A complex of the soluble interleukin-6 receptor and interleukin-6 is internalized via the signal transducer gp130

Lutz Graeve*, Tatjana A. Korolenko, Ulrike Hemmann, Oliver Weiergräber, Elke Dittrich, Peter C. Heinrich

Institut für Biochemie, Rheinisch-Westfälische Technische Hochschule Aachen, Pauwelstr. 30, 52057 Aachen, Germany

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Abstract In human body fluids a soluble form of the interleukin-6 receptor (sIL-6R) has been found which together with interleukin-6 (IL-6) acts agonistically on cells expressing the signal transducer gp130. The means by which the sIL-6R is removed from the circulation is unknown. Here, we show that a complex of ^{125}I-labelled recombinant sIL-6R and IL-6 is internalized by MDCK cells stably transfected with gp130 and by human hepatoma cells HepG2 that endogenously express the IL-6R and gp130. We further show that most of the internalized sIL-6R is degraded within lysosomes. Our studies suggest that cells expressing gp130 are capable of endocytosing an IL-6/sIL-6R complex, thereby removing both from the circulation.

Key words: Interleukin-6; Soluble interleukin-6 receptor; gp130; Endocytosis; Lysosome

1. Introduction

The interleukin-6 receptor complex comprises two subunits, an 80 kDa IL-6 binding protein (IL-6 receptor) and a 130 kDa signal transducing protein, gp130 [1–3]. Binding of IL-6 to its receptor leads to the formation of a gp130 dimer which transduces the signal into the cell [4]. For this process the cytoplasmic domain of gp130 was found to be essential [5]. In contrast, the intracellular as well as the transmembrane domain of the IL-6 receptor is not necessary for signalling [2,6,7]. Such soluble IL-6 receptors (sIL-6R) are found in body fluids and their concentrations seem to be elevated during inflammation [6,8–10]. The process by which the sIL-6R is formed is under investigation. After expression in COS-7 cells the membrane-bound form can be quantitatively released into the medium particularly upon treatment of the cells with 4β-phorbol 12-myristate-13-acetate [11]. This indicates that the sIL6-R is formed by limited proteolysis ('shedding') and that this process is regulated by protein kinase C [11–13]. Recently, however, an alternatively spliced mRNA coding for a sIL-6R was also detected in human T and B cells and macrophages [14,15]. The sIL-6R purified from human plasma also contains a protein formed through differential splicing [16].

After binding to its hepatic receptor, IL-6 is internalized and degraded intracellularly [17,18]. This leads to a downregulation of the surface expression of the IL-6 receptor [18]. We have recently demonstrated that the signal transducer gp130 is a crucial component for this endocytosis and that a di-leucine internalization motif within its cytoplasmic domain is important for both internalization and ligand-induced downregulation [19,20]. Since the sIL-6R binds IL-6-dependently to a soluble form of gp130 [21,22] and since the IL-6/sIL-6R complex acts agonistically on cells expressing gp130 at the cell surface, we wished to determine whether such a complex is also internalized.

2. Materials and methods

2.1. Chemicals

Human recombinant IL-6 (1.5×10^6 B-cell stimulatory factor-2 units/mg protein) was prepared as described by Arcone et al. [23] and kindly provided by J. Müllberg (Aachen, Germany). The biological activity of IL-6 was tested using the B9 proliferation assay [24]. Fetal calf serum (FCS) was from Seromed, Dulbecco's modified Eagle medium (DMEM) and DMEM/F12 media were from Gibco. Leupeptin and monensin were from Boehringer Mannheim and Calbiochem, respectively. Bolton and Hunter reagent (*N*-succinimidyl-3-(4-hydroxy-5-[^{125}I]iodophenyl) propionate); 74 TBq/mmol) was purchased from Amersham International.

2.2. Expression, purification and iodination of the sIL-6R

The construction of the baculovirus transfer vector pVLSIR1 that codes for the extracellular part of the human IL-6R, the production of recombinant baculoviruses, the expression of the sIL-6R into supernatants of virus-infected *S. frugiperda* cells and its purification were recently described [25]. The recombinant protein was iodinated with Bolton and Hunter reagent to a specific activity of about 1 MBq/μg [26]. The iodinated sIL-6R was stored in aliquots at −20°C and retained its biological activity as determined by the induction of haptoglobin in HepG2-IL-6 cells [25].

2.3. Cell culture

The establishment of stably transfected Madin-Darby canine kidney cells expressing gp130 was recently described [27]. The cells were cultured in DMEM containing 10% FCS, 60 mg/l penicillin and 100 mg/l streptomycin. Human hepatoma cells HepG2 and fibrosarcoma cells 2fTGH (a kind gift from Dr. S. Pellegrini, Pasteur Institute, Paris [28]) were grown in DMEM/F12 and DMEM, respectively, supplemented as above. For binding and internalization experiments, cells were seeded in 24-well plates and grown for 2–3 days until they reached about 90% confluency.

2.4. Binding and internalization studies

All studies were performed in binding medium: DMEM containing no bicarbonate, 0.2% bovine serum albumin and 20 mM HEPES, pH 7.2. ^{125}I-sIL-6R at a concentration of 10 ng/ml was incubated with the appropriate amount of IL-6 overnight at 4°C in binding medium. Before the experiment, cells were washed with ice-cold binding medium and 200 μl of the radioactive sample was added to each well. Binding was allowed for 2 h at 4°C. Surface-bound sIL-6R was eluted after three washes with binding medium by incubation for 5 min in 0.5 M NaCl, pH 1. This treatment released more than 94% of surface-bound ligand (data not shown). In internalization studies, cells were rapidly warmed to 37°C after the initial binding step and incubated for different times in a waterbath. After incubation, surface-bound

*Corresponding author. Fax: (49) (0241) 8888428.

Abbreviations: IL-6, interleukin-6; sIL-6R, soluble interleukin-6 receptor; MDCK, Madin-Darby canine kidney.

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ligand was eluted as above and in order to determine internalized ligand cells were lysed in 1 M NaOH.

2.5. Degradation studies

After binding of ^{125}I-sIL-6R/IL-6 to HepG2 cells at 4°C for 2 h the temperature was shifted to 37°C and cells were incubated for 1 h at 37°C. Ligand was removed and cells were washed three times with warm binding medium. Then 200 μl binding medium was added to each well and incubation continued for up to 6 h. Supernatants were removed and mixed with an equal volume of 20% trichloroacetic acid. Samples were cooled on ice and spun in an Eppendorf centrifuge. Radioactivity was measured in supernatants (trichloroacetic acid-soluble), sediments (trichloroacetic acid-precipitable) and cell lysates after dissolution in 1 M NaOH. Inhibition studies were performed by including 20 μg/ml leupeptin, 20 mM ammonium chloride or 100 μM monensin during the 6 h incubation [29,30].

3. Results

The interaction of a complex of IL-6 and the sIL-6R with cells expressing the signal transducer gp130 was investigated in MDCK cells that had been stably transfected with an expression vector coding for gp130 (MDCK-gp130) [27]. ^{125}I-sIL-6R at a concentration of 10 ng/ml was incubated with increasing concentrations of IL-6 (2 ng/ml–2 μg/ml) at 4°C for 18 h and then added to non-transfected MDCK and to MDCK-gp130 cells for 2 h. After washing, surface-bound ligand was eluted at pH 1 and radioactivity was measured in a γ-counter. MDCK-gp130 cells bound ^{125}I-sIL-6R in an IL-6 dependent manner (Fig. 1A, ●). Control MDCK cells also showed binding, however, at a sixfold lower level (Fig. 1A, ○). This is explained by the fact that MDCK cells express endogenous gp130 [27]. We conclude that a complex of IL-6 and the sIL-6R can bind to cell-surface gp130. This binding was saturated at 1 μg/ml IL-6. Therefore, this concentration was used throughout the internalization studies.

For these studies, cells after ligand binding at 4°C were shifted to 37°C for 30, 60, 90 and 120 min. Internalized IL-6/^{125}I-sIL-6R was measured as the radioactive fraction that became resistant to the acid wash. Within 60 min of incubation the amount of ligand bound at time-point 0 was endocytosed by MDCK-gp130 cells (Fig. 1B). Thus, we conclude that the IL-6/sIL-6R complex is effectively internalized via gp130.

In order to exclude the possibility that the observed internalization is an artifact of transfected cells overexpressing the signal transducer, we analyzed binding and internalization in two human cell lines, hepatoma cells HepG2 expressing the IL-6R and gp130 and fibrosarcoma cells 2fTGH expressing only gp130 [31]. The latter cells are IL-6 responsive when stimulated with IL-6 and the sIL-6R [31]. Both cell lines were able to bind (data not shown) and internalize ^{125}I-sIL-6R in an IL-6 dependent manner at concentrations similar to those found with MDCK-gp130 cells (Fig. 2). However, the amount of sIL-6R bound and endocytosed was considerably smaller in 2fTGH cells (Fig. 2, □) than in HepG2 cells (Fig. 2, ■), indicating that 2fTGH cells have lower levels of endogenous gp130.

In HepG2 cells we further studied the fate of internalized ^{125}I-sIL-6R. As indicated in Fig. 3A, cell-associated radioactivity decreased steadily during a 6 h chase (squares). Simultaneously, there was an increase in radioactive material released into the supernatant. Of this about 78% was secreted as trichloroacetic acid-soluble material (Fig. 3A, circles),

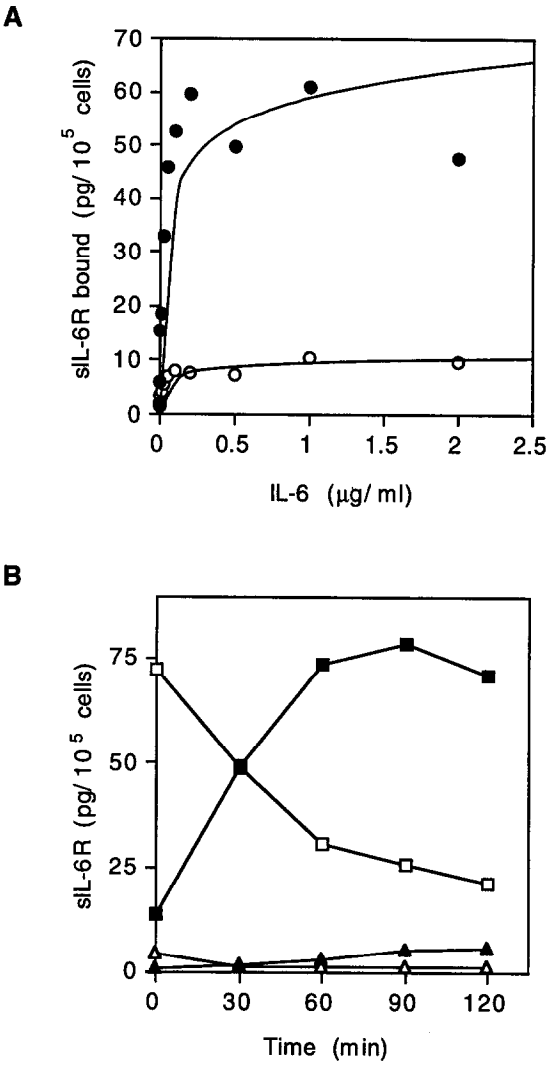

Fig. 1. (A) Binding of ^{125}I-sIL-6R. MDCK and MDCK-gp130 cells were incubated with 10 ng/ml ^{125}I-sIL-6R and different concentrations of IL-6 for 2 h at 4°C as indicated. Cells were washed three times with binding medium and surface-bound ligand was eluted with 0.5 M NaCl, pH 1 for 5 min. Eluted radioactivity was measured in a γ-counter. The data are representative of four independent experiments. (○) MDCK, (●) MDCK-gp130. (B) Internalization of ^{125}I-sIL-6R. MDCK and MDCK-gp130 cells were incubated with 10 ng/ml ^{125}I-sIL-6R and 1 μg/ml of IL-6 for 2 h at 4°C, shifted to 37°C for the times indicated, cooled on ice and washed three times with binding medium. Surface-bound ligand was eluted as above. Internalized ^{125}I-sIL-6R was determined after dissolving the cells in 1 M NaOH. Non-specific uptake was determined in the absence of IL-6 and subtracted. The data are representative of two independent experiments. Open symbols, surface-bound ligand; closed symbols, internalized ligand; squares, MDCK-gp130; triangles, MDCK.

whereas 22% was trichloroacetic acid-insoluble (Fig. 3A, triangles). Thus, obviously a large fraction of internalized ligand is degraded intracellularly.

To study whether the degradation of ^{125}I-sIL-6R occurs within the lysosomes, inhibitors of lysosomal function were added during the degradation study (Fig. 3B). The cysteine-proteinase inhibitor leupeptin at a concentration of 20 μg/ml slightly decreased the rate of degradation of ^{125}I-sIL-6R by about 30% (Fig. 3B, diamonds). However, at 6 h the lysosomotropic agent ammonium chloride at 20 mM as well as

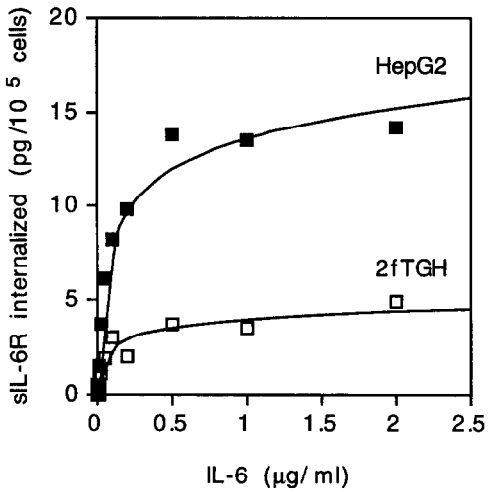

Fig. 2. Internalization of ^{125}I-sIL-6R by HepG2 and 2fTGH cells. Cells were incubated with 10 ng/ml ^{125}I-sIL-6R and different concentrations of IL-6 for 2 h at 4°C as indicated. Cells were then shifted to 37°C for 1 h, washed three times with ice-cold-binding medium and surface-bound ligand was eluted at pH 1. Internalized ligand was determined after dissolving the cells in 1 M NaOH. The data are representative of two independent experiments. (■) HepG2; (□) 2fTGH.

monensin at 100 μM significantly inhibited the degradation process by 75 and 80%, respectively (Fig. 3B, triangles and squares).

4. Discussion

Cytokines are important regulators of many different physiologic processes, such as cell proliferation, growth inhibition, cell death, cell differentiation and migration. Dysregulation of cytokine production often leads to pathological situations, such as inflammation, tumor growth and metastasis, and development of fibrosis. Therefore, cytokine synthesis and cytokine receptor levels must be tightly regulated. This is mainly achieved by a complex control of gene transcription. However, efficient mechanisms for removing cytokines from the blood circulation must also exist in order to protect the organism from over-stimulation.

For a number of cytokine receptors, soluble forms were found in plasma and other biological fluids. These soluble receptors are either formed by proteolytic cleavage or by differential splicing [32]. For the sIL-6R both mechanisms of formation have been described [12,14,15]. Whereas most soluble cytokine receptors are antagonists since they compete with membrane-bound receptors for binding of the ligand [32], the sIL-6R is peculiar because after binding its ligand it acts agonistically on cells that express the signal transducer gp130 [2,6,7]. The fact that essentially all cells investigated so far do express gp130 suggests that a complex of IL-6 and the sIL-6R has a much broader spectrum of target cells than IL-6 alone. Therefore, the means by which the organism controls the level of circulating soluble receptors are of interest.

We have recently reported that the sIL-6R after intravenous injection into rats rapidly accumulates in liver, muscle, skin and kidneys [25]. However, the mechanisms by which the cells of these organs bind the sIL-6R were not investigated. We show in this paper that cells expressing gp130 are capable of binding and internalizing the sIL-6R in the presence of IL-6, suggesting that the IL-6/sIL-6R complex is internalized via gp130. This is in agreement with recent results from our laboratory which have demonstrated that the cytoplasmic part of gp130 but not of the IL-6R is necessary for efficient IL-6 internalization and IL-6R down-regulation [19]. The identification of a di-leucine internalization motif within this intracellular part of gp130 indicates that the endocytosis of the IL-6/sIL-6R/gp130 complex occurs by receptor-mediated endocytosis via clathrin-coated pits [20]. It is well known that recep-

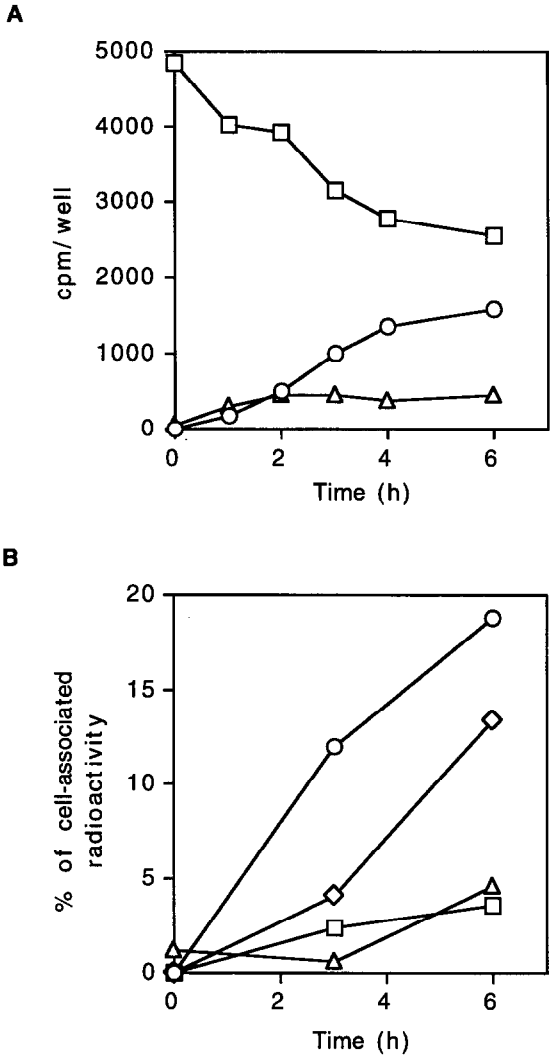

Fig. 3. (A) Degradation of ^{125}I-sIL-6R in HepG2 cells. Cells were incubated with 10 ng/ml of ^{125}I-sIL-6R for 2 h at 4°C and then for 1 h at 37°C in the presence and absence of 100 nM of IL-6. Cells were washed three times and incubated in binding medium for different times as indicated. Degradation of ^{125}I-sIL-6R was measured in the supernatant after trichloroacetic acid precipitation of the medium (acid-soluble radioactivity, circles). Acid-precipitable radioactivity (triangles) was measured in sediments. Cell-associated radioactivity (squares) was counted after dissolving the cells in 1 N NaOH. Non-specific degradation was determined in the absence of IL-6 and subtracted. The data are representative of three independent experiments. (B) The degradation study was performed as described above in either the absence (circles) or presence of 20 μg/ml of leupeptin (diamonds), 20 mM ammonium chloride (triangles) or 100 μM monensin (squares) during the final incubation at 37°C. Degradation was calculated as percentage of total cell-associated radioactivity at time point 0. The data are representative of two independent experiments.

tor-mediated endocytosis is important for the cellular uptake of many biologically active molecules. However, to our knowledge, this report is the first to demonstrate directly endocytosis of a soluble cytokine receptor/ligand complex.

35% of the internalized sIL-6R was degraded intracellularly over 6 h. This is a rather slow process when compared to the degradation of labeled IL-6 by HepG2 cells which is almost complete after 3 h (Graeve et al., unpublished observation). This indicates that the degradation of the sIL-6R is slower than that of IL-6. Future studies are necessary to elucidate the reason for this difference. The partial inhibition of the degradation of the IL-6/sIL-6R complex by leupeptin, ammonium chloride and monensin suggests that this degradation takes place in a lysosomal compartment.

So far, the endocytosis of IL-6 and the sIL-6R has only been studied in liver parenchymal cells and cells of mesenchymal origin. It is currently unknown whether the same process also occurs in hematopoietic cells like monocytes/macrophages and B- or T-lymphocytes. We hypothesize that essentially all cells are capable of endocytosing IL-6 and its soluble receptor as long as they express gp130 on their plasma membrane.

The importance of the endocytosis of cytokines and their soluble receptors for signalling events and for the understanding of pathological situations is still unclear. In a recent report, two chimeric receptors consisting of the extracellular part of the epidermal growth factor receptor and the intracellular part of c-Ros and containing either the transmembrane domain of the epidermal growth factor receptor or of c-Ros were found not only to differ in their internalization efficiencies but also to exhibit opposite effects on cell growth [33]. This study suggests that the internalization of signalling components has profound effects on the biological response. Whether this is also the case for the IL-6 signal transduction is currently under investigation.

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