Internalization of the interleukin 6 signal transducer gp130 does not require activation of the Jak/STAT pathway

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Signalling receptors often undergo receptor-mediated endocytosis. In many cases this internalization is stimulated by ligand binding and activation of intrinsic receptor tyrosine kinases, resulting in a receptor down-regulation. We have analysed whether internalization of the interleukin 6 signal transducer gp130 is dependent on the activation of receptor-associated Jak kinases. By using a chimaeric receptor system we found that receptor mutants that lack box1 and therefore are not capable of activating Jak and signal transducer and activator of transcription (STAT) proteins are still endocytosed efficiently. A chimaeric receptor with the recently identified dileucine internalization motif being replaced by two alanine residues was not efficiently internalized but still capable of recruiting STATs. Furthermore an antagonistic antibody that inhibits the signalling of all interleukin-6-type cytokines via gp130 was internalized as efficiently as an agonistic one that activates the Jak/STAT pathway. Our findings suggest that the endocytosis of gp130 is signal-independent.

INTRODUCTION

Interleukin 6 (IL-6) acts via a cell-surface receptor complex composed of two subunits: the 80 kDa IL-6 receptor (IL-6R, gp80) and a 130 kDa signal-transducing molecule, gp130 [1–3]. gp130 is also the signal-transducing component of the receptors for leukaemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, IL-11 and cardiotrophin 1 [4]. Binding of IL-6 to the IL-6R induces the homodimerization of two gp130 molecules and transduction of the signal into the cell [5]. The 277-residue cytoplasmic domain of gp130 contains two short segments within the membrane proximal region referred to as box1 and box2, which are conserved between members of the haemopoietic cytokine receptor family [6]. The box1 motif is required for the association of the Janus tyrosine kinases Jak1 and Jak2 [7,8]. Jak1, Jak2 and Tyk2, a third member of the Jak family, were found to be constitutively associated with gp130 and are activated and autophosphorylated on stimulation with IL-6 [9,10]. In turn, the Janus kinases are believed to mediate the tyrosine phosphorylation of gp130, thereby generating docking sites for signal transducer and activator of transcription (STAT) factors. Two members of the STAT family, STAT1 and STAT3, are rapidly tyrosine phosphorylated in response to IL-6 [9,11–13]. The activated STATs homo- or heterodimerize and translocate to the nucleus, where they bind to specific enhancers within promoter regions of IL-6-responsive genes [14,15].

After binding to its receptor, IL-6 is rapidly internalized and the IL-6R is down-regulated [16,17]. We have shown that the cytoplasmic domain of gp130, but not that of gp80, is essential for the efficient endocytosis of IL-6 and receptor down-regulation [18]. Moreover, we identified a dileucine motif (STQPLL) at positions 141–146 in the cytoplasmic tail of gp130 that is responsible for this process [19].

Currently it is unknown whether activation of the IL-6 signalling cascade induces endocytosis of the signal transducer or whether gp130 internalization is signal-independent. To address this question we constructed chimaeric receptors that consisted of the extracellular domains of the IL-5 receptor (IL-5R) α and β chains respectively, and the transmembrane and cytoplasmic parts of wild-type and mutant forms of gp130. This experimental system allowed us to examine the function of gp130 mutants either defective in signalling or internalization in COS-7 cells independently of the endogenous gp130. We also compared the internalization kinetics of agonistic and antagonistic monoclonal antibodies against gp130.

MATERIALS AND METHODS

Materials

Restriction enzymes, T4 DNA ligase and Klenow enzyme were purchased from Boehringer Mannheim (Mannheim, Germany) and New England Biolabs (Schwalmbach, Germany). Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany). Dulbecco's modified Eagle's medium was from Gibco (Eggenstein, Germany). Recombinant human IL-5 was prepared as described [20]. Dr. T. Taga (Osaka, Japan) kindly provided the expression vector pSVLgp130. The generation and characterization of monoclonal antibodies against human gp130 are described in [21]. FITC-conjugated mouse IgG antibody was purchased from Dakopats (Hamburg, Germany). $Na^{125}I$ was purchased from ICN (Meckenheim, Germany) and ^{125}I-labelled IL-5 was prepared with the Iodogen iodination agent (Pierce Chemicals, Rockford, IL, U.S.A.) as described [22].

Construction of expression vectors

Standard cloning procedures were performed as outlined by Sambrook et al. [23]. The construction of the gp130 mutant gp130AA is described in [19]. The mutant gp130Δbox1 was generated by deleting 69 nt between the unique restriction sites *Bst*EII and *Bsa*I. The chimaeric molecules IL-5Rα/gp130, IL-

Abbreviations used: EGF-R, epidermal growth factor receptor; EMSA, electrophoretic mobility-shift assay; IL, interleukin; IL-5R, IL-5 receptor; IL-6R, IL-6 receptor; STAT, signal transducer and activator of transcription.

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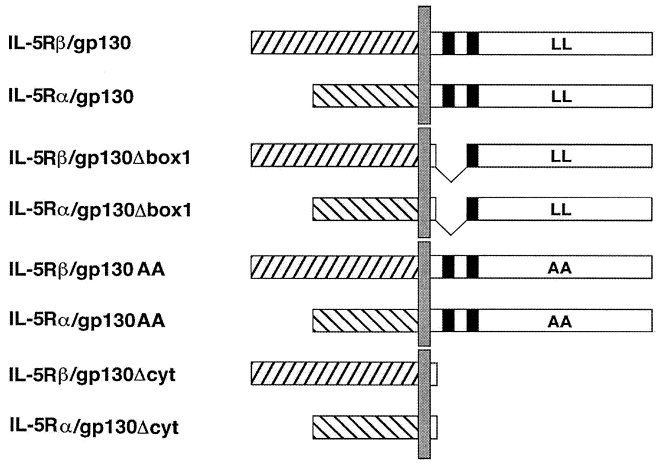

Figure 1 Schematic representation of IL-5R/gp130 chimaeras

The chimaeras are composed of the extracellular part of the IL-5R α or β chain (hatched boxes) and the transmembrane and cytoplasmic parts of the signal transducer gp130 (open boxes). Box1 and box2 are indicated by filled boxes; LL and AA represent the wild-type and mutated dileucine motifs respectively.

5Rα/gp130Δcyt, IL-5Rβ/gp130 and IL-5Rβ/gp130Δcyt are described in [24]. The molecules IL-5Rα/gp130AA, IL-5Rβ/gp130AA, IL-5Rα/gp130Δbox1 and IL-5Rβ/gp130Δbox1 were constructed by replacing the *Eco*RI/*Bam*HI fragment coding for the transmembrane and the cytoplasmic domains of IL-5Rα/gp130 or IL-5Rβ/gp130 respectively with one of gp130AA or gp130Δbox1. All chimaeric constructs used are depicted schematically in Figure 1.

Transfections

COS-7 cells (A.T.C.C. CRL 1651) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum (Seromed, Berlin, Germany), streptomycin (100 mg/l) and penicillin (60 mg/l) in a water-saturated air/CO_2 (19:1) atmosphere. Transfection of cells was performed with an Electro Square Porator T820 from BTX (San Diego, CA, U.S.A.). For internalization studies and electrophoretic mobility-shift assays (EMSAs) with chimaeric receptors, 2×10^6 COS-7 cells were co-transfected with 15 μg of pSVL vector containing the IL-5Rα/gp130 wild-type or mutant cDNA species and with 15 μg of pSVL vector containing the IL-5β/gp130 wild-type or mutant cDNA species. Mock transfections were done with 30 μg of empty pSVL vector. EMSAs after 'stimulation' with monoclonal antibodies B-R3 and B-S12 were performed with nuclear extracts of cells transfected with 20 μg of gp130 cDNA and 10 μg of pSVL-STAT3 cDNA (described in [25]). For immunofluorescence studies, 2×10^6 COS-7 cells were transfected with 30 μg of gp130 or gp130AA. All transfections were performed with one pulse of 99 μs length and 1.5 kV. Cells were used for further studies 2 or 3 days after transfection when reaching confluence.

EMSA

COS-7 cells transfected with chimaeric receptor cDNA species were grown in a 60 mm diameter dish for 2–3 days and stimulated with IL-5 (80 ng/ml) for 30 min. Nuclear extracts were prepared as described [13]. The protein concentration was determined with a Bio-Rad® protein assay. EMSAs were performed as described previously, with a double-stranded ^{32}P-labelled mutated m67SIE-oligonucleotide from the c-*fos* promotor (m67SIE, 5′-GAT CCG GGA GGG ATT TAC GG GAA ATG CTG-3′) [24]. The protein–DNA complexes were separated on a 4.5% (w/v) polyacrylamide gel containing 7.5% (v/v) glycerol in 0.25 × TBE (0.25 × TBE is 2.7 g/l Tris, 1.075 g/l boric acid and 0.5 mM EDTA, pH 8) at 20 V/cm for 4 h. Gels were fixed in methanol/acetic acid/water (1:1:8, by vol.) for 1 h, dried and autoradiographed.

Immunofluorescence studies

Approximately 10^5 COS-7 cells grown on coverslips for 48 h were incubated for 2 h at 4 °C with the monoclonal gp130 antibodies B-R3 and B-S12 (10 ng/ml). After removing unbound antibody, cells were shifted to 37 °C for 30 min. Cells were washed twice with PBS, fixed with 2% (w/v) paraformaldehyde, permeabilized with 0.2% saponin and incubated with a 1:200 dilution of an FITC-conjugated mouse IgG antibody. Coverslips were mounted on slides with Mowiol® 4-88 (Calbiochem, La Jolla, CA, U.S.A.) and analysed by confocal immunofluorescence microscopy (Confocal Laser Scan Microscope, Zeiss).

Internalization assay for IL-5R/gp130 chimaeras

Cells transfected either with the IL-5R/gp130 constructs or with an empty control vector were incubated with 1 nM ^{125}I-IL-5 for 2 h at 4 °C. Internalization was initiated by rapidly warming the cells to 37 °C without removing unbound ligand. After different durations of incubation, cells were placed on ice and washed three times with PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$. Surface-bound ^{125}I-IL-5 was removed by subjecting the cells to PBS/HCl/0.1% BSA (pH 3.0) for 7 min (acid wash) followed by an additional wash with PBS. This treatment released more than 90% of surface-bound ligand. Internalized ^{125}I-IL-5 was determined after lysis of the cells in 1 ml of 1 M NaOH. Specific binding and internalization was calculated by subtracting the values obtained with control-vector-transfected cells from the values obtained with cells expressing the chimaeric receptors.

Internalization assay for antibodies

Transfected cells were incubated with 1 μg/ml of ^{125}I-labelled B-R3 or B-S12 for 2 h at 4 °C. Internalization was performed as described above. To remove surface-bound ^{125}I-antibodies, cells were first subjected to an acid wash and then incubated with 0.5 ml (1 μg/ml) of proteinase K (Fluka, Basel, Switzerland) for 10 min to remove residual surface-bound antibodies. After 3 min of centrifugation, internalized ^{125}I-antibodies were determined in the cell pellet. Specific binding and internalization were calculated by subtracting the values obtained with control-vector-transfected cells from the values obtained with cells expressing gp130 wild-type or gp130AA.

RESULTS

IL-5 is efficiently internalized via a chimaeric receptor complex composed of the extracellular domains of the IL-5R α and β chains and the transmembrane and cytoplasmic domains of gp130

We first analysed whether a chimaeric IL-5R/gp130 complex is internalized and down-regulated similarly to the authentic IL-6R complex. Such a chimaeric IL-5R/gp130 complex binds IL-5 with an affinity similar to that seen with the wild-type IL-5R complex expressed in COS-1 cells (K_d 1 nM) [24]. COS-7 cells were

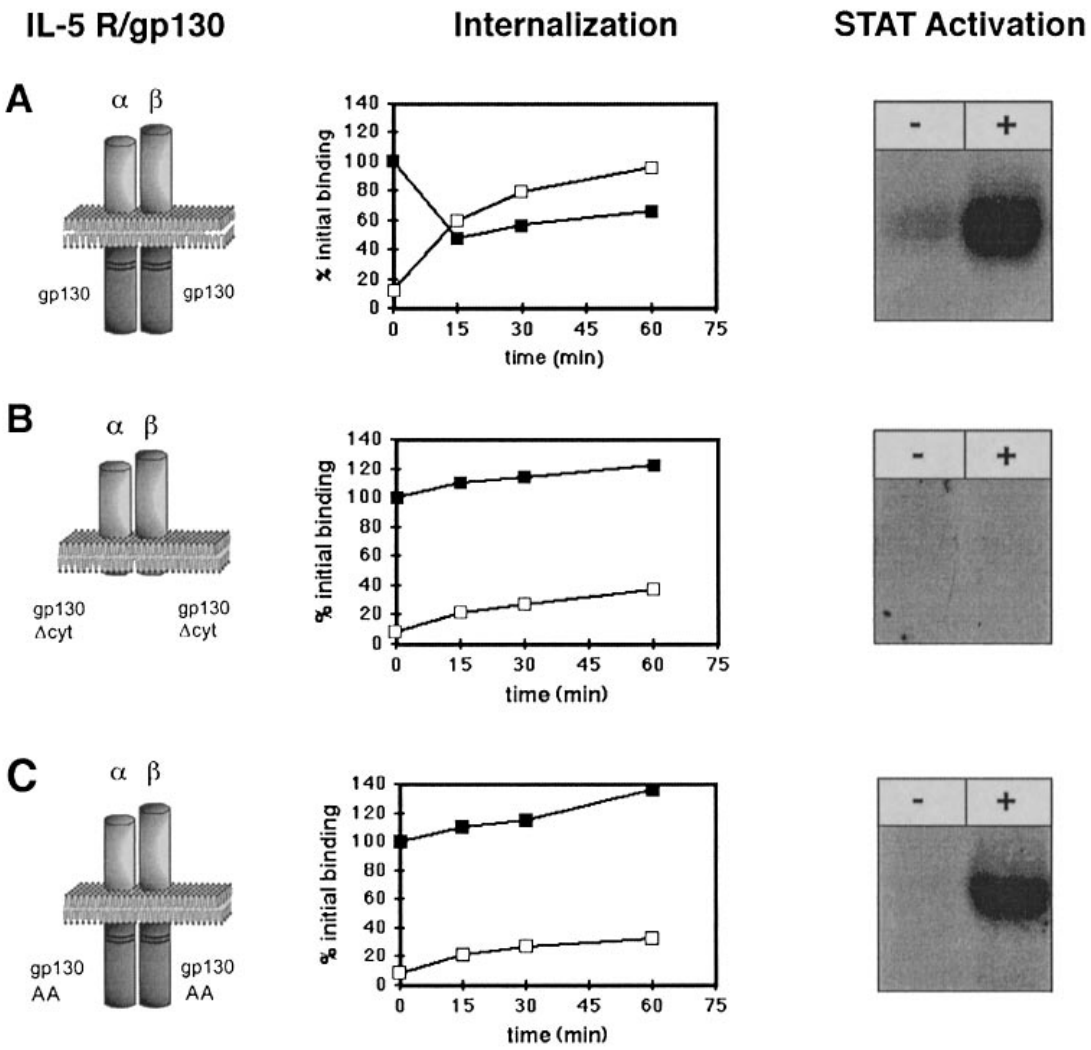

Figure 2 Internalization of surface-bound ^{125}I-IL-5 and activation of STAT1 by IL-5 via chimaeric receptor complexes

Left panels: schematic diagrams of the chimaeric receptor complexes. Middle panels: internalization of chimaeric receptors; 2 or 3 days after transfection, cells were washed twice with ice-cold binding medium. After incubation with 1 nM ^{125}I-IL-5 for 2 h at 4 °C, cells were warmed to 37 °C for the indicated times without removing unbound ligand. Cells were placed on ice again and washed three times with PBS to eliminate unbound ligand. Surface-bound ^{125}I-IL-5 (■) was determined after subjecting cells to an acid wash. Internalized ^{125}I-IL-5 (□) was measured after lysis of the cells in 1 M NaOH. Values obtained for binding and internalization by mock-transfected cells were subtracted. Results are expressed as percentages of the amount of IL-5 bound at zero time (see the Results section). Values are the means of three independent experiments. The sum of the surface-bound and internalized IL-5 at later time points adds to more than 100%, indicating that during the incubation period a pool of intracellular receptors translocated to the cell surface. Right panels: activation of STAT by chimaeric receptors. COS-7 cells were transfected with 15 μg of each of the respective α-chain and β-chain expression constructs. After 3 days, cells were stimulated with 80 ng/ml IL-5 for 30 min, and nuclear extracts were analysed as described in the Materials and methods section. Symbols: − and +, controls and stimulated cells respectively. The bands shown represent STAT1.

transfected with the respective expression vectors, plated on 24-well dishes and after reaching confluence incubated with 1 nM of ^{125}I-IL-5 for 2 h at 4 °C. Cells were shifted to 37 °C for 0–60 min after which surface-bound and internalized ligand was determined. Cells expressing the chimaeric IL-5R/gp130 complex bound approx. $[14.3 \pm 1.24$ (S.D.)$] \times 10^3$ molecules of ^{125}I-IL-5 per cell ($n = 4$). Non-specific binding by mock-transfected cells was $(0.81 \pm 0.24) \times 10^3$ molecules per cell ($n = 4$) and was subtracted to obtain specific binding. ^{125}I-IL-5 was efficiently internalized by the transfected COS-7 cells, with 50% of initially bound ligand being found in the cell lysate after 15 min (Figure 2A). The number of surface receptor complexes was down-regulated to 48% during this period. This rate of endocytosis is comparable with that observed for IL-6 with the authentic receptor components [18]. Mock-transfected cells did not show any internalization of IL-5 (results not shown). The cytoplasmic domain of gp130 was found to be essential for the internalization of IL-6 [18]. Therefore we analysed the internalization of ^{125}I-IL-5 by a chimaeric complex in which both the α-chain and β-chain chimaeras lacked the cytoplasmic domain (IL-5Rα/gp130Δcyt + IL-5β/gp130Δcyt). As expected, COS-7 cells expressing these chimaeras did not internalize ^{125}I-IL-5 efficiently (10% of initially bound within 15 min; Figure 2B) and receptor down-regulation did not occur. ^{125}I-IL-5 bound to this truncated receptor complex with the same affinity as to the complete chimaeras [24]. Interestingly, transfectants expressing these truncation mutants consistently showed a 2–3-fold increase in binding sites $[(38.2 \pm 14.1) \times 10^3$ sites per cell; $n = 3]$. This was due to an increased surface expression of both chimaeric subunits as assessed by FACS analyses (results not shown).

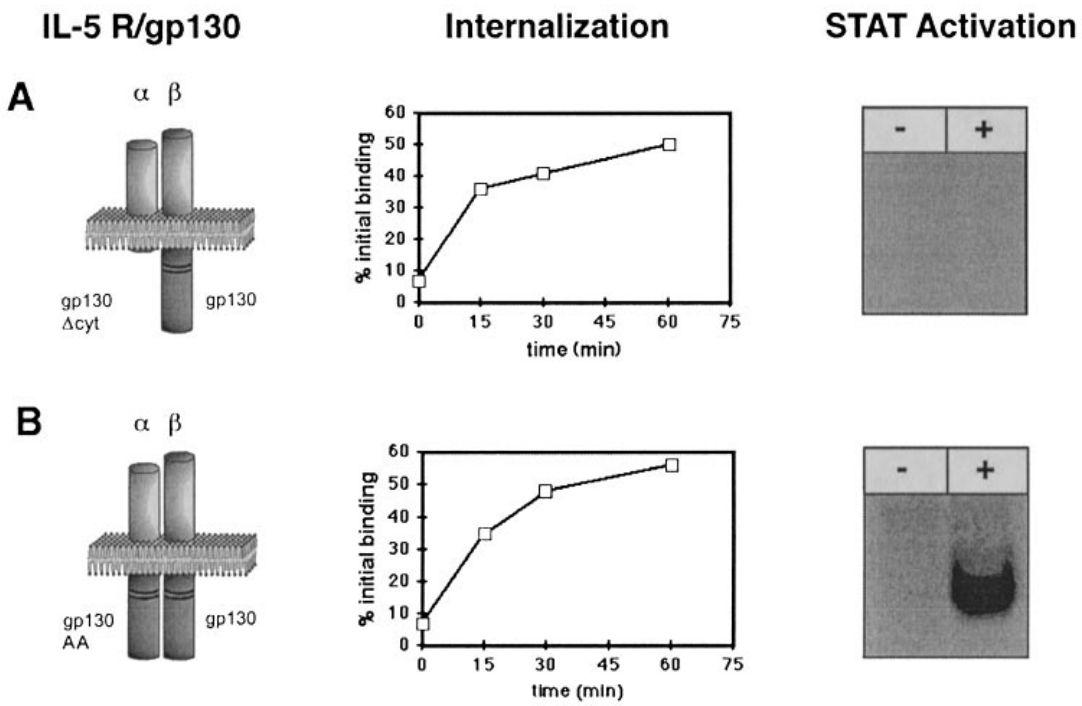

Figure 3 Internalization of surface-bound ^{125}I-IL-5 and activation of STAT1 by IL-5 via chimaeric receptor complexes

Left panels: schematic diagrams of the chimaeric receptor complexes. Middle panels: internalization of chimaeric receptors. Only the values of internalized ligand are shown. Right panels: activation of STAT by chimaeric receptors. Studies were performed as described in the legend to Figure 2.

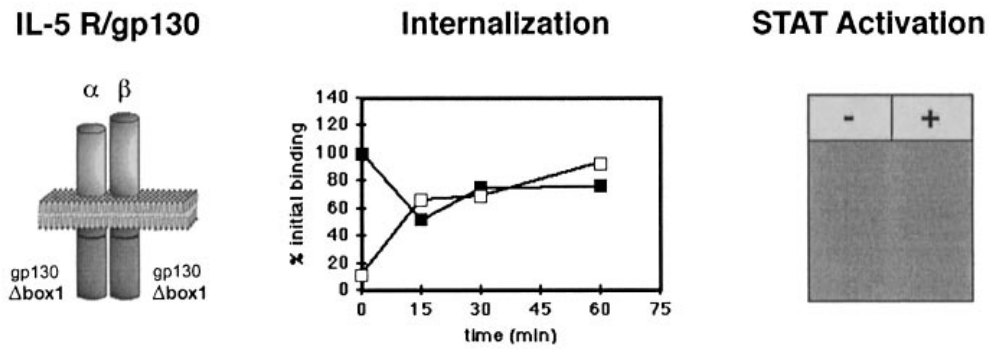

Figure 4 Internalization of surface-bound ^{125}I-IL-5 and activation of STAT1 by IL-5 via a chimaeric IL-5R/gp130Δbox1 receptor complex

Left panel: schematic diagram of the chimaeric receptor complex. Middle panel: internalization of chimaeric receptors. Right panel: activation of STAT by chimaeric receptors. Studies were performed as described in the legend to Figure 2.

Because we were interested in studying the role of the Jak/STAT pathway for the endocytotic process, we analysed whether the chimaeric receptors were capable of activating STAT factors. Activation of STATs by tyrosine phosphorylation results in their dimerization, which can be conveniently assayed by an EMSA [13,14]. As previously shown [24] stimulation of the IL-5Rα/gp130+IL-5β/gp130 chimaeras with IL-5 resulted in an activation of a nuclear factor binding to a STAT1/STAT3 consensus motif, which very probably represents STAT1 because this is the most prominent factor activated in COS-7 cells after stimulation via the endogenous gp130 [13] (Figure 2A). Activation of STAT1 was not observed with the truncated chimaeras (Figure 2B). These results suggested that the chimaeric IL-5R/gp130 system would allow us to study how mutations affecting the signal transduction via the Jak/STAT pathway influence the internalization process and vice versa.

gp130 dileucine internalization motif is necessary for efficient endocytosis of IL-5 via the chimaeric receptor complex

A dileucine motif within the cytoplasmic domain of gp130 is crucial for the efficient endocytosis of the IL-6/IL-6R complex [19]. We verified that this motif also mediates the internalization of the chimaeric receptor complex. An IL-5R/gp130 chimaeric complex where in both chains the dileucine motif at position 145/146 of the gp130 intracellular part was replaced by

alanine residues (IL-5Rα/gp130AA + IL-5Rβ/gp130AA) was as inefficient in IL-5 endocytosis as the receptor complex with truncated intracellular parts (Figures 2B and 2C). Also with these transfectants the number of initial binding sites was increased [$(33.4 \pm 9) \times 10^3$ sites per cell; $n = 3$]. Interestingly, the complex with the alanine mutants was still capable of activating STAT1 (Figure 2C). We conclude that internalization is not a prerequisite for signalling via the Jak/STAT pathway.

The chimaeric system also allowed us to investigate whether a single dileucine motif within a dimeric receptor complex would be sufficient for endocytosis. To address this question we co-expressed the IL-5Rβ/gp130 construct with either IL-5Rα/gp130Δcyt (Figure 3A) or IL-5Rα/gp130AA (Figure 3B). In both cases we observed significant internalization of ^{125}I-IL-5 (Figures 3A and 3B). Nevertheless only the complex IL-5Rα/gp130AA + IL-5Rβ/gp130 was capable of activating STAT1 (Figure 3B). This result indicated that internalization was not strictly dependent on signal transduction. In both cases the percentage of surface-bound ligand internalized was apparently smaller than in Figure 2 (note the different scales). However, this is due to the way in which the results are presented. Both chimaeric subunits without a dileucine motif, IL-5Rα/gp130Δcyt [$(31.2 \pm 2.7) \times 10^3$ sites per cell; $n = 3$] and IL-5Rα/gp130AA [$(36.1 \pm 5.6) \times 10^3$ sites per cell; $n = 3$] showed a higher surface expression than IL-5Rα/gp130 or IL-5Rβ/gp130. With asymmetric chimaeras this results in an excess of α-surface binding sites over internalization-competent β subunits and only a smaller percentage of binding sites will be internalized than in the situation where both subunits are equally abundant, as in Figure 2 [24].

Signalling via the Jak/STAT pathway is not necessary for efficient internalization of the chimaeric receptor complex

It has been shown that two regions within the membrane-proximal part of gp130 designated box1 and box2 are responsible for the interaction with the Jak tyrosine kinases. Receptor mutants lacking box1 were not capable of binding and activating Jaks [7,8]. To study whether activation of the Jak/STAT pathway is necessary for the efficient internalization of the gp130 signal transducer, we constructed chimaeric receptors lacking 23 residues including box1 (IL-5Rα/gp130Δbox1, IL-5Rβ/gp130Δbox1; Figure 1). Co-expression of these constructs in COS-7 cells resulted in receptor complexes incapable of activating STAT1 (Figure 4). These complexes endocytosed ^{125}I-IL-5 as efficiently as did the chimaeric IL-5R/gp130 complex (compare Figures 2A and 4).

IL-6 signal transducer gp130 is internalized independently of the activation of the Jak/STAT pathway

The results described above suggest that internalization and signal transduction via the Jak/STAT pathway are two independent processes. To corroborate this finding by an alternative method we took advantage of two monoclonal antibodies directed against epitopes within the extracellular part of human gp130. One of these antibodies, B-S12, was agonistic, i.e. in several bioassays it was capable of eliciting a biological response in the absence of IL-6 [21]. The most likely explanation for this property is that the B-S12 cross-links two gp130 molecules and thereby activates the associated Jak kinases. A second antibody, B-R3, was found to inhibit the responses to all IL-6-type cytokines, probably by interfering with the formation of signal-competent dimers [26]. To demonstrate the agonistic action of B-S12 in our system, we transiently expressed gp130 and STAT3 in COS-7 cells, incubated them with different concentrations of B-S12 and B-R3 for 15 min and prepared nuclear extracts. Incubation with B-S12 but not with B-R3 resulted in the typical three-band pattern in the EMSA representing activated STAT1 and STAT3 (Figure 5) [13].

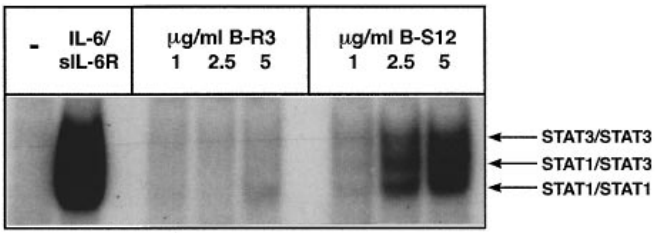

Figure 5 Activation of STAT1 and STAT3 by monoclonal antibody B-S12 via gp130

COS-7 cells were co-transfected with 20 μg of the gp130 and 10 μg of the STAT3 expression construct. After 3 days, cells were incubated for 30 min with indicated concentrations of B-R3 or B-S12, and nuclear extracts were prepared. For comparison COS-7 were stimulated with recombinant IL-6 (20 ng/ml) and soluble IL-6R (1 μg/ml) for 15 min. EMSAs were performed with the labelled m67SIE probe. The positions of the three dimers formed are indicated [11].

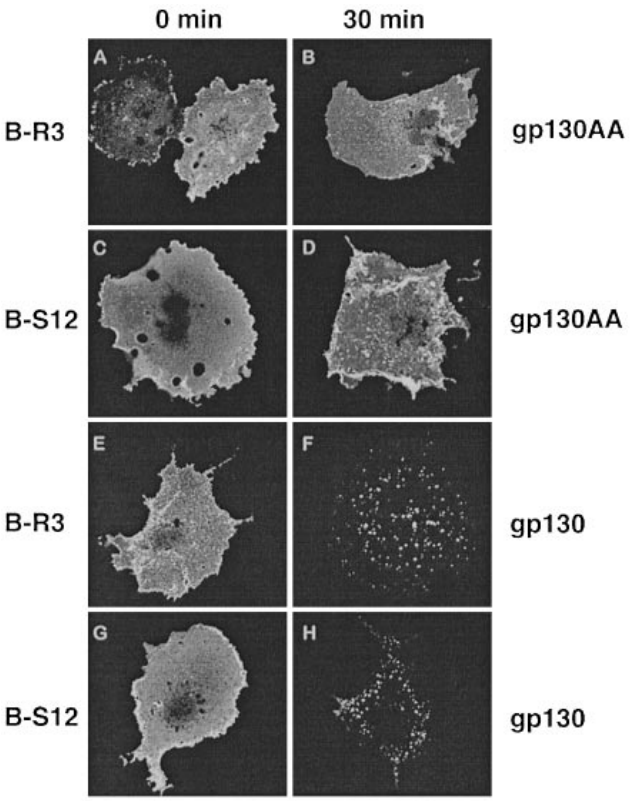

Figure 6 Internalization of monoclonal antibodies B-R3 and B-S12 by COS-7 cells expressing gp130AA or gp130

At 3 days after transfection, COS-7 cells were incubated with B-R3 (**A**, **E**) or B-S12 (**C**, **G**) for 2 h at 4 °C. Prebound antibodies were internalized for 30 min at 37 °C (**B**, **D**, **F**, **H**). Cells were fixed and permeabilized, then stained with an FITC-conjugated mouse IgG antibody. Representative optical cuts are shown. (**A**–**D**) Staining of cells transiently expressing gp130AA; (**E**–**H**) staining of cells transiently expressing gp130.

To study the effect of these two antibodies on the internalization of wild-type gp130, indirect immunofluorescence studies were performed. COS-7 cells expressing gp130 and

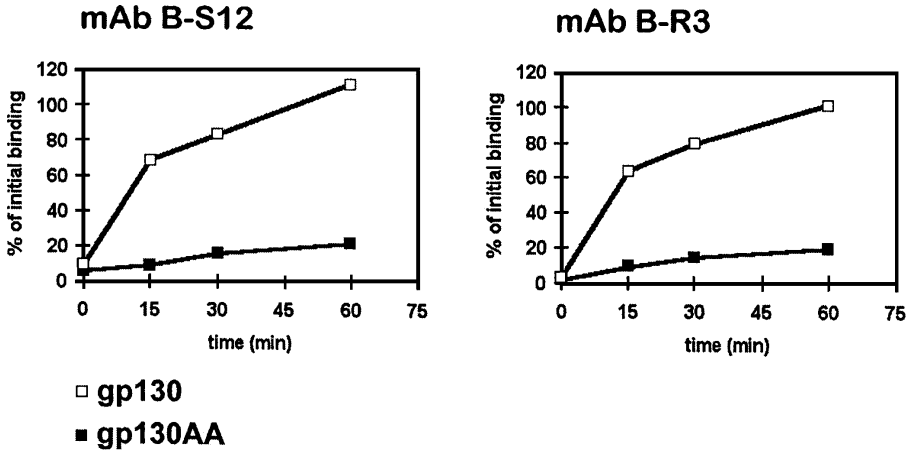

Figure 7 Internalization of surface-bound ^{125}I-labelled monoclonal antibody (mAb) B-S12 or ^{125}I-mAb B-R3 via gp130 or gp130AA

At 3 days after transfection with gp130 or gp130AA cDNA species, cells were washed twice with ice-cold binding medium. After incubation with 1 μg/ml ^{125}I-labelled monoclonal antibody for 2 h at 4 °C, cells were warmed to 37 °C for the indicated durations without removal of unbound antibodies. Surface-bound radiolabelled antibodies were removed by an acid wash followed by an incubation with 1 μg/ml proteinase K for 10 min. After 3 min of centrifugation, internalized ^{125}I-labelled antibodies were determined as the radioactivity located in the cell pellet. Values obtained for binding and internalization by mock-transfected cells were subtracted. Results are expressed as percentages of the amount of radiolabelled antibodies bound at zero time. Values are means for at least three independent experiments. Symbols: □, internalization via gp130; ■, internalization via gp130AA.

gp130AA were incubated at 4 °C with either B-S12 or B-R3, shifted to 37 °C for 30 min and fixed. After permeabilization cells were stained with an FITC-labelled secondary antibody and processed for laser scanning immunofluorescence microscopy. With both monoclonal antibodies there was a bright staining of the cell surface at time point 0 (Figures 6A, 6C, 6E and 6G). After 30 min at 37 °C both antibodies gave similar staining in cells expressing the gp130AA mutant (Figures 6B and 6D). In contrast, cells expressing wild-type gp130 showed a punctate intracellular staining pattern irrespective of which antibody was used, indicating that both were efficiently internalized (Figures 6F and 6H).

To obtain more quantitative data, both antibodies were radioiodinated and internalization studies were performed. Both B-S12 and B-R3 were endocytosed to a similar extent via gp130 (Figure 7, □). Only a marginal internalization was observed in cells expressing gp130AA (Figure 7, ■). Thus both antibodies were endocytosed via gp130 with the same efficiency independently of whether or not the Jak/STAT pathway was induced.

DISCUSSION

Many hormone, growth factor and cytokine receptors undergo receptor-mediated endocytosis via clathrin-coated pits, a process that often results in a down-regulation of surface receptors [27]. However, the molecular mechanisms that direct signalling receptors to the endosomal/lysosomal compartment are still being elucidated. In many cases at least two signals seem to be necessary for the efficient endocytosis of signalling receptors. One signal that is directly involved in the interaction of the cytoplasmic domain of the receptor with components of the endocytic machinery either contains a tyrosine and a hydrophobic amino acid at position +3 or comprises a dileucine motif [19,28,29]. The second signal necessary for efficient receptor internalization and down-regulation in many cases is the activation of the endogenous receptor tyrosine kinase [30–34]. However, the link between these two signals is not clear at the moment.

Compared with the receptors that contain an endogenous tyrosine kinase domain the situation in the IL-6R system is more complicated. First, the receptor complex consists of two proteins, namely gp80, which binds the ligand, and gp130, which transduces the signal and mediates endocytosis and down-regulation of the ternary receptor complex [18]. Secondly, the tyrosine kinases involved in signal transduction are not part of the signal transducing chain but independent tyrosine kinases that are constitutively associated with the membrane-proximal cytoplasmic part of the signal transducer [9,10]. In respect of IL-6 three tyrosine kinases are activated: Jak1, Jak2 and Tyk2.

It was the major goal of our study to investigate whether activation of the Jak/STAT pathway is necessary for the efficient endocytosis and down-regulation of the IL-6R complex. For this purpose, chimaeric receptor constructs were used that allowed the activation of the Jak/STAT pathway independently of the endogenous gp130, which is expressed in essentially all cell lines. These chimaeric receptors consist of the extracellular parts of the IL-5R α and β chains and the transmembrane and intracellular parts of gp130. Internalization of IL-5 via the chimaeric complex was found to be as efficient as internalization of IL-6 via its authentic receptor (Figure 2) [18] and dependent on the dileucine motif within the gp130 intracellular part (Figure 2). Thus the chimaeric complex was a system suitable for addressing the question of how signal transduction and endocytosis are linked.

Expression of chimaeras that lacked 23 membrane-proximal amino acid residues including box1 resulted in a receptor complex that was incapable of activating STAT1 (Figure 4). Because box1 was found to be crucial for binding Jak kinases [7,8] and because all STAT recruitment motifs are still present in these mutants, this result suggests that in cells expressing the Δbox1 chimaeras the Jaks are not activated after IL-5 stimulation. However, this mutant receptor complex was internalized as efficiently as the IL-5R/gp130 chimaera (Figure 4). Thus activation of the Jak/STAT pathway is not required for the efficient endocytosis of the chimaeric complex.

Incubation of transfected cells expressing authentic gp130 with the agonistic antibody B-S12 led to an activation of STAT1 and STAT3, whereas incubation with monoclonal antibody B-R3 did not. However, both antibodies were internalized with comparable

efficiencies (Figure 7) and this internalization was still dependent on the dileucine motif of gp130 because mutant gp130AA was not capable of efficiently endocytosing either B-S12 or B-R3. This experiment strongly suggests that the endocytosis of gp130 is not stimulated by activation of the Jak/STAT pathway.

This result was somewhat unexpected because recent studies had shown that mutation of a serine residue within a putative casein-kinase II phosphorylation site immediately upstream of the gp130 dileucine motif resulted in a decreased internalization rate, suggesting that phosphorylation of this site might lead to an enhanced recognition of the internalization signal by the endocytic machinery [19]. However, so far we have no evidence for a phosphorylation of this serine residue on ligand binding. An alternative explanation, which can be reconciled with our new results, would be that this serine residue is not phosphorylated but is part of the dileucine internalization motif itself.

Our result is also different from those obtained with other signalling receptors. Both the epidermal growth factor receptor (EGF-R) and the insulin receptor require activation of the intrinsic receptor tyrosine kinase for efficient endocytosis [30–34]. However, for EGF-R this requirement is only obvious at subsaturating concentrations of EGF [35,36]. In a recent paper it was directly demonstrated that an active tyrosine kinase is required for the recruitment of EGF-R into clathrin-coated pits [37]. For the platelet-derived growth factor receptor it was reported that tyrosine kinase activity promotes, but is not a prerequisite for, ligand-induced internalization and degradation of the ligand–receptor complex [38]. From the large family of haemopoietic cytokine receptors only the IL-2 receptor has been studied in some detail in this respect. Similarly to the IL-6 system, it was found that the IL-2 receptor internalizes in the absence of ligand; however, ligand binding increases internalization [39,40]. The γ chain of the IL-2 receptor complex was found to be essential for endocytosis [41]; the role of the β chain and the involvement of Jak kinases in this process has not been studied so far.

In combination with our previous results [16,18,19,42] the present results support the following model of IL-6R internalization and down-regulation. First IL-6 binds to gp80, which by itself is not efficiently internalized; then gp130 binds to the IL-6/gp80 complex and promotes its internalization. This results in a depletion of gp80 molecules at the cell surface and the observed down-regulation of IL-6-binding sites.

At the moment it is not clear whether the rate of internalization of gp130 molecules increases on ligand-binding/dimerization. The observation that mutants lacking the cytoplasmic domain or the dileucine motif consistently show a remarkable increase in surface expression suggests that either unligated gp130 is internalized at a higher rate than bulk membrane proteins or that the post-endocytic fate of internalized gp130 (recycling versus lysosomal degradation) depends on the presence of the dileucine motif. Future studies with Fab fragments and reversible biotinylation protocols will address this question.

In conclusion, we have demonstrated in COS-7 cells that the efficient endocytosis of the IL-6 signal transducer gp130 does not require activation of the Jak/STAT pathway. We cannot exclude the possibility that the activation of a different signalling pathway is necessary for gp130 internalization. However, to our knowledge there has so far been no evidence of a biological signal elicited via gp130 that does not require the activation of Jak kinases.

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