desensitization in SOCS3 deficient cells. The endogenous SOCS3 gene of murine embryonal fibroblasts from SOCS3-floxed mice was eliminated by transient adenoviral expression of Cre-recombinase [25]. Knocking out of the SOCS3 gene was confirmed by PCR with specific primers for the floxed or deleted SOCS3 gene locus (Figure 8A). Pre-treatment of $SOCS3^{fl/fl}$ cells with IL-6/sIL-6R led to desensitization after a second stimulus, as observed for wild-type cells (Figure 8B, left-hand panel). In contrast, $SOCS3^{-/-}$ cells express constitutively active STAT3 even in the absence of IL-6/sIL-6R. No desensitization could be observed in these SOCS3-deficient cells; i.e. the enhanced basal STAT3 activity is hardly affected by additional (pre-)stimulation with IL-6/sIL-6R, suggesting a crucial role of SOCS3 for repression of basal STAT3 activity (Figure 8B, right-hand panel).

DISCUSSION

Signal transduction can be negatively regulated by the inactivation of already activated signalling molecules, but also by counteracting further activation of signalling components (Figure 1, scheme). Thus the phenomenon in which cells which have been stimulated become refractory to a second stimulation that follows is defined as desensitization.

We have observed that STAT3 activation in response to short-term stimulation with IL-6 is transient, i.e. STAT3 is rapidly inactivated. On the other hand, continuous stimulation does not lead to permanent STAT3 activation, indicating that IL-6 signalling becomes refractory and thus insensitive towards further stimulation (Figure 1). We have also shown that after short-term treatment with IL-6, the HepG2 cells are less sensitive to a second stimulation and require some time to recover and to become fully sensitive to the cytokine again (Figure 1B).

In this study the molecular mechanisms of desensitization of IL-6 signal transduction were analysed. Alterations in cell-surface expression of both subunits of the IL-6R complex, gp130

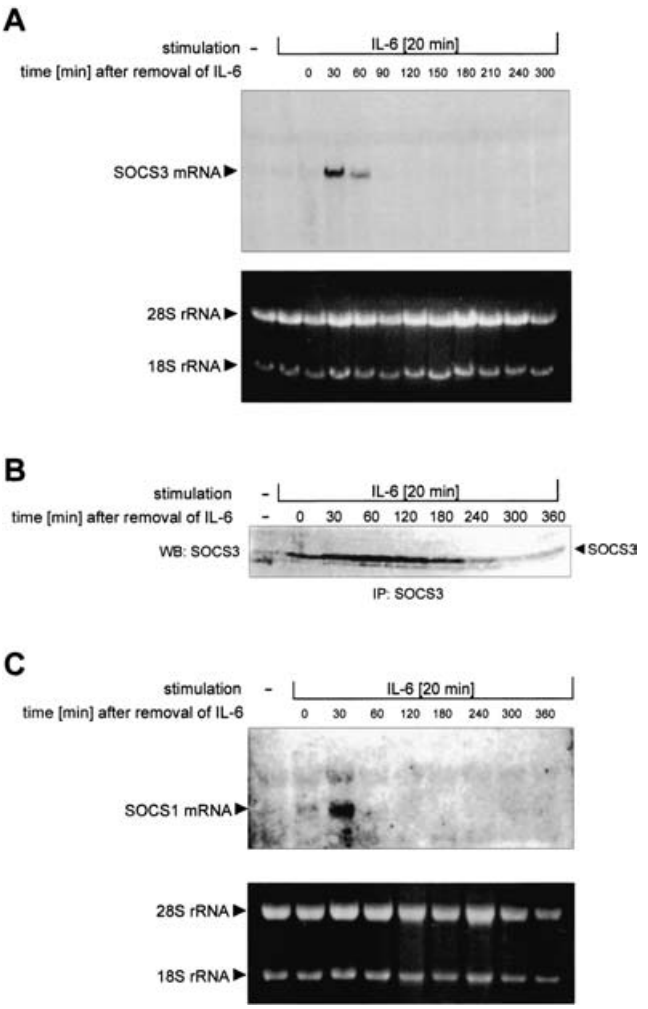

Figure 7 Kinetics of SOCS1 and SOCS3 mRNA induction

(**A**) HepG2 cells were stimulated with 100 units/ml IL-6 for 20 min. Medium was removed after stimulation and cells were left in fresh, IL-6-free medium for the times indicated. Total mRNA was isolated and analysed by Northern blotting with a specific probe for SOCS3 (**A**) or SOCS1 (**C**). As loading controls, the gel was stained with ethidium bromide (lower panels). (**B**) To detect SOCS3 protein, equal amounts of lysates from similarly treated HepG2 cells were incubated with antibodies against SOCS3. After immunoprecipitation protein–antibody complexes were separated by SDS/PAGE and analysed by Western blotting with biotinylated antibodies specific for SOCS3. Staining was performed with streptavidin-coupled HRP.

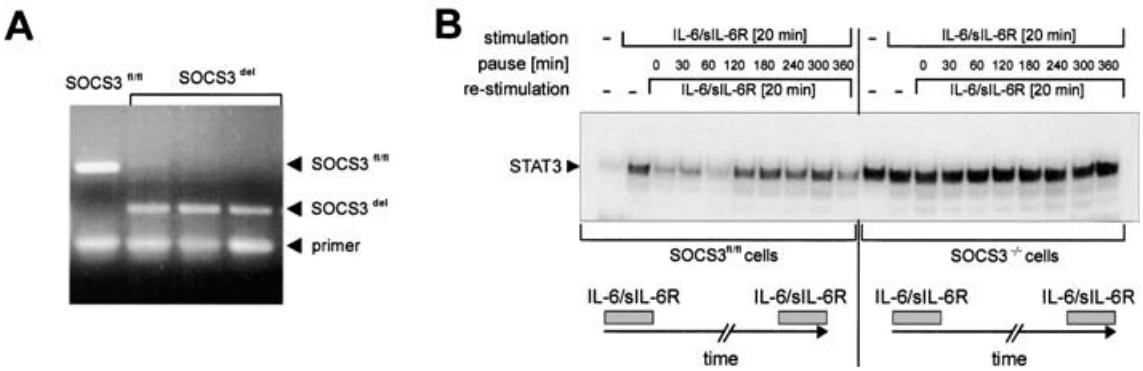

Figure 8 Constitutive STAT3 activation in SOCS3-deficient cells

(**A**) SOCS3-deficient MEFs were generated by infecting MEFs from homozygous SOCS3-floxed mice [25] with Cre-recombinase-expressing adenovirus. Infection was repeated four times until no SOCS3-floxed alleles were detectable by genomic PCR using as described previously [25]. The primer pairs used where specific for the SOCS3$^{fl/fl}$ or the SOCS3$^{-/-}$ genotype respectively. The lane on the left represents the PCR product prior to recombination. The following three lanes represent the PCR products of three independent cell lines after recombination in response to infection with Cre-recombinase-expressing adenovirus. For the following experiments the cells shown in the right-hand lane were used. (**B**) SOCS3$^{fl/fl}$ and SOCS3$^{-/-}$ cells were stimulated with 100 units/ml IL-6 plus 1 μg/ml sIL-6R for 20 min (lane 2 of both panels). For the kinetic analyses (lanes 3–10 of both panels) medium was removed after stimulation and cells were left in fresh, IL-6-free medium for pausing for the times indicated. Finally cells were treated a second time with 100 units/ml IL-6 plus 1 μg/ml sIL-6Rα for 20 min before they were harvested. STAT3 activation was determined as described in the legend to Figure 1(**A**).

and the IL-6R (gp80), are not mediating desensitization after stimulation of HepG2 cells with IL-6, since neither depletion of IL-6 surface receptors (Figure 2A) nor impaired IL-6 binding (Figure 2C) was detected in response to pre-stimulation. Thus the addition of agonistic sIL-6Rα does also not affect desensitization (Figure 2B). This is in line with our previous observation that gp130 is ligand-independently (constitutively) internalized [17–19].

Furthermore, we found that not only the signalling through the pool of activated receptors but also through those receptors which have not been activated before is attenuated (*trans*-desensitization; Figure 3). To be able to distinguish and to stimulate independently both receptor populations we expressed two sets of chimaeric gp130 receptors (EpoR–gp130 and IL-5R–gp130) which could be activated by two different cytokines (Epo and IL-5; Figure 3). The results of these experiments suggest that desensitization targets both signalling through pre-activated as well as through non pre-activated receptors, indicating *trans*-desensitization. This further corroborates the finding that receptor internalization is not causative for desensitization. From these observations it is concluded that not a specific modification of activated receptors but *trans*-acting inhibitory factors are responsible for desensitization of IL-6 signalling. Such *trans*-acting proteins may affect signal transduction at all levels of the signalling cascade. We found STAT3 activation as well as gp130 and JAK1 phosphorylation to be attenuated by desensitization (Figure 4). Reduced STAT3 activation is likely to be the result of reduced JAK1 activity. Potential inhibitors of JAK1 activity are the protein tyrosine phosphatase SHP2, which may dephosphorylate and thus inactivate JAKs, and the JAK inhibitors SOCS1 and SOCS3.

Desensitization was also observed in cells expressing a mutated SHP2 (Figure 6A), indicating that the tyrosine phosphatase is not essential for desensitization, although SHP2 has recently been confirmed to counteract IL-6 signalling [22]. Instead, IL-6-induced feedback inhibitors of the SOCS family could be potential mediators of desensitization. Both SOCS1 and SOCS3 are known to inhibit IL-6 signalling in human hepatoma cells *in vitro* [23]. Indeed, the decrease of IL-6 signalling in response to permanent stimulation depends on protein synthesis [46]. Similarly, desensitization was overcome by the blockade of *de novo* protein synthesis by the transcriptional inhibitor actinomycin D (Figure 6B). Actually, the kinetics of SOCS1 and SOCS3 mRNA induction by IL-6 as well as the appearance of SOCS3 protein roughly correspond to the kinetics of desensitization (Figure 7). Therefore, the IL-6-induced feedback inhibitors SOCS1 and SOCS3 are potential candidates to mediate desensitization. Since SOCS3 binds to tyrosine-759 of gp130 to inhibit IL-6 signalling [23,24] we tested whether this tyrosine motif is crucial for desensitization (Figure 5). The crucial requirement of tyrosine-759 for the inhibition of signalling after pre-stimulation suggests that SOCS3 may be a mediator of desensitization. Recent studies in SOCS3-deficient mice showed that SOCS3 is a central regulator for IL-6 signalling *in vivo* and suggest that SOCS3 and SOCS1 have reciprocal functions in IL-6 and interferon-γ signal transduction [25–27]. Thus SOCS3 could also play a central role for desensitization of IL-6 signalling. Indeed, cells lacking the SOCS3 gene show enhanced basal STAT3 activity and IL-6 pre-stimulation did not counteract STAT3 activation in these cells. These observations indicate a crucial role of the feedback inhibitor SOCS3 for repression of basal STAT3 activity (Figure 8). The lack of down-regulation of STAT3 activity in these cells may also suggest a potential role of SOCS3 for desensitization. The constitutive activation of STAT3 may be a direct consequence of impaired desensitization as a consequence of the SOCS3 knockout. Actually, these results additionally suggest that, although SHP2 and SOCS3 are both potent inhibitors for IL-6 signal transduction they play different roles for desensitization of IL-6 signalling. During the reviewing process of this paper, Mahboubi et al. [47] described desensitization of signalling by OSM (oncostatin M). In contrast to the observations made with IL-6, neither SOCS3 nor SHP2 is crucial for desensitization of signalling by OSM. This difference between IL-6 and OSM signalling could be due to the differences in IL-6- and OSM-receptor-complex composition.

Finally, our data stress further that inactivation and desensitization of the signal-transduction pathway has to be analysed independently, since both can be mediated by different inhibitory signalling components.

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