Termination of IL-6-induced STAT activation is independent of receptor internalization but requires de novo protein synthesis

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Abstract The interleukin-6 (IL-6) receptor complex comprises the IL-6 receptor (IL-6R, gp80) and the signal transducer gp130. Binding of IL-6 to its receptor results in dimerization of gp130, activation of the Jak/STAT pathway, and in a down-regulation of IL-6 binding sites by endocytosis. The STAT activation after stimulation is transient, being maximal after 15–30 min and disappearing after 60–90 min. The mechanism which leads to the termination of the signal is still unknown.

In this paper we have studied whether the down-modulation of the STAT signal requires the endocytosis of the receptor complex. Our results suggest that the desensitization of the IL-6 signal is not due to internalization of the receptor complex but requires de novo protein synthesis.

Key words: Endocytosis; gp130; Interleukin-6; Desensitization; Jak/STAT signaling

1. Introduction

Interleukin-6 (IL-6) is a pluripotent cytokine that – depending on the cell type – stimulates proliferation, inhibits cell growth and regulates gene expression (for a recent review see [1]). The IL-6 receptor complex comprises a specific binding subunit (IL-6 receptor, gp80) and a signal transducer gp130. Binding of IL-6 to its cognate receptor triggers dimerization of gp130 which results in the phosphorylation and activation of Jak1, Jak2 and Tyk2 [1–4]. These kinases phosphorylate gp130 on tyrosine residues within its cytoplasmic domain thereby creating recruitment sites for STAT1/STAT3 and SH2 domain containing phospho-tyrosine phosphatase-2 (SHP-2). STATs are tyrosine-phosphorylated, dimerize and translocate to the nucleus where they bind to enhancer elements in the promoter regions of IL-6 responsive genes [1]. The role of SHP-2 in IL-6-dependent signaling is still controversial. It is reported to act as an adapter for the cross-talk to the MAP kinase pathway which is also involved in a number of IL-6 responses [5,6]. Furthermore, a limiting effect on IL-6-dependent gene activation was demonstrated [7].

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Abbreviations: STAT, signal transducer and activator of transcription; IL, interleukin; Jak, Janus kinase; MAPK, mitogen activated protein kinase; SOCS, suppressor of cytokine signaling; PIAS, protein inhibitors of activated STATs; EPO, erythropoietin; EGF, epidermal growth factor; sIL-6R, soluble IL-6 receptor

Binding of IL-6 to gp80 also results in down-regulation of IL-6 binding sites within 30–60 min via endocytosis of gp80 [8]. We have recently shown that this internalization is mediated via gp130 and strictly dependent on a di-leucine motif within the gp130 cytoplasmic domain [9]. Whereas internalization of gp80 is ligand-dependent, gp130 undergoes constitutive endocytosis most likely via clathrin coated pits [10].

The STAT activation upon IL-6 stimulation as detected in an electrophoretic mobility shift assay or a phospho-tyrosine blot is biphasic. After a rapid activation which is detectable within a few minutes and peaks around 15–30 min, the STAT dimer disappears at around 1 h and reappears – although to a lesser extent – from 3 h up to 16 h and longer [11]. The mechanism of the observed down-modulation of the IL-6 signal after 30 min is unknown. Possible mechanisms involve: desensitization via (i) endocytosis and down-regulation of gp80, (ii) activation of a phospho-tyrosine phosphatase such as SHP-2, and (iii) induction of negative feedback inhibitors such as suppressors of cytokine signaling (SOCS) and protein inhibitors of activated STATs (PIAS) [12–16].

In this study we have addressed the question whether endocytosis of gp130 is responsible for the rapid down-modulation of STAT activation. To answer this question we compared the kinetics of STAT activation/inactivation in cell lines expressing internalization-competent and -deficient forms of gp130. Our results suggest that desensitization of the IL-6 signal is not mediated via the endocytosis of the IL-6 receptor complex but requires de novo protein synthesis.

2. Materials and methods

2.1. Cell culture and transfections

HepG2 cells were grown in DMEM/F12 mix supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l). Ba/F3 cells, a murine pre-B lymphocyte line, were cultured in DMEM containing 10% fetal calf serum and 5% conditioned medium from X63Ag-653 BPV-mIL-3 myeloma cells as a source of IL-3 [17]. All cells were grown at 37°C and 5% CO_2 in a water-saturated atmosphere.

Ba/F3-gp130 wild-type and mutant cell lines were stably transfected by electroporation of 28 μg of pSVLgp130$_{WT}$ or pSVLgp130$_{AA}$ respectively and 2 μg of pSV2neo into 3.5×10^6 cells in 0.8 ml medium at 200 V and 70 ms. Selection with G418 (1 mg/ml) in IL-3-conditioned medium was initiated 24 h after transfection. Selected Ba/F3 clones were screened for the presence of cell surface gp130 by flow cytometry.

2.2. Cell surface half-life measurement of gp130

Ba/F3 cells stably expressing human gp130$_{WT}$ and gp130$_{AA}$ were incubated at 37°C for different times in culture medium containing 50 μM cycloheximide to prevent de novo synthesis of gp130. Subsequently, the cells were cooled to 4°C, washed, and cell surface expression of gp130 was assessed by flow cytometry.

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2.3. Flow cytometry

Ba/F3-gp130 and Ba/F3-gp130$_{AA}$ cells were harvested, washed, and resuspended in cold PBS supplemented with 5% fetal calf serum and 0.1% sodium azide (PBS/azide); cells were then fixed in 2% paraformaldehyde for 20 min on ice. 1×10^6 cells in 200 μl of PBS/azide were incubated with 2 μg of a gp130 specific monoclonal antibody (mAb) [18] for 30 min, washed twice, and the gp130-bound antibodies visualized using a 1:50 dilution of an R-phycoerythrin-conjugated anti-mouse IgG-F(ab′)$_2$ (Dianova) for 30 min on ice. Subsequently, cells were washed and resuspended in PBS/azide. 2×10^4 cells/sample were analyzed using a FACScalibur (Becton Dickinson). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent antibody binding. Specific binding was calculated by subtracting the base line cell fluorescence intensity which was determined with cells incubated solely with the secondary antibody from the fluorescence intensity obtained with both, primary and secondary antibodies: (MFI of cycloheximide treatment) divided by (MFI of cells at time point 0)$\times$100.

2.4. Cell lysis and immunoprecipitations

For immunoprecipitations of SHP-2, Ba/F3-gp130 and Ba/F3-gp130$_{AA}$ cells were stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μg/ml) for 0–120 min and lysed in 1 ml of lysis buffer (50 mM Tris, pH 7.4; 150 mM sodium chloride; 1 mM EDTA; 0.5% Nonidet P-40; 1 mM sodium fluoride; 15% glycerol; 1 mM sodium vanadate; 0.5 mM EDTA; 0.25 mM PMSF; 5 μg/ml aprotinin; 1 μg/ml leupeptin; 1 μg/ml pepstatin). 1 μg of a polyclonal anti-SHP-2 antiserum was then added to the lysates at 4°C for 18 h to precipitate SHP-2. After incubation with protein A-sepharose (2.5 mg/ml lysis buffer) for 1 h at 4°C, the samples were washed with washing buffer (50 mM Tris, pH 7.4, 150 mM sodium chloride, 15% glycerol, 1 mM EDTA, 0.5% Nonidet P-40), boiled in reducing sample buffer and separated on a 10% SDS–polyacrylamide gel.

2.5. Western blotting and immunodetection

The electrophoretically separated proteins were transferred to polyvinylidene difluoride membranes by the semi-dry Western blotting method. Non-specific binding was blocked with 10% bovine serum albumin in TBS-N (20 mM Tris/HCl, pH 7.4, 137 mM NaCl, 0.1% Nonidet P-40) for 20 min. Phosphorylation of SHP-2 was detected by an anti-phospho-tyrosine antibody (4G10) (Upstate Biotech) at a concentration of 1 μg/ml in TBS-N for 1 h. After extensive rinsing with TBS-N, the immunoblots were incubated for 1 h with a horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody. The immunoblots were developed using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.6. Electrophoretic mobility shift assay (EMSA)

Ba/F3-gp130, Ba/F-gp130$_{AA}$, and HepG2 cells were grown for 2–3 days and stimulated with IL-6 for different times. Nuclear extracts were prepared as described [19]. The protein concentration was determined with a Bio-Rad™ protein assay. EMSAs were performed as described previously using a double-stranded ^{32}P-labelled mutated m67SIE oligonucleotide from the c-fos promoter (m67SIE: 5′-GAT CCG GGA GGG ATT TAC GGG GAA ATG CTG-3′) [20]. The protein–DNA complexes were separated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE 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.

3. Results

HepG2 cells were stimulated with IL-6 at 1 μg/ml and the time-course of STAT activation was monitored over 16.5 h employing an electrophoretic mobility shift assay (Fig. 1, left panel). A strong STAT3 signal was detected after 30 min that disappeared almost completely after 1.5 h. At later time points a second, less intensive wave of activation was seen until 16.5 h confirming previous observations that STAT activation after IL-6 is biphasic [11]. We have shown that in HepG2 cells within 60 min IL-6 binding sites are down-regulated after

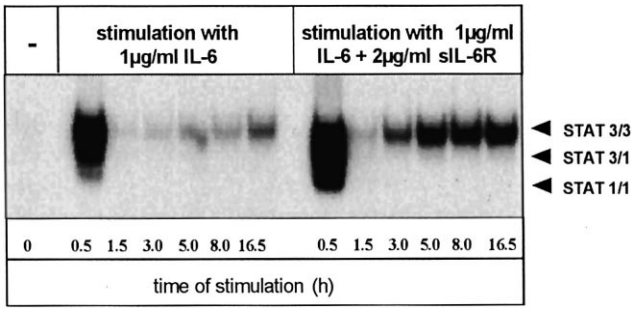

Fig. 1. Transient STAT activation of HepG2 cells after stimulation with IL-6 (left panel) and IL-6/sIL-6R (right panel). HepG2 cells were stimulated with 1 μg/ml IL-6 or 1 μg/ml IL-6 and 2 μg/ml sIL-6R respectively for the indicated time and nuclear extracts were prepared. EMSAs were performed with the labelled m67SIE probe. The positions of the three dimers formed are indicated.

stimulation with saturating amounts of IL-6 [8], raising the possibility that the observed down-modulation of STAT activity after 1.5 h is due to the depletion of plasma membrane IL-6R (gp80). In order to test this, we stimulated HepG2 cells with IL-6 and 2 μg/ml of recombinant soluble IL-6R and again monitored the kinetics of STAT activation/inactivation. The soluble IL-6R in combination with IL-6 acts agonistically on cells expressing gp130 [21]. As shown in Fig. 1 (right panel), the STAT signal over the whole time-course is increased under these conditions so that even STAT1 is now prominently activated. This is most likely due to a surplus of gp130 over IL-6R molecules at the plasma membrane of HepG2 cells [21]. However, this activation pattern is still biphasic and qualitatively not different from the one in the left panel. We have recently demonstrated that gp130 is endocytosed constitutively and not down-regulated by stimulation via IL-6/sIL-6R [10]. In addition, although the complex of IL-6 and sIL-6R is internalized by HepG2 cells [22], only marginal amounts of this complex will be depleted from the culture medium within 1 h. Thus, at 1.5 h of stimulation gp130 and IL-6/sIL-6R are still present at the cell surface, nevertheless the signal is turned-off as well. Therefore, we conclude that endocytosis of the IL-6 receptor complex might not be the crucial process for the observed down-modulation of STAT activation.

In order to further analyze the relationship between endocytosis and signal desensitization, studies with murine Ba/F3 pre-B cells stably transfected with gp130$_{WT}$ and gp130$_{AA}$ – in which the di-leucine internalization motif was replaced by two alanines [9] – were performed. In transfected Ba/F3 cells, gp130$_{WT}$ but not gp130$_{AA}$ is efficiently internalized as assessed by flow cytometry (Fig. 2a). As shown in Fig. 2b, the biphasic STAT activation pattern is observed in both cell lines after stimulation with IL-6/sIL-6R. The quantitative difference of the initial STAT activation between Ba/F3-gp130$_{WT}$ and Ba/F3-gp130$_{AA}$ is due to the increased surface expression of gp130$_{AA}$ (data not shown). In addition, phosphorylation of SHP-2 was compared in these two cell lines (Fig. 2c). Also SHP-2 is only transiently phosphorylated and no difference was observed in activation of SHP-2 in these two cell lines.

Additional experiments were performed in A375 melanoma cells stably expressing internalization-competent and -deficient forms of EPO receptor/gp130 chimeras. In these cells, activation of the Jak/STAT pathway results in an inhibition of proliferation [23,11]. After EPO stimulation, STAT and

SHP-2 activation were comparable in A375 cells expressing internalization-competent and -deficient receptor chimeras and in both cell lines proliferation was inhibited to the same extent (data not shown). Taken together, our results strongly suggest that endocytosis is not the mechanism of down-modulation of STAT activity.

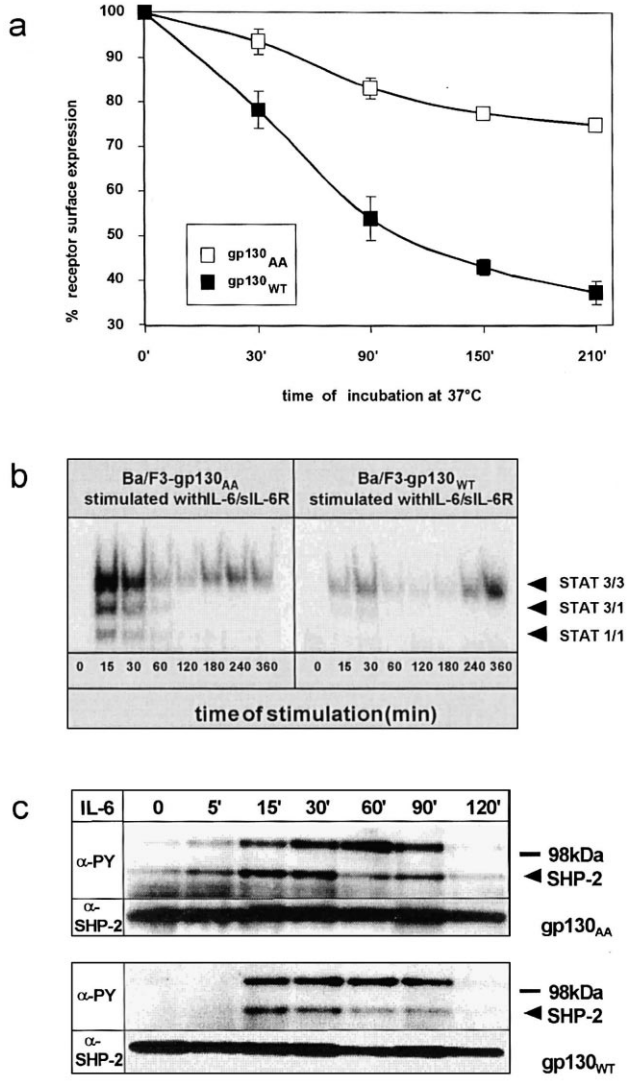

Fig. 2. a: Cell surface half lives of $gp130_{WT}$ or $gp130_{AA}$ in stably transfected Ba/F3 cells. Cell surface expression on cells treated for different times with 50 μM cycloheximide was assessed by flow cytometry using an anti-gp130 monoclonal antibody. Time 0 corresponds to a 30 min incubation in cycloheximide containing medium, since this is the time required for newly synthesized gp130 to reach the cell surface [19]. Antibody binding was calculated as described in Section 2. The means and S.D. of three independent experiments are shown. b: STAT activation of stably transfected Ba/F3 cells expressing $gp130_{WT}$ and $gp130_{AA}$ after stimulation with IL-6/sIL-6R. The cells were stimulated with 20 ng/ml IL-6 and 1 μg/ml sIL-6R for the indicated time and nuclear extracts were prepared. EMSAs were performed with the labelled m67SIE probe. The positions of the three dimers formed are indicated. c: Kinetics of SHP-2 phosphorylation in stably transfected Ba/F3 cells expressing $gp130_{WT}$ and $gp130_{AA}$. Cells were stimulated with 20 ng/ml IL-6 and 1 μg/ml sIL-6R, lysed and immunoprecipitations using an anti-SHP-2 antiserum were performed as described in Section 2. Aliquots were separated on an SDS gel and the immunoblot was developed using an anti-phospho-tyrosine monoclonal antibody and reprobed with an anti-SHP-2 antiserum. The prominent band at 100 kDa most likely represents Gab-1 [32].

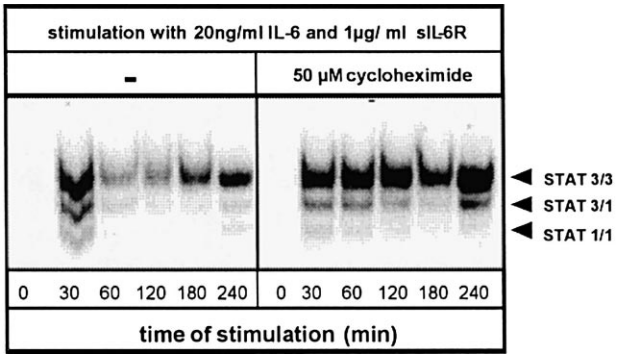

Fig. 3. STAT activation of Ba/F3-$gp130_{AA}$ cells. The cells were treated for different times with 50 μM cycloheximide or left untreated and stimulated with 20 ng/ml IL-6 and 1 μg/ml sIL-6R for the indicated time and nuclear extracts were prepared. EMSAs were performed with the labelled m67SIE probe. The positions of the three dimers formed are indicated.

Recently, it was demonstrated that proteins of the SOCS family are negative feedback regulators of cytokine signaling [14–16]. These proteins are induced by cytokines and bind to the receptors and the Jaks, respectively, thereby inhibiting their activity. If these or related proteins are involved in the STAT desensitization observed in different cell systems, this down-modulation should be sensitive to inhibitors of de novo mRNA and protein synthesis. We addressed this question using the Ba/F3-$gp130_{AA}$ cells. These cells are especially useful for this study since inhibition of protein synthesis in these cells does only marginally (25%) affect gp130 surface expression (Fig. 2a). In contrast, in $gp130_{WT}$ expressing cells, gp130 surface protein levels are rapidly declining after cycloheximide due to the constitutive internalization and short half life of this protein (Fig. 2a). As shown in Fig. 3, stimulation of Ba/F3-$gp130_{AA}$ cells with IL-6/sIL-6R in the presence of cycloheximide resulted in a sustained STAT activation over the whole 4 h time-course. Thus, we conclude that (a) de novo synthesized protein(s) are responsible for the down-modulation of STAT activity after the initial activation peak.

4. Discussion

Cytokines and growth factors elicit their intracellular signals via plasma membrane receptors that usually activate phosphorylation cascades ultimately leading to altered growth behavior or gene expression. Receptor activation has to be spatially and temporally controlled in order to avoid overstimulation that might result in cell transformation and tumor growth. Therefore, efficient mechanisms to turn-off a signal cascade must exist. Means to desensitize an activated receptor are: (i) phosphorylation or dephosphorylation, (ii) translocation into endosomes via its internalization, (iii) increased degradation or diminished synthesis, and (iv) induction of negative feedback inhibitors [12–16,24].

In this study we have studied possible mechanisms which are responsible for the rapid attenuation of Jak/STAT signaling after the initial trigger by the IL-6 signal transducer gp130. We focussed on the role of endocytosis in this process since the observed desensitization is parallel in time to the endocytosis and down-regulation of gp80 [8]. Furthermore,

recent experiments had revealed that truncated gp130 chimeras which still contain a STAT recruitment site but lack an internalization motif result in a prolonged STAT activation [11]. This suggested that attenuation of the signal requires internalization. However, these mutants also lacked a recruitment site for SHP-2, a tyrosine phosphatase that possibly is also involved in desensitization [7]. The results of this study unequivocally demonstrate that endocytosis is not necessary for attenuation to occur. This was first demonstrated in hepatoma cells HepG2, where the desensitization not only was observed after stimulation with IL-6 but also after IL-6/sIL-6R treatment (Fig. 1). In the latter case the down-regulation of the plasma membrane IL-6R is compensated by the large excess of agonistic soluble receptor complexes. Since surface gp130 is not down-regulated by ligation [10] and thus present at the cell surface at all times, signaling-competent receptor complexes still can be formed. Therefore, other mechanisms than IL-6R down-regulation have to be responsible for desensitization. This conclusion is further supported by our findings that signal attenuation even occurs after stimulation of internalization-deficient gp130. These were created by replacing the crucial di-leucine residue of the internalization motif by alanines, a mutation that strongly reduces the internalization capability of gp130 ([9,25] and this study (Fig. 2). However, no effect on the time-course of signal attenuation was observed with $gp130_{AA}$.

Similar findings were obtained in A375 melanoma cells that express an EPOR/$gp130_{WT}$ or EPOR/$gp130_{AA}$ chimeric receptor [11] (data not shown). Neither STAT nor SHP-2 phosphorylation nor EPO-induced cell growth inhibition were different. Thus, signal generation as we have previously shown [25] as well as signal termination (this study) are independent of the endocytosis of gp130. Since signaling also does not affect the rate of gp130 endocytosis, we conclude that signaling and internalization of the IL-6 receptor complex are not interdependent. In this respect, gp130 behaves more like G-protein linked receptors such as the β2-adrenergic receptor or the neurokinin receptor-1 than like receptor tyrosine kinases such as the epidermal growth factor receptor [26]. The β2-adrenergic receptor is efficiently internalized after ligand binding but neither inhibitors of endocytosis nor mutations within the receptor that prevent agonist-induced endocytosis affect homologous desensitization [27,28]. In contrast, the EGF receptor signaling is uniformally enhanced in cells in which endocytosis via clathrin coated pits is blocked by over-expression of a dominant-negative form of dynamin [24] and EGF receptors with a point mutation of the kinase domain that abolishes kinase activity are only internalized at a basal rate [29].

What then is the mechanism of gp130 desensitization? The tyrosine phosphatase SHP-2 is known to be recruited to tyrosine 759 of gp130 after phosphorylation by Jaks of this residue. Mutation of Tyr-759 leads to a loss of SHP-2 recruitment and results in an enhanced transcription of reporter genes [7]. However, in cell lines expressing wild-type gp130 or the Tyr-759 mutant the time-course of STAT activation and inactivation is similar although it differs in a quantitative fashion. At 1.5 h the signal in both cell lines is strongly down-regulated. Thus, SHP-2 is also not the determining factor in gp130 desensitization. Haspel et al. reported on the inactivation of STAT1 by a nuclear protein tyrosine phosphatase [30,31]. However, the identity of this phosphatase and its regulation are presently unknown. Furthermore, it is not clear whether this phosphatase also dephosphorylates STAT3.

Ba/F3-$gp130_{AA}$ cells stimulated with IL-6/sIL-6R displayed a sustained STAT activation in the presence of cycloheximide. This suggests that de novo synthesized proteins are responsible for the down-modulation of STAT activity. In this respect, it is interesting that two classes of inhibitors for Jak/STAT signaling have recently been discovered, the SOCS proteins and PIAS-1/PIAS-3. SOCS are a growing family of immediate early genes that are rapidly transcribed after STAT activation and code for proteins that inhibit the Jak/STAT pathway by direct interaction with the receptors or active Jaks thereby creating a negative feedback loop [14–16]. PIAS-1/PIAS-3 are proteins that directly bind activated STAT1/STAT3 and inhibit their binding to cognate DNA elements [13]. Whether and how PIAS activity is regulated is currently unknown. Our finding that inhibition of protein synthesis completely abolishes signal attenuation makes it likely that members of the SOCS family are responsible for this down-modulation (Fig. 3).

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