Constitutive internalization and association with adaptor protein-2 of the interleukin-6 signal transducer gp130

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Abstract The transmembrane protein gp130 is the common signalling receptor subunit for the interleukin-6 (IL-6)-type cytokines. It has recently been shown that the cytoplasmic domain of gp130 contains a dileucine internalization motif and that endocytosis of gp130 occurs signal-independent. Here, we have studied whether gp130 itself undergoes constitutive internalization or whether its endocytosis is stimulated by formation of the IL-6/IL-6R/gp130 complex. Using two different assays, we found that gp130 is internalized independent from IL-6/IL-6R stimulation. In addition, we show that gp130 is constitutively associated with the cell surface adaptor complex AP-2. Our findings strongly suggest endocytosis of gp130 to be constitutive.

Key words: Interleukin-6; Signal transducer; Constitutive endocytosis; Adaptor protein-2

1. Introduction

Polypeptide hormones, growth factors and cytokines activate intracellular signalling cascades via plasma membrane receptors. In many cases, ligand binding results in the dimerization of receptor molecules which then triggers activation of intracellular kinases and phosphorylation of signalling components. In addition, many ligand-receptor complexes undergo endocytosis and degradation within lysosomes. Receptor-mediated endocytosis of signalling proteins serves at least two important functions: removal of the ligand from the circulation and modulation of cell surface receptor expression.

Two modes of internalization have been observed in well-characterized receptor systems like the insulin and the epidermal growth factor receptor: (i) a basal constitutive endocytosis and (ii) a ligand-triggered increased endocytosis depending on an activated receptor tyrosine kinase which usually results in a receptor down-regulation [1–4].

Endocytosis of plasma membrane receptors in most cases occurs via clathrin coated vesicles. Prerequisite for the inclusion into clathrin coated vesicles are amino acid motifs within the cytoplasmic domain of the receptors that are recognized by adaptor proteins of which three are currently known, AP-1, AP-2 and AP-3. Each adaptor complex forms a heterotetramer consisting of two larger and two smaller subunits. Whereas AP-1 is crucial for the formation of clathrin coated vesicles at the trans-Golgi network, AP-2 serves this function at the plasma membrane. The function of AP-3 is currently unclear since it does not associate with clathrin [5,6]. Two sorts of amino acid motifs are found as internalization signals, tyrosine-based motifs and dileucine motifs. Both motifs were found to interact with the medium chains (μ1 and μ2) of AP-1 and AP-2, respectively [7,8]. However, for dileucine-based motifs an interaction with the β-chain of AP-1 has also been described [9].

In recent years we have studied the endocytosis of interleukin-6 (IL-6), a multifunctional cytokine. IL-6 signals via a cell surface receptor complex consisting of IL-6, the IL-6 receptor gp80 (CD126) and gp130 (CD130) (reviewed in [10]). IL-6 first binds to gp80 and then induces the dimerization of two molecules of gp130. Thereby, associated Janus tyrosine kinases (Jak) are activated and phosphorylate gp130 and transcription factors of the STAT family. Phosphorylated STATs dimerize and translocate to the nucleus where they transactivate IL-6 target genes [10–12]. After binding, IL-6 is internalized efficiently and surface gp80 is down-regulated [13]. We have previously demonstrated that the IL-6 signal transducer gp130 contains a dileucine motif within its cytoplasmic domain necessary for the endocytosis of the IL-6 receptor complex [14]. Since gp80 per se is internalized very inefficiently, the observed down-regulation of gp80 after IL-6 stimulation can be explained by the formation of the ternary receptor complex in which gp130 not only mediates signal transduction but also promotes efficient endocytosis [15].

Activation of the Jak/STAT pathway via the IL-6 receptor complex or agonistic antibodies against gp130 was found not to be necessary for endocytosis to occur [16]. These findings suggested that signalling and internalization are independent processes. However, currently it is not clear whether gp130 itself undergoes constitutive endocytosis or whether its internalization is stimulated by formation of the IL-6/gp80/gp130 complex. To answer this question we compared the internalization of free and complexed gp130 using two different protocols and studied whether gp130 associates with the plasma membrane adaptor complex AP-2.

2. Materials and methods

2.1. Reagents

Restriction enzymes and T4 ligase were obtained from Boehringer Mannheim (Mannheim, Germany). Dulbecco's modified Eagle's medium was purchased from Gibco (Eggenstein, Germany), and fetal calf serum was from Seromed (Berlin, Germany). Recombinant IL-6 was prepared as described [17]. Soluble human IL-6R (sIL-6R) was prepared in insect cells infected with recombinant baculoviruses [18].

2.2. Cell culture

COS-7 cells were grown in DMEM, HepG2 cells in DMEM/F12-Mix, each supplemented with 10% fetal calf serum, streptomycin (100 mg/l) and penicillin (60 mg/l). Ba/F3 cells, a murine pre-B lymphocyte

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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 [19]. All cells were grown at 37°C at 5% CO_2 in a water-saturated atmosphere.

2.3. Expression vectors

The vector pSVLgp130 was a kind gift of Drs. Taga and Kishimoto (Osaka, Japan). The expression vector pCAGGS was kindly provided by Dr. Hirano (Osaka, Japan); the vector pCAGGS-gp130 was constructed by subcloning the *Xba*I-*Bam*HI fragment of the pSVLgp130 plasmid containing the gp130 cDNA into the *Xba*I-*Bgl*II sites of pCAGGS.

2.4. Transfections

2×10^6 COS-7 cells in 0.8 ml DMEM were transiently transfected with 30 μg of pCAGGS-gp130 with the Electro Square Porator T820 (BTX, San Diego, CA, USA) using one pulse of 99 μs and 1500 V. Cells were processed for cell surface biotinylation two or three days after transfection when reaching confluency.

Ba/F3 cells were stably transfected by electroporation of 28 μg of pSVLgp130 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.

2.5. Cell surface half-life measurement of gp130

Ba/F3 cells stably expressing human gp130 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.

2.6. Flow cytometry

Ba/F3-gp130 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 the gp130 specific monoclonal antibody (mAb) B-R3 [21] 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, Hamburg, Germany) 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, USA). Mean fluorescence intensity (MFI) was recorded and used in the calculation of percent antibody binding: (MFI of cycloheximide treatment) divided by (MFI of cells at time point 0)$\times$100.

2.7. Reversible biotinylation assay

A cleavable biotin analogue, sulfosuccinimidyl 2-(biotinamino)ethyl-1,3 dithiopropionate (NHS-SS-biotin, Pierce, USA) was used to label cell surface proteins [20]. The cells were washed three times with biotin buffer (120 mM NaCl, 20 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 8.5). NHS-SS-biotin (0.5 mg/ml in biotin buffer, freshly diluted from a frozen stock of 200 mg/ml in DMSO) was added and labelling was carried out for 45 min. After washing, warm medium was added and cells were transferred to 37°C for 10–30 min. This incubation was performed in the absence or presence of IL-6 (20 ng/ml) and sIL-6R (1 μg/ml). For controls and each time point, one 10-cm diameter dish with 1×10^7 cells was used; two dishes of each stimulated and non-stimulated cells were kept on ice. Incubation was stopped by transferring the cells back to 4°C. Following a 60-min incubation with a reducing solution (310 mg glutathione in 17 ml H_2O, 1 ml of 1.5 M NaCl, 0.12 ml of 50% NaOH, 2 ml of serum) to remove cell surface-bound biotin, free SH groups were quenched in 5 mg/ml iodacetamide in biotin buffer for 30 min. Cell extracts were immunoprecipitated and analyzed as described below.

2.8. Immunoprecipitations

For immunoprecipitation of biotinylated gp130, surface labelled COS-7 cells were washed twice with PBS and solubilized for 30 min in 1 ml of lysis buffer (1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). Insoluble material was removed by centrifugation for 10 min at 12 000$\times g$. The supernatants were incubated with 1 μg of gp130-specific mAb B-S12 [21] for at least 8 h at 4°C. Subsequently, the lysates were treated for 2 h at 4°C with protein A-Sepharose (5 mg/ml in lysis buffer), to which rabbit anti-mouse IgG was previously bound. After centrifugation of the immunocomplexes, the Sepharose beads were washed three times with lysis buffer. The samples were heated to 55°C in non-reducing gel electrophoresis sample buffer, and the eluted proteins were separated on an SDS-polyacrylamide (7.5%) gel.

For co-immunoprecipitations of gp130 and AP-2, 1×10^8 HepG2 cells were lysed in 10 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, pH 7.5). Ten μg of mAb B-S12 were then added to the lysates to precipitate gp130. After incubation with protein A-Sepharose as described above, the samples were boiled in reducing sample buffer and separated on a 7.5% SDS-polyacrylamide gel.

2.9. Western blotting and immunodetection

The electrophoretically separated proteins were transferred to polyvinylidene difluoride (PVDF) 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. The blots were incubated with a gp130-specific mAb (B-P4 [21]) or a specific antiserum directed against α-adaptin (Upstate Biotechnology, USA), respectively, 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 or a goat anti-rabbit IgG secondary antibody, respectively.

To detect biotinylated gp130, the immunoblot was incubated with horseradish peroxidase-conjugated streptavidin (Pierce, USA; 1 mg/ml in TBS-N) for 1 h. The immunoblots were developed using the enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions.

3. Results

In order to analyze whether the internalization of gp130 is constitutive or stimulated by ligand binding, a reversible surface biotinylation assay was performed on COS-7 cells transiently over-expressing the IL-6 signal transducer [20]. Two to three days after transfection, cell surface proteins were labelled with an NHS-SS-biotin at 4°C. Afterwards cells were transferred to 37°C for 10–30 min and then incubated at 4°C in a reducing solution resulting in the cleavage of the biotin label in proteins still located at the cell surface. Gp130 was immunoprecipitated, separated by SDS-PAGE, transferred to a PVDF membrane and biotinylation was detected using horseradish peroxidase-conjugated streptavidin. Fig. 1 shows a prominent signal for biotinylated gp130 in the control lane

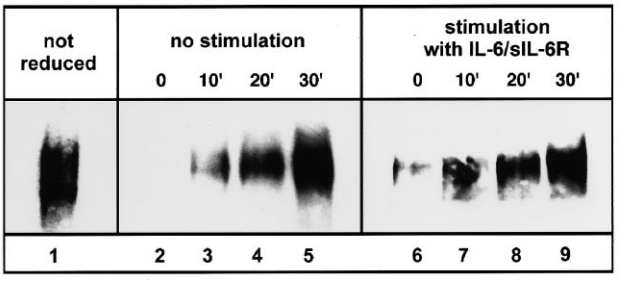

Fig. 1. Endocytosis of biotinylated gp130 in COS-7 cells. The cell surface proteins were labelled with NHS-SS-biotin as described in Section 2. Cells were incubated at 37°C for different time points as indicated. The cells were lysed, and gp130 was immunoprecipitated, separated on a 7.5% non-reducing SDS-gel, and blotted on a PDVF membrane, and biotinylated gp130 detected using horseradish peroxidase-conjugated streptavidin and the ECL system. Lane 1 represents non-reduced control cells; lanes 2–5: unstimulated cells; lanes 6–9: cells stimulated with IL-6 (20 ng/ml) and sIL-6R (1 μg/ml).

(no reduction; lane 1). After reduction this label is almost completely lost (lanes 2 and 6). Upon incubation at 37°C an increasing amount of biotinylated gp130 becomes resistant to reduction irrespective of whether a ligand (IL-6 and soluble IL-6 receptor) is present or not, indicating that gp130 is constitutively removed from the cell surface by internalization (compare lanes 2–5 and 6–9).

In order to corroborate this finding using a different approach, we studied the half-life of surface-expressed gp130 in the absence or presence of IL-6/sIL-6R complexes using flow cytometry. To this end, we used non-adherent murine Ba/F3 cells which have been stably transfected with human gp130 (Ba/F3-gp130) [19]. These cells were incubated with cycloheximide at a concentration of 50 μM to block further protein synthesis and cells were either incubated with IL-6/sIL-6R or left unstimulated. After different times at 37°C, cells were cooled down to 4°C and processed for flow cytometry. Fig. 2 shows the time course of the reduction of cell surface gp130 as measured by the mean fluorescence intensity. The gp130 signal disappears with a half-life of about 2 h irrespective of whether a ligand was present or not, supporting the notion that internalization as well as surface receptor half-life are ligand-independent.

Recently, it was shown that not only tyrosine-based but also dileucine-based internalization motifs are recognized by the adaptor complexes AP-1 and/or AP-2 [7]. Endocytosis of gp130 is dependent on the dileucine internalization motif identified previously [15]. We next studied whether gp130 interacts with the plasma membrane adaptor AP-2 in HepG2 cells – which express gp130 endogenously in considerable amounts [22]. Gp130 was immunoprecipitated from unstimulated HepG2 cells and two equal aliquots were analyzed by SDS-PAGE. After transfer to a PVDF membrane the immunoblot was developed either using a gp130-specific monoclonal antibody or an antiserum directed against the α-subunit (α-adaptin) of AP-2. As shown in Fig. 3, a protein of about 100 kDa, which is specifically recognized by the α-adaptin antiserum, was co-immunoprecipitated with gp130. Upon IL-6 stimulation, no increased association of gp130 with α-adaptin was detectable (data not shown).

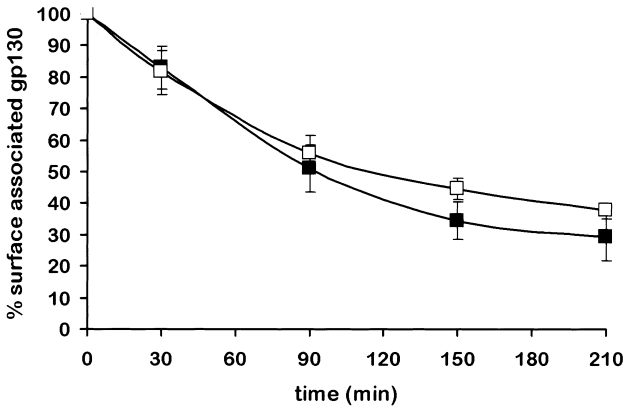

Fig. 2. Cell surface half-lives of gp130 in IL-6/sIL-6R-stimulated (■) or unstimulated (□) Ba/F3-gp130 cells. Cell surface expression on cells treated for different times with 50 μM cycloheximide was assessed by flow cytometry using mAb B-R3. Time 0 corresponds to a 30-min incubation in cycloheximide containing medium, since this is the time required for newly synthesized gp130 to reach the cell surface [22]. Antibody binding was calculated as described in Section 2. The means and S.D. of four independent experiments are shown.

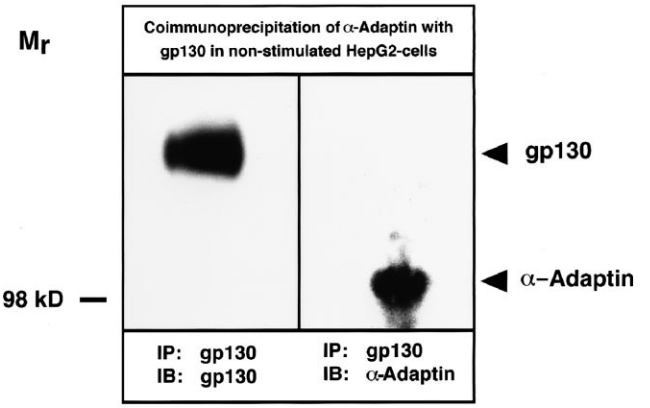

Fig. 3. α-Adaptin co-immunoprecipitates with gp130 in HepG2 cells. 1×10^8 unstimulated HepG2 cells were lysed and gp130 was immunoprecipitated (IP) as described in Section 2. Equal aliquots were separated on an SDS-gel and the immunoblot (IB) was developed using either a gp130 specific mAb (left lane) or an antiserum specific for α-adaptin (right lane). A protein migrating at the identical position was also detected when whole cell extracts were directly immunoprecipitated with an anti-α-adaptin antibody (data not shown).

4. Discussion

In this paper we demonstrated that gp130, the signal transducer of the IL-6 receptor complex, undergoes constitutive internalization independent of the presence of a ligand. In this respect, gp130 more resembles nutrient receptors like the low-density lipoprotein receptor and the transferrin receptor than other signalling receptors such as insulin receptor or epidermal growth factor receptor which exhibit an increased internalization rate upon ligand binding depending on an active intrinsic tyrosine kinase [1–4,23,24]. In accordance with this, we recently found that activation of Janus kinases is not necessary for gp130 endocytosis to occur [16].

In this study we also demonstrated a constitutive interaction of gp130 with the plasma membrane adaptor AP-2. This adaptor complex is an important component of clathrin coated vesicles. It is thought to mediate clathrin coat assembly by binding to the cytoplasmic tails of proteins containing internalization signals and recruiting clathrin [5]. Thus, it seems very likely that the constitutive endocytosis of gp130 occurs via clathrin coated vesicles. Recently, an association of gp130 with caveolae has been described in the human kidney epithelial tumor cell line TCL-598 [25]. However, this might be a special situation since in these cells gp130 was found to be associated with the urokinase-type plasminogen activator which is bound to the membrane by a glycosylphosphatidylinositol anchor.

The internalization kinetics as determined by the biotinylation procedure (Fig. 1) and the half-life of total surface gp130 as determined by flow cytometry (Fig. 2) were distinct. Although this could reflect the different cell types used, it seems more likely that this observation indicates that internalized gp130 at least in part recycles back to the cell surface. This behavior is also typical for nutrient receptors where each molecule was found to recycle many times before it is degraded in lysosomes [24]. Interestingly, IL-6/sIL-6R complexes did not show any significant effects on the gp130 surface half-

life suggesting that not only internalization but also recycling are independent of receptor ligation.

In conclusion, our data indicate that the trafficking of the IL-6 signal transducer gp130 is largely independent of ligation and dimerization and thus constitutive.

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