Cross-regulation of cytokine signalling: pro-inflammatory cytokines restrict IL-6 signalling through receptor internalisation and degradation

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Summary

The inflammatory response involves a complex interplay of different cytokines which act in an auto- or paracrine manner to induce the so-called acute phase response. Cytokines are known to crosstalk on multiple levels, for instance by regulating the mRNA stability of targeted cytokines through activation of the p38-MAPK pathway. In our study we discovered a new mechanism that answers the long-standing question how pro-inflammatory cytokines and environmental stress restrict immediate signalling of interleukin (IL)-6-type cytokines. We show that p38, activated by IL-1β, TNFα or environmental stress, impairs IL-6-induced JAK/STAT signalling through phosphorylation of the common cytokine receptor subunit gp130 and its subsequent internalisation and degradation. We identify MK2 as the kinase that phosphorylates serine 782 in the cytoplasmic part of gp130. Consequently, inhibition of p38 or MK2, deletion of MK2 or mutation of crucial amino acids within the MK2 target site or the di-leucine internalisation motif blocks receptor depletion and restores IL-6-dependent STAT activation as well as gene induction. Hence, a novel negative crosstalk mechanism for cytokine signalling is described, where cytokine receptor turnover is regulated in trans by pro-inflammatory cytokines and stress stimuli to coordinate the inflammatory response.

Key words: Cytokine, Signalling, Crosstalk, Internalisation, Interleukin-6

Introduction

The inflammatory response is a highly coordinated answer of the body to trauma, tissue injury or infection. It involves a complex interplay of different cell types releasing cytokines which act in an auto- or paracrine manner to induce the so-called acute phase response. This reaction starts with the release of pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF) α. Later, the major mediator of hepatic acute phase protein expression IL-6 is expressed and exerts pro- as well as anti-inflammatory activities (Bode and Heinrich, 2001).

It is mandatory that the function of these cytokines is stringently regulated since dysregulated IL-6 signalling, involving the Janus kinase/signal transducer and activator of transcription (JAK/STAT), MAPK (mitogen activated protein kinase) and the PI3K/Akt pathways, leads to severe inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, and also cancer (e.g. plasmacytoma). IL-6 signal transduction is controlled at different levels: it affects IL-6-dependent gene expression through activation of target gene promoters (Zhang and Fuller, 1997; Zhang and Fuller, 2000; Bode et al., 2001a) or by enhancing the IL-6-induced expression of the SOCS3 feedback inhibitor (Bode et al., 1999; Yang et al., 2004). IL-1β counteracts signal transduction of IL-6-type cytokines at different levels: it affects IL-6-induced gene expression through acting on target gene promoters (Zhang and Fuller, 1997; Zhang and Fuller, 2000; Bode et al., 2001a) or by enhancing the IL-6-induced expression of the SOCS3 feedback inhibitor (Bode et al., 1999; Yang et al., 2004). IL-1β also rapidly counteracts IL-6-mediated STAT3 activation (Ahmed and Ivashkiv, 2000; Ahmed et al., 2002). This rapid inhibition of IL-6-initiated STAT3 activation has been suggested to be independent of de novo protein synthesis.
and SOCS3 induction. Even activation of Janus family tyrosine kinases, essential for IL-6-mediated signalling, appears to be dispensable. Instead, a contribution of the p38 stress kinase-dependent pathway was proposed (Ahmed et al., 2002). However, the underlying molecular mechanisms involved in this rapid stress-induced effect on IL-6-type cytokine signalling have not been identified so far.

An indispensable prerequisite for any cytokine stimulation of a given cell type is the availability of the receptor complex on the cell surface. During differentiation of cells, as well as during cellular responses to exogenous stimuli, the expression of cytokine receptors on the cell surface is tightly regulated. Unlike a number of growth factor receptors, which are internalised only in response to ligand binding (Hari and Roth, 1987; McClain et al., 1987; Glenney et al., 1988; Sorkin et al., 1991), the common receptor subunit of all IL-6-type cytokines, gp130, appears to internalise constitutively (Thiel et al., 1998) through a di-leucine motif-dependent mechanism (Dittrich et al., 1994) and is subsequently degraded (Wang and Fuller, 1994; Blanchard et al., 2001). Gp130 is expressed ubiquitously. Therefore it has so far been a generally accepted idea that the responsiveness of individual cell types to IL-6-type cytokines is mainly determined by the expression of a specific alpha-receptor (i.e. IL-6Rα, IL-11Rα, CNTFRα) or by the expression of a second specific signal-transducing subunit (i.e. LIF-R, OSM-R, WSX1).

Here we describe a novel molecular mechanism for the restriction of IL-6-type cytokine signal transduction by pro-inflammatory cytokines and cellular stress: we show that activation of the stress-activated protein kinase p38 by IL-1β, TNFα or environmental stress leads to the MAPK-activated protein kinase 2 (MK2)-dependent serine phosphorylation of gp130 on serine 782 and increased receptor internalisation and degradation resulting in the net loss of surface-bound gp130 molecules and consequently in reduced IL-6-dependent STAT3 activation and gene induction. The identification of gp130 as a new target of the p38-MK2 axis unravels a novel mechanism of cytokine crosstalk.

Results
IL-1β negatively regulates STAT activation by IL-6-type cytokines independent of NF-κB and de novo protein biosynthesis

To study the mechanisms involved in the rapid inhibition of IL-6 signalling by IL-1β we used HepG2 hepatoma cells, which are very responsive to IL-6 since they express the membrane-bound IL-6 receptor as well as the signal transducer gp130. Preincubation of HepG2 cells with IL-1β results at any time point and at any dose tested in a reduction of IL-6-induced STAT3 tyrosine phosphorylation (Fig. 1A). Binding of activated STAT3 to an optimised STAT-recruiting DNA probe is severely compromised in IL-1β-pretreated cells (Fig. 1B, lanes 2 and 4). To determine whether the IL-1β-activated transcription factor NF-κB is involved in this fast inhibitory effect on STAT3 activation, we made use of HepG2 cells stably expressing a non-degradable IκBα mutant. These cells have been extensively characterised and lack NF-κB activation in response to IL-1β (Fig. 1C, compare lanes 1-5 with lanes 6-10 and Yang et al., 2004). Fig. 1D shows that expression of the non-degradable IκBα in HepG2-IκBα cells did not affect the early inhibition of STAT3 DNA-binding after IL-1β treatment (Fig. 1D, compare lanes 3 with 4 and 7 with 8, and supplementary material Fig. S1 for quantification). We recently reported that IL-1β enhances IL-6-induced SOCS3 expression by stabilising SOCS3 mRNA (Yang et al., 2004). However, we could show that IL-1β was able to inhibit IL-6-mediated STAT3 activation independent of de novo protein synthesis (Fig. 1E).

![Fig. 1. IL-1β inhibits IL-6-induced STAT3 activation independently of NF-κB activation and de novo protein synthesis.](image-url)

(A) HepG2 cells were stimulated with IL-6 (100 IU/ml) for the indicated times (left panel) or for 20 minutes (right panel) with the concentrations indicated, and/or IL-1β (100 IU/ml). For co-stimulation, IL-1β was added 10 minutes prior to the addition of IL-6. After stimulation, cell lysates were prepared and subjected to western blot analysis using antibodies against Y705-phosphorylated STAT3 (pY-STAT3; upper panel). The blots were stripped and re-probed with antibodies against STAT3 (lower panel). (B) HepG2 cells were stimulated for 20 minutes with IL-6 (100 IU/ml) and/or IL-1β (100 IU/ml) as indicated. For co-stimulation, IL-1β was added 10 minutes prior to addition of IL-6. Nuclear extracts were prepared and 6 μg of protein was subjected to EMSA with the 32P-labelled optimised STAT3 binding site (m67SIE). (C) HepG2-mock or HepG2-IκBα cells were stimulated with 100 IU/ml IL-1β for the times indicated. The nuclear protein (6 μg) was subjected to EMSA using the 32P-labelled IκB-site containing DNA fragment. (D) HepG2-mock or HepG2-IκBα cells were stimulated, lysed and analysed as described for A. (E) HepG2-mock cells were pretreated with DMSO or cycloheximide [CHX (25 μM)] for 40 minutes. Subsequently IL-1β (100 IU/ml) was added where indicated. Ten minutes later IL-6 (100 IU/ml) was added for the times indicated, cells were lysed and analysed for STAT3 tyrosine phosphorylation as described for A.
IL-1β-mediated suppression of IL-6-induced STAT3 phosphorylation requires activation of the p38 MAPK pathway

In addition to activating the NF-κB pathway, IL-1β is a known activator of the stress-activated MAP kinase p38 (Dunne and O’Neill, 2003). Therefore, we analysed whether p38 MAPK activation plays a role in the negative regulation of IL-6-induced STAT3 activation. In these experiments, STAT3 Y705 phosphorylation was determined by intracellular FACS analyses (Fig. 2A) and by western blotting of whole cell lysates (Fig. 2B). Whereas IL-1β efficiently inhibited the IL-6-induced STAT3 phosphorylation by approx. 50% in DMSO-treated cells, the inhibitory effect of IL-1β was absent in cells treated with the p38-specific inhibitor SB202190 (Fig. 2A,B). Efficient inhibition of p38 kinase by SB202190 was demonstrated by lack of phosphorylation of the downstream target protein MK2 in the presence of the inhibitor (Fig. 2B, lower panels, lanes 5-8).

In an alternative approach we determined IL-6-induced translocation of STAT3 from the cytoplasm to the nucleus by confocal microscopy. As expected, treatment of HepG2 cells with IL-6 for 20 minutes led to an obvious nuclear accumulation of STAT3 (Fig. 2C, first panel). Pretreatment with IL-1β for 15 minutes blocked IL-6-induced STAT3 translocation almost completely (third panel) and again, in the presence of SB202190 the inhibitory action of IL-1β was efficiently prevented and thereby accumulation of STAT3 in the nucleus restored (Fig. 2C, fourth panel).

**Activation of the p38 MAPK pathway by various stimuli results in loss of surface expressed gp130**

Initiation of any cytokine signalling cascade requires appropriate availability of cytokine receptors at the cell surface. Since the negative regulatory effect of IL-1β described above occurred rapidly we focussed on the influence of IL-1β on the expression of IL-6-type cytokine receptors on the plasma membrane. Indeed, within the first 15 minutes IL-1β induced a fast and persistent reduction of gp130 cell surface localisation to approximately 50% of the initial amounts present (Fig. 3A). Simultaneous presence of IL-6 did not further influence gp130 plasma membrane localisation (not shown). Of note, IL-1β specifically affected gp130 and not the IL-6 α-receptor, the oncostatin M (OSM) receptor (Fig. 3B) or the LIF receptor (not shown). In accordance with data presented above, this negative effect of IL-1β was not dependent on NF-κB activation, since HepG2 cells stably expressing the non-degradable IκBα mutant or lacking the IκB kinase subunit IKKγ, due to siRNA knock down, displayed a similar loss in gp130 expression (supplementary material Fig. 4).
S2A,B). However, pharmacological inhibition of p38 activity significantly reduced the downregulation of surface bound gp130 by IL-1β in HepG2 (Fig. 3C). Pre-treatment with a specific JNK inhibitor had no effect (supplementary material Fig. S2C).

The suppressive effect of IL-1β on gp130 cell surface expression was not restricted to HepG2 cells, but was also detectable in HeLa cells as well as primary human dermal fibroblasts (Fig. 3D). In addition to IL-1β, other pro-inflammatory cytokines such as TNFα, as well as cellular stress, e.g. exposure to UV light or arsenic-containing chemicals (phenyl arsine oxide, PAO) activate p38 (supplementary material Fig. S2D). Therefore, we investigated whether these stimuli also induce gp130 downregulation. Indeed, TNFα (Fig. 3D) as well as PAO treatment and UV irradiation (Fig. 3E) potently downregulated gp130 cell surface expression. As observed previously for IL-1β, these properties were significantly reduced in the presence of the p38 inhibitor SB202190 (Fig. 3D,E). Again, OSMR expression was not significantly affected (supplementary material Fig. S2E).

**IL-1β induces gp130 internalisation and subsequent lysosomal degradation**

The loss of gp130 surface expression as a result of stress could be explained by an increased gp130 internalisation or by altered post-endocytic processes, i.e. degradation rather than recycling. To monitor receptor internalisation we carried out classical antibody uptake experiments. Receptor uptake was quantified in a FACS-based assay; either indirectly by measuring loss of surface receptor bound antibodies (Fig. 4A) or alternatively by measuring intracellular accumulation of prebound FITC-labelled gp130 antibodies (Fig. 4B). Both approaches revealed an accelerated internalisation of gp130 in response to IL-1β. Again, IL-1β did not significantly increase uptake of OSMR antibodies (supplementary material Fig. S3A).

The increased uptake of FITC-labelled gp130 antibody in response to IL-1β was also confirmed by live-cell imaging; cell-surface-expressed receptors were stained by FITC-labelled antibodies added to the culture media of living cells. This resulted

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**Fig. 3. IL-1β stimulation and other p38-activating stimuli induce loss of gp130 from the plasma membrane.** (A,B) IL-1β reduces steady state gp130 cell surface expression without affecting IL-6R or OSMR. HepG2 cells were treated with 100 IU/ml IL-1β for the indicated periods of time. Cell surface-stained gp130 (A), IL-6R (B) and OSMR (B) were analysed by FACS using monoclonal antibodies directed against their extracellular regions. (A) The purple filled histogram represents surface staining with an isotypic control antibody, gp130 cell surface expression in the presence of any cytokine is represented by the green histogram whereas the pink histogram represents cell surface expression of gp130 in the absence of any cytokine. (B) The bar diagrams present processed data of the FACS analyses (grey bars, unstimulated; hatched bars, stimulated with IL-1β). Background fluorescence was subtracted and the value obtained for untreated cells was set to 100. Values are medians ± s.e.m. (n=4 for gp130, n=3 for IL-6R and OSMR). ***P<0.001, **P<0.01, *P<0.05 (two-tailed, paired t-test). (C) HepG2 cells were preincubated with solvent (DMSO) or the p38 inhibitor SB202190 (10 μM) for 30 minutes before stimulation with IL-1β (100 IU/ml) for 30 minutes. Cell surface expression of gp130 was determined as described for A (values are medians ± s.e.m., n=4; *P<0.05 vs untreated, **P<0.01) (D) HeLa cells (left panel) or HDF cells (right panel) were pretreated with DMSO or SB202190 (10 μM) and stimulated with 100 IU/ml IL-1β or 5 ng/ml TNFα as described in B. Gp130 cell-surface expression was determined as described in A (grey bars, unstimulated; hatched bars, stimulated with IL-1β; light grey bars, stimulated with TNFα). Values are medians ± s.e.m.; n=4 for HeLa cells, n=4 for IL-1β-treated HDF, n=2 for TNFα-treated HDF; **P<0.01, *P<0.05 vs untreated, ***P<0.001. (E) HeLa cells were pretreated with DMSO or SB202190 (10 μM) prior to stimulation with phenylarsine oxide (PAO, 1 μM; hatched bars) or UV irradiation (100 J/m²; light grey bars) for 30 minutes or left untreated (dark grey bars). Gp130 cell-surface expression was determined as described in A (values are medians ± s.e.m., n=6, ***P<0.001, **P<0.01 vs untreated, ***P<0.001, **P<0.01).
Fig. 4. Reduction of gp130 cell surface expression is mediated by receptor internalisation. (A) HepG2 cells labelled with 1 µg/ml B-R3 (anti-gp130) antibody were either left untreated (blue squares) or were stimulated with IL-1β (red triangles; 100 IU/ml) for 5, 10 or 20 minutes at 37°C. Non-internalised gp130 was analysed by FACS. Background fluorescence was subtracted and the value obtained from untreated cells was set to 100 (median ± s.e.m., n=3 for 5 and 30 minutes; n=4 for 0, 10 and 20 minutes; *P<0.05). (B) Uptake of FITC-labelled B-R3 (anti-gp130, Diaclone) antibody in response to IL-1β (100 IU/ml) treatment of HepG2 cells for 15 or 30 minutes at 37°C (red triangles). Internalised FITC–B-R3 labelled gp130 was monitored by FACS and normalised to uptake after 30 minutes under control conditions (blue squares; median ± s.e.m., n=3, *P<0.05). (C) Live-cell imaging of untreated and IL-1β-treated HepG2 cells. After preincubation with or without 10 µM SB202190 for 15 minutes, cells were labelled with FITC–B-R3 anti-gp130 at 10% CO2 and 37°C for 40 minutes in the absence or presence of IL-1β (100 IU/ml). Membranes were stained with Trypan Blue (red fluorescence) and confocal laser scanning analysis was carried out (green fluorescence, excitation at 488 nm; red fluorescence, excitation at 543 nm; and the merged pictures). (D) Confocal microscopy live-cell imaging of HepG2 cells. Incubation was performed in the presence of FITC–BR-3 anti-gp130 and Alexa Fluor 555-transferrin at 10% CO2 and 37°C for 40 minutes with or without IL-1β (100 IU/ml). (E) Immunofluorescence demonstrates colocalisation of anti-gp130-positive vesicles with EEA1. HepG2 cells were preincubated in ice-cold binding medium containing 1 µg/ml FITC–BR-3 anti-gp130 for 45 minutes. After washing to eliminate unbound antibodies the cells were incubated at 37°C for 30 minutes in the presence or absence of IL-1β (100 IU/ml). Subsequently, the cells were fixed in 3.7% formaldehyde, permeabilised in 0.1% Triton X-100 and stained with goat-anti-EEA1 antibody and Cy3-anti-goat secondary antibody. (F) Internalised gp130 localises to the lysosome. Live-cell imaging of HepG2 cells was carried out as described for C. Incubation was performed with FITC-labelled anti-gp130 antibody at 10% CO2 and 37°C for 40 minutes with or without IL-1β (100 IU/ml). For the last 15 minutes LysoTracker Red DND-99 was added and laser scanning was carried out. Representative pictures are depicted for all fluorescence data. (G) HepG2 cell were treated with cycloheximide (25 µM). After stimulation for up to 360 minutes with IL-1β (100 IU/ml) whole cellular extracts were prepared and analysed for gp130 by western blotting using a gp130-specific antibody (upper panel) and p38 activity was monitored using an activation-specific antibody for p38 (middle panel). Tubulin was stained to control loading of the gel (lower panel). Representative blots are depicted.
in a clearly visible signal which colocalised with Trypan Blue, which is a membrane stain. Only a minor proportion of the antibodies was constitutively taken up into vesicles over time (Fig. 4C, upper panel). Upon IL-1β treatment, however, we observed a striking loss of gp130 membrane staining, documented in steady-state pictures (Fig. 4C, middle panel) and multiple time series (supplementary material Movie 1). Consequently, hardly any colocalisation with Trypan Blue staining was detectable.

To examine the role of p38 MAPK in this process, we conducted live-cell imaging in the presence of SB202190. The p38 inhibitor efficiently prevented IL-1β-induced gp130 uptake and thus plasma membrane staining was retained (Fig. 4C, lower panel). This result was confirmed by the FACS-based internalisation assay (supplementary material Fig. S3B). A general effect of IL-1β on the internalisation machinery could be excluded by determination of the ratio of FITC-gp130-positive vesicles over constitutively internalised Cy3-transferrin-positive vesicles (supplementary material Fig. S3C).

To elucidate the fate of gp130 we analysed colocalisation of internalised gp130 (FITC-labelled antibodies with endocytosed transferrin (Alexa-Fluor-555-labelled antibodies; Fig. 4D), the early endosomal marker EEA1 (Fig. 4E) as well as the lysosomal marker LysoTracker Red DND-99 (Fig. 4F). After IL-1β stimulation we observed some colocalisation of gp130-positive vesicles and transferrin-positive vesicles (Fig. 4D), however, a much larger number of vesicles co-localised with the early endosomal marker EEA1, a process that was IL-1β dependent (Fig. 4E). Additionally, we observed a broad co-localisation with the lysosomal marker (Fig. 4F) indicating that IL-1β induced both internalisation and lysosomal degradation of gp130.

To corroborate these findings biochemically, we examined whether IL-1β affects the decay of gp130 overall. Therefore, HepG2 cells were incubated with cycloheximide to block de novo protein biosynthesis. The amount of gp130 remaining after 1-6 hours in the absence or presence of IL-1β was examined by western blotting (Fig. 4G). Indeed, we observed accelerated degradation of gp130 in the presence of IL-1β, further supporting a role for IL-1β, not only in internalisation but also in subsequent degradation of gp130.

**The loss of gp130 cell surface expression depends on receptor internalisation and requires the serine residue S782 in the cytoplasmic region of gp130**

Next, we determined the receptor requirements contributing to p38-dependent internalisation. Overexpression of a dominant-negative mutant of dynamin 2 (Dyn2-K44A) – which inhibits clathrin-dependent internalisation (Damke et al., 1994) – prevented stress-induced loss of coexpressed gp130 in COS-7 cells (Fig. 5A). It is well established that the constitutive internalisation of gp130 depends on a di-leucine motif (L786 L787) in its cytoplasmic tail (Dittrich et al., 1994; Thiel et al., 1998). Indeed, mutation of this amino acid sequence leads to an internalisation-deficient receptor which is no longer downregulated upon treatment with PAO (Fig. 5B). Previous studies on LIF signalling suggested that serine residues, most prominently S782, located upstream of the di-leucine motif in gp130 (Fig. 5B, upper panel) might be involved in the regulation of the internalisation process (Gibson et al., 2000). As shown in Fig. 5C, phosphorylation of S782 was indeed induced by the pro-inflammatory cytokine IL-1β treatment of HepG2 cells with IL-1β for 5 minutes (Fig. 5C lane 3) or 10 minutes (lane 7) led to a strong phosphorylation of endogenous gp130 at S782. Importantly, this IL-1β-induced phosphorylation could be blocked by the p38 inhibitor SB202190 (lanes 5 and 9). By contrast, IL-6 itself did not lead to S782 phosphorylation of gp130 (lanes 2 and 6). Also, in HepG2 cells phosphorylation of gp130 in response to LIF could not be observed (supplementary material Fig. S4A).

Based on these observations we speculated that S782 in gp130 serves as a stress sensor, limiting gp130 cell surface expression. To prove this hypothesis, we constructed new receptor mutants in which S782 was replaced by aspartate or alanine to mimic constitutive phosphorylation of S782 or to prevent phosphorylation of S782, respectively. In order to allow assessment of these mutants in cells endogenously expressing gp130, we generated chimeric receptors containing the extracellular region of the erythropoietin receptor fused to the gp130 transmembrane and intracellular part containing the above mentioned mutations of S782 or of the di-leucine internalisation motif. Additionally, these chimeric receptors contained an N-terminal HA-tag to allow detection. Cell surface expression of each gp130 construct was monitored in stably transfected HepG2 cells. In response to IL-1β the surface expression of the wild-type construct was markedly decreased, whereas the internalisation-deficient gp130 (LL/AA) construct as well as the non phosphorylatable S782A mutant were resistant to IL-1β treatment (Fig. 5D). By contrast, the expression of the phosphorylation mimic S782D was reduced already in the absence of IL-1β and did not decrease further upon IL-1β treatment (Fig. 5D). As expected, only the wild-type receptor and the LL/AA mutant were phosphorylated in response to IL-1β (Fig. 5D, lower panel). Furthermore, similar observations were made for PAO- or TNFα-treated HEK293 cells stably expressing IL-5Rα-gp130 chimeric receptors (supplementary material Fig. S4B,C).

Since lower steady state expression levels of the S782D mutant could indicate a faster internalisation or an overall lower expression of this mutant, we carried out internalisation assays using live-cell imaging and flow cytometry. In live-cell imaging experiments uptake of Alexa-Fluor-555-labelled anti HA-antibody was assessed 15 minutes after transfer of labelled cells from 4°C to 37°C. Single cell analysis at the 0- and 15-minute time points indicated a constitutive internalisation of wild-type gp130 (Fig. 5E, first panel), the S782D mutant (third panel) and to a minor extent also of the S782A mutant (fourth panel). The internalisation-deficient mutant gp130 LL/AA remained at the cell surface (second panel). Quantitative fluorescence analysis at the cell surface and the overall fluorescence analysis was performed for the same cell by single cell analyses at 0 and 15 minutes. Cumulative results for 20-25 cells per cell line are presented in supplementary material Fig. S4D. Flow cytometry allowed quantification of a larger number of cells and confirmed a faster constitutive internalisation of the S782D mutant compared with wild-type gp130 or the gp130 S782A mutant (Fig. 5F).

Taken together, these data clearly show that inflammatory cytokines and stress stimuli lead to a p38-mediated phosphorylation of S782 within the cytoplasmic part of gp130 and that phosphorylation of this serine is crucial for reducing the surface expression of gp130.

**MK2 mediates phosphorylation of S782 of gp130**

Since S782 in gp130 is not part of a p38 consensus site, it is very unlikely that p38 phosphorylates this residue directly. However, p38 itself is an activator of kinases belonging to the family of MAPK-activated protein kinases (MK), e.g. MK2, MK3 and MK5 (for a review, see Gaestel, 2006). These enzymes typically phosphorylate substrates containing a φxRxxS/T consensus sequence, where φ
Cross-regulation of cytokine signalling represents a bulky, hydrophobic amino acid. As demonstrated in Fig. 6A, S782 is part of such a consensus sequence. Furthermore, we could demonstrate that IL-1β or PAO treatment results in MK2 activation in HepG2 and HeLa cells (supplementary material Fig. S5A,B). We generated two new IL-5Rα-gp130 chimeric proteins containing mutations within the MK2 consensus sequence. In these

![Graph](https://via.placeholder.com/150)

**Fig. 5.** Reduction of gp130 cell surface expression in response to cellular stress requires an intact di-leucine internalisation motif as well as serine residue 782. (A) COS-7 cells were either transiently MOCK-transfected or transfected with an expression vector encoding dominant negative dynamin. Co-transfection of a GFP-expression vector allowed screening for transfected cells. 48 hours post-transfection, cells were incubated for 45 minutes with PAO (1 μM; hatched bars) or DMSO alone (grey bars). Gp130 cell surface expression in GFP-positive cells was evaluated by FACS as described in legend to Fig. 3A (values are means ± s.e.m., n=4, **P<0.01, *P<0.05). (B) Upper panel: schematic representation of the intracellular region of gp130; the sequence surrounding the di-leucine internalisation motif is enlarged below. Lower panel: COS-7 cells transiently expressing gp130-GFP fusion proteins were incubated with PAO (1 μM; hatched bars) or DMSO (grey bars) for 45 minutes. The cells were stained with anti-gp130 antibody (B-R3) followed by PE-conjugated secondary antibody. GFP-expressing cells were gated and fluorescence of the gated cells was analysed in the FL2 channel (PE, surface expression). Cell surface expression was determined by FACS analysis as described for Fig. 3A (values are medians ± s.e.m., n=4, ***P<0.001, *P<0.05). (C) HepG2 cells were pretreated with DMSO or SB202190 (10 μM) for 40 minutes as indicated. Subsequently, cells were stimulated with IL-6 (100 IU/ml), IL-1β (100 IU/ml) or left untreated for 5 or 10 minutes. Cellular extracts were prepared and western blots were incubated with antibodies against gp130 phosphorylated at S782 (pS-gp130) (lower panel). The blot was stripped and re-probed with antibodies against gp130 (lower panel). **(D)** Stably transfected HepG2 cells were incubated for 30 minutes with IL-1β (100 IU/ml; hatched bars). Chimeric HA-tagged EpoR-gp130 receptors were stained with an anti-HA antibody and cell surface expression was evaluated as described in legend to Fig. 3A by FACS (upper panel; means ± s.e.m., n=3, **P<0.01, *P<0.05). Additionally, cellular extracts were prepared and western blots were incubated with antibodies against gp130 phosphorylated at S782 (pS-gp130) (lower panel). The blot was stripped and re-probed with antibodies against the HA-tag (lower panel). **(E)** Receptor internalisation in stably transfected HepG2 cells was visualised by live cell imaging. HA-tagged EpoR-gp130 was stained with an Alexa-Fluor-555-labelled anti-HA antibody at 4°C for 1 hour, cells were transferred into a live cell chamber and receptor distribution was documented by multiple times series (shown for 0 and 15 minutes). (F) Constitutive internalisation of the HA:EpoR-gp130 receptor was analysed for the indicated time periods in transfected HepG2 cells by uptake of anti-HA antibodies as described in Materials and Methods (medians ± s.e.m., n=4, ***P<0.001, **P<0.01, *P<0.05 vs untreated; #P<0.05). Red lines indicate the linear regression of the values obtained.
constructs alanine replaced either phenylalanine 777 (F777A) or arginine 779 (R779A; Fig. 6A). HEK293 clones stably expressing these mutants were treated with PAO and the surface expression of the chimeras was monitored by FACS analyses. In two clonal cell lines expressing the wild-type construct, PAO treatment resulted in a pronounced receptor downregulation, whereas the receptor surface expression in gp130-F777A- or -R779A-expressing cell lines remained unchanged (Fig. 6A). Additionally, no serine phosphorylation of gp130 was observed when these mutants were stimulated with PAO (not shown).

These results strongly suggest that an MK family kinase phosphorylates gp130 in response to cellular stress. To further narrow down the potential kinase involved, we examined murine embryonic fibroblasts (MEFs) deficient for MK2 or MK5 (Shi et al., Journal of Cell Science 123 (6)).
2003). MEFs from control mice responded as expected with a strong serine phosphorylation of gp130 after IL-1β and PAO treatment (Fig. 6B). Preincubation of the cells with SB203580 strongly reduced the serine phosphorylation initiated by IL-1β and PAO (lanes 3 and 5) as clearly demonstrated by quantitative analysis of the western blots (supplementary material Fig. S5C) indicating that also in murine cells the effect is mediated by p38 MAPK. The inhibitor was slightly less efficient at dampening PAO-induced serine phosphorylation of gp130, indicating that additional, yet-to-be-identified serine kinases might be activated in response to PAO. In MK2-deficient MEFs gp130 serine phosphorylation was strongly impaired (Fig. 6B, lanes 7 and 9; supplementary material Fig. S5C) whereas it remained clearly detectable in MK5-deficient MEFs (Fig. 6B, lanes 12 and 14; supplementary material Fig. S5C) after both stimuli. This correlated well with the intact MK2 phosphorylation in MK5-deficient MEFs compared to wild-type MEFs (Fig. 6B, lower panels).

Consistent with the strongly impaired gp130 serine phosphorylation in MK2-deficient MEFs we also observed a reduced IL-1β- and PAO-mediated loss of gp130 cell surface expression in these cells whereas no difference could be detected between wild-type and MK5–/– MEFs (Fig. 6C,D). Internalisation assays in wild-type MEFs, MK2 –/– cells and MK2 –/– cells reconstituted with MK2 confirmed these results (supplementary material Fig. S5D). Additionally, a low molecular mass inhibitor of MK2 strongly impaired IL-1β-dependent internalisation of gp130 and retained plasma membrane localisation (Fig. 6E, lower panel). Taken together, these experiments provide strong evidence that MK2 is the kinase responsible for the downregulation of gp130 upon stress and inflammatory cytokine stimulation.

S782 of gp130 contributes to IL-1β-mediated inhibition of IL-6-induced STAT3 activation and gene expression

After having shown that pro-inflammatory cytokines and cellular stresses result in a MK2-dependent phosphorylation of gp130 at S782, which initiates rapid loss of surface expressed gp130, we assumed that this molecular mechanism contributes to the rapid IL-1β-mediated inhibition of IL-6-type cytokine-induced STAT3 tyrosine phosphorylation. We therefore tested whether IL-1β-mediated inhibition of gp130-dependent STAT3 activation is abrogated in MK2-deficient fibroblasts. Since fibroblasts do not express membrane-bound IL-6R the cells had to be stimulated with the agonistically acting soluble IL-6R. Indeed, IL-1β reduced IL-6- and sIL-6R-induced STAT3 activation in wild-type MEFs (Fig. 7A, lanes 1-4), but not in MK2-deficient MEFs (lanes 5-8), whereas in MK5-deficient cells the IL-1β effect was unaffected (lanes 9-12).

The observation that the S782D mutant of gp130 displays a much lower cell surface expression than the S782A mutant (Fig. 5D) led us to test whether decreased cell surface expression is reflected in reduced gene induction through this receptor mutant. We utilized EpoR-gp130 receptor chimeras which upon Epo stimulation induce signalling similar to the wild-type IL-6 receptor complex independent of the presence of endogenous gp130. As shown in Fig. 7B an α1-antichymotrypsin promoter-driven as well as an α2-macroglobulin promoter-driven luciferase gene is much better transcribed in response to activation of the gp130-S782A construct than the gp130-S782D mutant, indicating that decreased cell surface expression indeed reduces the responsiveness of cells to the respective ligand.

To check whether our observation also holds true for the regulation of IL-6-induced endogenous gene expression by IL-1β we analysed induction of endogenous γ-fibrinogen mRNA by real-time PCR in HepG2 cells. As shown in Fig. 7C (columns 1-4) IL-1β significantly reduces γ-fibrinogen expression which was initiated through the EpoR-gp130 chimeric receptor. To test whether this inhibitory activity
of IL-1β is mediated through the newly identified MK2-dependent pathway we knocked down MK2 expression by siRNA (Fig. 7C, left panel). In HepG2 cells lacking MK2 expression through siRNA knock-down, no inhibitory effect of IL-1β on IL-6-dependent γ-fibrinogen expression could be observed (columns 5-8).

These data strongly suggest that phosphorylation of S782 within gp130 by MK2 contributes to IL-1β-dependent inhibition of gp130-mediated STAT3 activation and gene induction.

**Discussion**

The present study identifies a previously unobserved phosphorylation of gp130, the common signal transducing receptor subunit of IL-6-type cytokines, by pro-inflammatory cytokines (IL-1β, TNFα) and cellular stress (UV, PAO), which results in accelerated internalisation and degradation of gp130. Therefore, our finding significantly contributes to the understanding of how pro-inflammatory stimuli and cellular stress rapidly downregulate IL-6-type cytokine-mediated signal transduction and gene expression (Andus et al., 1988; Ahmed and Ivashkiv, 2000; Bode et al., 2001b; Ahmed et al., 2002).

The regulatory phosphorylation occurs on S782 in the intracellular region of gp130, close to the di-leucine motif crucial for internalisation (Dittrich et al., 1994). We demonstrate that phosphorylation of gp130 at S782 induces loss of up to 50% of cell surface expressed gp130 within 30 minutes (Fig. 3A). Both phosphorylation of S782 and the presence of the di-leucine motif are essential for the downregulation. Our studies clearly show that IL-1β initiates the following processes: gp130 internalisation is significantly accelerated (50% internalisation in response to IL-1β after 30 minutes versus 25% constitutive internalisation; Fig. 4A) and gp130 translocates from the plasma membrane to early endosomes and finally to the lysosomal compartment (Fig. 4E, F) where it is degraded (Fig. 4G).

Interestingly, one important prerequisite for di-leucine-mediated internalisation through the membrane adaptor AP-2 is an acidic amino acid in position –4 to the di-leucine motif (Pieters et al., 1993; Pond et al., 1995; Dietrich et al., 1997). Gp130 does not contain such an acidic residue at position –4, but instead the serine residue 782. Thus, phosphorylation of this serine might generate the negative charge required for increasing the affinity for the AP-2 adaptor complex. Indeed, replacement of S782 by aspartate to mimic the phosphorylation decreased the steady state cell surface expression level of gp130 by 50% compared with the S782A mutant, which can no longer be phosphorylated (Fig. 5D).

Serine 782 in gp130 has previously been shown to be phosphorylated by stimulation of 3T3-L1 pre-adipocytes with leukaemia inhibitory factor (LIF) (Gibson et al., 2000). The authors suggested that this phosphorylation contributes to LIF-mediated receptor internalisation. Mutation enhanced expression of target genes. No similar observation has been reported so far for any other receptor tyrosine kinase family this mechanism seems to be specific for the EGFR since neither the insulin receptor nor the HGF receptor c-Met were internalised in response to TNFα, IL-1β or UV (Zwang and Yarden, 2006). It will be interesting to investigate whether MK2, as a downstream substrate of p38, plays a role in growth factor internalisation as well. Here we demonstrate that within the IL-6-type cytokine receptor family only gp130 is susceptible to p38 and/or MK2-mediated internalisation whereas the IL-6R, the OSMR (Fig. 3B) and LIFR (not shown) were not affected. This is in line with the fact that the serine 782 motif within an MK2 target sequence is not conserved in these receptors although the internalisation motif in the LIFR is also neighboured by a serine at position –4. So far, no involvement in receptor internalisation has been attributed to this serine residue. It is conceivable, however, that similar mechanisms might apply for other cytokine receptor families.

Fig. 7 demonstrates a significant contribution of gp130 internalisation to the restriction of IL-6-mediated signal transduction: MK2-deficient cells are resistant to IL-1β-mediated inhibition of STAT3 tyrosine phosphorylation (Fig. 7A) and receptors harbouring a serine 782 to aspartate mutation show a severely impaired reporter gene induction upon stimulation (Fig. 7B). Most importantly, knockdown of MK2 renders cells resistant to IL-1β-mediated inhibition of endogenous IL-6-induced gene expression (Fig. 7C).

Recently, in vivo studies identified the threshold of STAT3 signalling initiated by IL-6-type cytokines as a critical determinant for pathological consequences. Gp130-dependent STAT3 hyperactivation correlates with splenomegaly, exaggerated hepatic acute-phase response and dysregulated haematopoietic homeostasis. These effects could be completely rescued by reducing STAT3 expression levels by knockdown of one STAT3 allele (Jenkins et al., 2005b; Jenkins et al., 2005a).

Thus, on the one hand the inhibitory activity of IL-1β on IL-6 signalling dampens potential additive pro-inflammatory effects of both cytokines, resulting in prevention of overshooting immunological reactivity such as autoimmune arthritis or inflammatory bowel disease (for a review, see Kubo et al., 2003). On the other hand, IL-1β delays the anti-inflammatory effects of IL-6 to strengthen the pro-inflammatory processes in the initial phase of an inflammation.

Since the negative effect of IL-1β and TNFα on IL-6-induced acute phase protein gene expression was first described in 1988 by Andus et al. (Andus et al., 1988), many studies on the underlying molecular mechanisms of IL-1β-dependent repression of IL-6...
signalling have been performed. Our novel findings can explain various recent observations for the inhibition of gp130-mediated signalling: Ahmed et al. reported that IL-1β inhibits IL-6-mediated STAT3 activation in a very rapid manner, independent of de novo protein synthesis (SOCS), independent of the Y759 residue in gp130 and independent of modifications of the STAT3 serine phosphorylation (Ahmed and Iavashkiv, 2000; Ahmed et al., 2002). Interestingly, IL-1β-mediated inhibition of IL-6 signalling could be strongly impaired by inhibiting p38 activity. Additionally, overexpression of a constitutive active MKK3 or MKK6 almost completely abolished IL-6-mediated gene induction (Bode et al., 2001b; Ahmed et al., 2002). These observations suggested that IL-1β acts very early in the signalling pathway, however, the exact target could not be identified. Here, we finally identify gp130 itself as the target of pro-inflammatory cytokine intervention in IL-6 signal transduction.

Materials and Methods

Cytokines

Recombinant OSM and TNFα were purchased from Peprotech (London, UK). Recombinant IL-6 and sIL-6R were prepared as described by Arcone et al. and Weiergräber et al. (Arcone et al., 1991; Weiergräber et al., 1995). The specific activity of IL-6 was 2×10⁶ U/mg cell-stimulatory factor-2 units/mg protein. Recombinant IL-1β was purchased from Roche. Recombinant erythropoietin (Epo) was a generous gift from B. Hilger and K. H. Selliger (Roche, Mannheim, Germany).

Antibodies

Antibodies against Y705-phosphorylated STAT3 (pY-STAT3; 9131, 9138), MK2 (3042), T222-phosphorylated MK2 (p-TK2; 3044) and tubulin (2148) were obtained from Cell Signaling Technology (Frankfurt/Main, Germany). Antibodies against gp130 (M20, sc-656), the OSMR (AN-A2, sc-9992), p38 MAPK (C-20, sc-535) and EEA1 (C-15, sc-6414) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against S782-phosphorylated gp130 (pS-pgp130) were from Santa Cruz Biotechnology or BD Biosciences (558096). The mouse monoclonal antibody against STAT3 (610189) was purchased from BD Biosciences. The antibody used for FACS analysis to monitor human gp130 (B-R3) and IL-6Rα (B-R6) cell surface expression was obtained from Diagnol (Hützel Diagnostika GmbH, Cologne, Germany) and for murine gp130 (D023-3) from MBL (Japan). The antibody against the extracellular region of the IL-5Rα was a kind gift from Jan Tavernier (Ghent, Belgium). Antibodies against active double-phosphorylated p38 (T180/Y182; pT-P- p38) were from Promega (Madison, WI, USA). HRP-coupled secondary antibodies as well as IgG1 antibodies for control were from DAKO (Hamburg, Germany). Monoclonal HA antibodies to the HA-tagged extracellular domain of the EpoR and gp130 receptors were from Covance (HA.11, MM-101R).

Suppliers

Aprotinin, pepstatin, and gentamycin were from Sigma-Aldrich (Taufkirchen, Germany). DMSO, SB202190, SB203580 and Jak-Inhibitor I (Bio-Rad, München, Germany). The oligonucleotides used were synthesised by Operon (Carlsbad, CA, USA). Real-time PCR was performed with the Rotor-Gene 6000 Analytory (Corbett Research Pty Ltd, Mortake, NSW, Australia).

Buffers

RIPA buffer: 50 mM Tris-HCl (pH 7.5) 150 mM NaCl, 0.5% NP-40, 15% glycerol, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO4, protease inhibitor cocktail (10 μg/ml of each aprotinin, pepstatin (Sigma-Aldrich), and leupeptin (PM Biomedicals, Illkirch, France). Triton cell lysis buffer: 20 mM Tris-HCl (pH 7.5) 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM PMSF (phenylmethyl sulphonyl fluoride). Washing buffer: 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10 mM NaF, 0.1% Triton X-100. TBE: 20 mM Tris base, 20 mM boric acid, 0.5 mM EDTA (pH 8.0). Binding medium: Phenol Red-free DMEM-F12 medium, supplemented with 0.2% BSA and 10 mM HEPES buffer.

Media for cell culture

 Dulbecco’s modified Eagle’s medium (DMEM) was from Invitrogen-Gibco (Paisley, UK) and foetal calf serum (FCS) from PAA (Pasching, Austria).

HepG2 and HepG2 derivatives were grown in DMEM-nut mix F12; normal human dermal fibroblast (NHDF), Hela, HEK293 and COS-7 cells were grown in DMEM. Medium was supplemented with 10% FCS, streptomycin (100 μg/ml) and penicillin (60 μg/ml). MEF cells were cultured in DMEM (High Glucose, GlutaMAX) supplemented with 10% heat inactivated FCS.

Cell lines

HepG2, COS7 and HeLa cells were purchased from DSMZ (Braunschweig, Germany). InBx expressing HepG2 cells were supplied by Stephan Ludwig, University of Münster, Germany. HEK293 cells were purchased from the ATCC (Wesel, Germany). Primary human dermal fibroblasts were kindly supplied by Hans F. Merek and Yvonne Marquardt from the Department of Dermatology (Medical School RWTH Aachen).

Cell culture

HepG2-mock and HepG2-iKBα were prepared as described previously and generously supplied by S. Ludwig (Yang et al., 2004). Murine embryonal fibroblasts (MEF) deficient for MK2 or MK5, as well as the corresponding wild-type cells were described previously (Kotlyarov et al., 1999). Primary human dermal fibroblasts (HDF) cells were isolated from foreskin as described previously (Drew et al., 2005). Stably transfected HepG2 cells were generated by retroviral infections.

Immunoprecipitation and immunoblot analyses

For immunoprecipitation, HepG2 cells from a confluent culture on a 10 cm plate were lysed in 500 μl RIPA buffer. Before adding antibodies, protein amounts were adjusted to be equal (1 mg protein). Buffers were supplemented with a protease inhibitor cocktail. For MEF cells a modified Triton-X-100-containing lysis buffer was used. Immune complexes were washed in washing buffer and subsequently separated by SDS-PAGE and transferred to a poly(vinylidene difluoride; PVDF) membrane. Antigens were detected by incubation with specific primary antibodies and horseradish-peroxidase (HRP)-coupled secondary antibodies and developed with an enhanced chemiluminescence kit. For the analysis of whole cell extracts, cells were lysed as described above and 20 μg protein prepared by SDS-PAGE. For detection of activated STAT3, the protein amount was reduced to 10 μg, whereas for detection of S782-phosphorylated gp130 the amount was increased to 30 μg. Blotting and staining were carried out as for immunoprecipitated proteins.

Nuclear extract preparation and electrophoretic mobility shift assays (EMSA)

Preparation of nuclear extracts and EMSAs were performed as described previously (Wegenka et al., 1993). The protein concentration of the nuclear extracts was determined and 6 μg protein was incubated with a surplus of double-stranded 12P-labelled xsite (5'-GATCCATGGGGAGATTCCTCCCCCCATG-3') for binding NF-κB or a labelled optimized STAT3-binding site from the cfas-promoter m67SIE (5'-GATCCCCGAGGATTTACGGAAATACGCTG-3'). The protein-DNA complexes were isolated on a 4.5% polyacrylamide gel containing 7.5% glycerol in 0.25-fold TBE at 20 V/cm for 4 hours. Gels were fixed in 10% methanol, 10% acetic acid and 80% water for 1 hour, dried, and autoradiographed.

Expression vectors

Vectors for transfection of COS-7 cells (Fig. 5B): pSVL-gp130-eGFP encodes wild-type human gp130 fused at the C-terminus to the enhanced GFP protein, whereas pSVL-gp130-LA/AA-eGFP results from an exchange of the two leucine residues, 786L and 787L, for alanine within the il-leucine internalisation motif of gp130. Vectors for transfection of HEK293 cells (Fig. 6A, supplementary material S4B-C): pCAG-GS-IL-5Rα-gp130 encodes a chimeric receptor composed of the extracellular part of the IL-5Rα and the transmembrane and cytoplasmic part of gp130. Vectors of this vector, pCAG-GS-IL-5Rα-gp130-LA/AA and pCAG-GS-IL-5Rα-pCAG-IL5Rα-gp130-S782A, harbour a mutated di-leucine motif (IL786 L787 to AA) or a mutation of the newly identified MK2-phosphorylation site (S782 to A), respectively. pCAG-GS-IL-5Rα-gp130-F777A and pCAG-GS-IL-5Rα-pCAG-IL5Rα-S779A harbour single amino acid substitutions of phenylalanine 777 or arginine 779 to alanine in the MK2 consensus sequence of gp130.

Expression vectors for transient transfection of HepG2 cells (Fig. 7B): pCDNA3-1-Epo/gp130-S782A and pCDNA3-1-Epo/gp130-S782D encode chimeric receptors composed of the extracellular part of the EpoR and the transmembrane and cytoplasmic part of gp130. The vectors encode receptors which carry a mutation of the MK2 phosphorylation target residue 782 to alanine or aspartate, respectively. The chimeric receptor systems have been carefully characterised in previous studies in our laboratory. Vectors to express dominant negative dynamin (Fig. 5A) were kindly supplied by S. L. Schmid ( Scripps Research Institute, La Jolla, CA).
Transient and stable transfections

COS-7 and HepG2 cells were transiently transfected with 1-μg receptor expression plasmid using either the DEAE/dextran method as described previously (Herrmanns et al., 1999) or FuGene6 according to the manufacturer’s instructions. HEK293 Flp-In™-T-Rex™ (Invitrogen, Karlsruhe, Germany) were transfected using FuGene6 (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions.

Stably transfected HepG2 cells expressing the EpoR-gp130 chimera were washed in 3 ml 0.5% BSA-PBS, centrifuged and resuspended in 100 μl incubation. Primary antibody [phospho-STAT3 (1:100, Cell Signaling #9138) or anti-gp130 (1:200, BD Biosciences, San Jose, CA)] was added to HepG2 cells (30-40% confluent) and incubated at room temperature for 30 minutes. After washing, the bound supernatant was added together with 8 μg/ml polybrene (hexadimethrine bromide) to HepG2 cells (30-40% confluent). The following day medium was changed to DMEM-5% FCS and the temperature in the incubator reduced to 32°C. The retrovirus-containing supernatant was harvested 48 hours post-transfection in 1 ml Plates with 10 cm coverslips. After protein precipitation the supernatant was added together with 8 μg/ml polybrene to HepG2 cells (30-40% confluent). The following day medium was changed to DMEM-F12 plus 10% FCS. Cells were maintained in the presence of puromycine.

siRNA knock-down

siRNA knock-down was performed ON-TARGETplus SMART pools siRNA from Dharmacon (Schwerte, Germany). The pool L-005316-00-0020 was used to knock down human MK2, whereas the pool L-003767-00-0010 was used to knock down human P38α. To control the negative siRNA control duplex OR-0030-neg05 from Eurogentec (Seraing, Belgium) was used. siRNA was transfected using the DharmaFECT 1 transfection reagent T-2001-03 as suggested by the supplier. In detail, 3×10⁵ cells were seeded per well. The following day, 75 nM siRNA was dissolved in 100 μl OptiMEM (serum free). DharmaFECT1 was diluted 1:50 in OptiMEM at room temperature and incubated for 5 minutes before mixing the 100 μl diluted DharmaFECT1 and the 100 μl siRNA solution. While incubating the siRNA for 20 minutes, cells were washed with serum-free OptiMEM and dispensed with 800 μl of medium per well. siRNA solution was added and incubated for 5 hours at 37°C in the CO₂ incubator before adding 500 μl OptiMEM and 30% FCS. Before beginning the experiment, the cells were cultivated for additional 72 hours.

Isolation and analysis of mRNA

RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). cDNA was produced with help of the Omniscript RT Kit from Qiagen and mRNA content determined by real-time PCR. The probes Hs00241037 FGG and Hs99999909-m1 HPRT from Applied Biosystems (Carlsbad, CA) were selected to detect g-ribonuclease and HPRT mRNA.

Flow cytometry

5×10⁶ cells were harvested in ice-cold PBS-EDTA and resuspended in 100 μl ice-cold FACS buffer (PBS containing 5% FCS, 0.1% NaN₃) containing 0.2-1.0 μg monoclonal antibody raised against the extracellular part of the respective receptors or the HA-tag (anti-human gp130, anti-mouse gp130, anti-OSMR, anti-IL6R, anti-HA). After 30 minutes cells were washed in 3 ml FACS buffer and subsequently stained with mouse IgG-specific R-phycoerythrin-conjugated goat antibodies for an additional 30 minutes. Cells were analysed using the FACSCalibur or FACScanto (Becton Dickinson).

For intracellular FACS analysis cells were stimulated in 6 cm plates and afterwards detached using 0.25% EDTA. The cells were fixed by adding 4.5 ml ice-cold methanol for 30 minutes. Afterwards cells were blocked in 300 μl 2% PFA-PBS and rested for an additional 10 minutes. Cells were resuspended in 0.5 ml 2% PFA-PBS and rested for an additional 10 minutes. After centrifugation the cell pellet was left untreated or they were stimulated with IL-1β (100 IU/ml) for 15 or 30 minutes at 37°C. Control cells were left on ice during this incubation period to monitor initial gp130 cell surface localisation. After stimulation, the cells were harvested in ice-cold PBS-EDTA and incubated in FACS buffer with an R-phycocerythrin-conjugated goat anti-mouse antibody for 30 minutes on ice. Non-internalised gp130 was analysed by FACS. To monitor the appearance of intracellular, endocytosed gp130, cells were preincubated in ice-cold binding medium containing 1 μg/ml FITC-labelled control antibody or B-R3 (anti-gp130) antibody for 45 minutes. The cells were incubated with or without IL-1β (100 IU/ml) for 15 or 30 minutes at 37°C. After stimulation, the cells were harvested in ice-cold PBS-EDTA and washed for 5 minutes using 2 M glycine buffer (pH 2.5) to remove all cell surface-bound antibodies.

Immunofluorescence

HepG2 cells grown on coverslips were processed as described for the internalisation assays, however, binding medium also contained fluorescently labelled transferrin. After incubation at 37°C for 20 minutes, cells were fixed in 3.7% formaldehyde and mounted with Mowiol. For detection of EE1-positive vesicles, prior to mounting, cells were permeabilised in 0.1% Triton X-100 and stained using goat anti-EE1, followed by Cy3-labelled anti-goat secondary antibody.

Live-cell imaging

HepG2 cells were seeded on coverslips 1 day before investigation. During investigation cells were cultured in binding medium. Incubation was performed in the presence of FITC-labelled anti-gp130 antibody at 10% CO₂ and 37°C for 40 minutes with or without IL-1β (100 IU/ml). Recycling endosomes and lysosomes were stained with transferrin–Alexa-Fluor-555 and LysoTracker, respectively, according to the manufacturer’s instructions. To visualise the plasma membrane the incubation medium was changed, and cells were stained with Trypan Blue (red fluorescence) and confocal laser scanning analysis was carried out. When multiple time points were examined all pictures were taken using the automated acquisition protocol.

Internalisation assays were performed as described above. Briefly, cells were incubated for 1 hour at 4°C with Alexa-Fluor-555-labelled anti-HA antibody in binding medium. Subsequently, cells were transferred into the live-cell chamber of the LSM and analysed for 15 minutes under cell culture conditions.

Reporter gene assay

HepG2 cells were transiently transfected with 0.75 μg receptor expression plasmid, 0.8 μg pGL3ct2M-Luc or pGL3-ct1ACT-Luc reporter and 0.4 μg β-galactosidase plasmid (pCH110, GE Healthcare) using FuGene6 transfection reagent (Roche) according to the manufacturer’s instructions. pGL3-ct2M-Luc contains the promoter region –215 to +8 of the rat α2-macroglobulin promoter and pGL3-ct1ACT-Luc the promoter region –379 to +25 of the human α1-antichymotrypsin promoter upstream of the luciferase-encoding sequence of plasmid pGL3 (Promega). One day after transfection, cells were split onto 12-well plates and 1 day later stimulated with Epo (7 IU/ml) for 16 hours. Luciferase activity was determined with the Luciferase Assay System (Promega) and normalised to the activity of coexpressed β-galactosidase.

Statistical analysis

Unless stated differently, all data are expressed as medians + s.e.m. for at least three separate experiments (one-tailed, paired Student’s t-test). Probabilities of P<0.05, P<0.01 and P<0.001 was considered significantly (*), very significantly (**) and extremely significantly (***) different, respectively. All analyses were performed using the statistical software GraphPad Prism (GraphPad Prism for Windows, Version 3.00, GraphPad Software, Inc., CA).

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Supplementary material available online at http://jcs.biologists.org/cgi/content/full/123/6/947/DC1

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