The human interleukin-6 (IL-6) receptor exists as a preformed dimer in the plasma membrane

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Abstract The recently solved X-ray structure of the extracellular portion of the interleukin-6 (IL-6) receptor (IL-6R) revealed an IL-6R dimer in the crystal lattice which probably represents a physiological dimer. Performing coprecipitation experiments with two differently tagged IL-6R variants expressed in COS-7 cells, we show that an IL-6R dimer exists in the plasma membrane in the absence of IL-6. Ligand binding does not seem to affect the dimerization status. When lysates of COS-7 cells expressing only one of the IL-6R variants are mixed, spontaneous dimerization occurs. Thus, the IL-6R dimer observed in the crystal structure represents a physiologically occurring phenomenon.

Key words: Interleukin-6; Cytokine receptor; Plasma membrane; Dimer

1. Introduction

Interleukin-6 (IL-6) activates cells by binding to the specific IL-6 receptor α-chain followed by recruitment of two signal-transducing gp130 molecules, the β-receptors. Homodimerization of gp130 triggers activation of intracellular signalling cascades including the JAK/STAT- and ras/MAP-kinase pathways [1], whereas the IL-6 receptor (IL-6R) is not involved in signal transduction as exemplified by a biologically active soluble IL-6R [2]. In difference to IL-6, a viral interleukin-6 homolog, vIL-6, can directly induce a gp130 homodimer without requirement for the IL-6Rα [3]. The exact stoichiometry of the IL-6 receptor (IL-6R) complex is controversial and may consist of a tetramer of IL-6, IL-6Rα, and gp130 in a 1:1:2 stoichiometry or a hexamer in a 2:2:2 stoichiometry or both [4,5]. Receptors of the IL-6 family are type I cytokine receptors that are characterized by a cytokine binding module (CBM) constituted by two fibronectin type III-like domains of 100 amino acid length in roughly perpendicular orientation to each other [6], a configuration confirmed by the solved X-ray structure of the CBM of gp130 [7]. The NH_2-terminal domain contains four conserved cysteine residues, the COOH-terminal one a conserved WSXWS motif that is part of a structurally relevant 'tryptophane-zipper' motif [8]. In the viral IL-6/gp130 complex, the CBM of gp130 contacts the site II receptor binding epitope of vIL-6, whereas an additional Ig-like domain at the NH_2-terminus of the gp130 CBM is required to contact the site III epitope of vIL-6 [9]. The CBM of the IL-6R binds to site I of IL-6, whereas the NH_2-terminal Ig-like domain is dispensable for biological activity [10], but may have a role in post-translational processing of the receptor [11]. The complete crystal structure of the extracellular domains of the IL-6R was solved recently [12]. Similar to the gp130 CBM, the two fibronectin domains of the IL-6R CBM are oriented perpendicularly. Surprisingly, the crystal structure revealed dimer formation of two IL-6R molecules along the long-axis of the two fibronectin domains of the CBM. The large surface area buried between the contact sites suggested, that this dimer may also exist on the cell surface. Here, we show that dimerization of the IL-6R indeed occurs not only on the cell surface, but also in solution.

2. Materials and methods

2.1. Preparation of IL-6R mutants

The human IL-6R was tagged at the COOH-terminus either with a protein-C tag (IL-6R-PC, amino acid sequence including the last five COOH-terminal residues of the IL-6R: FFPR-SL-EDQVDPR-LIDGK) or a *Haemophilus influenza* tag (IL-6R-HA, FFPR-SR-YPYDVPDYA). Recombinant human IL-6 was prepared as reported before [13]. For immunoprecipitation and Western blot analysis, the murine monoclonal antibody (mAB) MT-18, which recognizes the Ig-like domain of the IL-6R [10,14], the anti-PC-tag mAB HPC4, the anti-HA-tag mAB 12CA5 (both obtained from Roche Molecular Biochemicals, Mannheim, Germany) and the anti-HA-tag rabbit polyclonal antibody sc805 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. The anti-HA-tag mAB 12CA5 was used only for immunoprecipitation, the anti-HA-tag antibody sc805 only for Western blot.

2.2. Immunoprecipitation and Western blot analysis

COS-7 cells were grown in Dulbecco's minimal essential medium supplemented with fetal calf serum (10%), penicillin and streptomycin. Cells were transfected with either IL-6R-PC or IL-6R-HA or both using the expression vector p409 [15]. Cells were lysed in 50 mM Tris, pH 7.5, 150 mM NaCl, and 3 mM sodium orthovanadate, containing a cocktail of protease inhibitors (Boehringer Mannheim, Germany), phenylmethylsulfonyl fluoride (1 mM) and 1% Brij-96. Insoluble material was pelleted and the supernatants immunoprecipitated with the anti-PC tag, anti-HA tag or the MT-18 antibody. The complexes were isolated with protein A–Sepharose CL-4B (Pharmacia, Uppsala, Sweden), subjected to SDS–PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Life Science, England). The membranes were incubated with an appropriate primary antibody before being labelled with a secondary antibody coupled to peroxidase. Subsequently, the membranes were developed using the Amersham ECL plus kit.

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Abbreviations: CBM, cytokine binding module; FNIII, fibronectin-type-III; IL-6, interleukin-6; IL-6R, IL-6 receptor; vIL-6, viral interleukin-6

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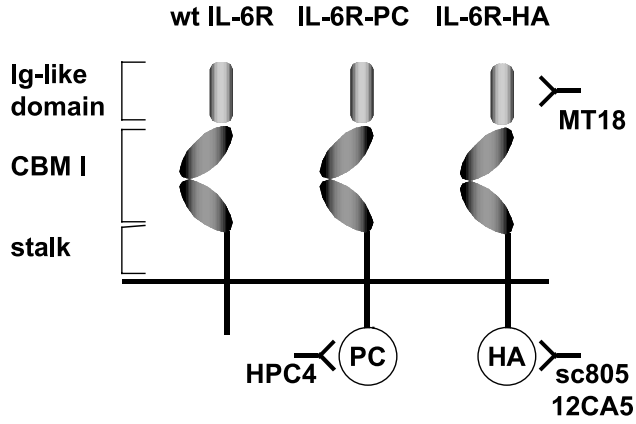

Fig. 1. Schematic representation of the IL-6R mutants prepared including the recognition sites of the antibodies used for immunoprecipitation and Western blot.

3. Results

The receptor mutants used and the recognition site of the antibodies applied are depicted in Fig. 1. To validate the specific recognition of the tags by the antibodies used, COS-7 cells were transfected with IL-6R-PC, IL-6R-HA or both and lysed. 5 μl of the lysate of a 10 cm dish were subjected to SDS–PAGE and blotted either with the anti-PC antibody HPC4 or the anti-HA antibody sc805 (Fig. 2A). The anti-PC antibody HPC4 only recognized the PC-tagged IL-6R mutant, but not the HA-tagged one. Similarly, the sc805 recognized only the HA-tagged IL-6R. A possible concentration effect of the tagged IL-6R mutants by immunoprecipitation with the IL-6R antibody MT18 did not change this pattern (Fig. 2B). In all experiments, the IL-6R appeared as a double band, reflecting differential glycosylation of the IL-6R on the secretory pathway [11,16].

To ascertain the possible existence of IL-6R dimers in the cell membrane, COS-7 cells double transfected with IL-6R-PC and IL-6R-HA were lysed, the lysates precipitated with the anti-PC antibody HPC4, subjected to SDS–PAGE and blotted with the anti-HA antibody sc805 (Fig. 3A). A clear band was detectable after this procedure, reflecting HA-tagged molecules coprecipitated with the anti-PC tag antibody. Similarly, the PC-tagged IL-6R was coprecipitated when the lysates were precipitated with the anti-HA tag antibody 12CA5. The bands were, however, much weaker, since the anti-HA tag antibodies did not immunoprecipitate well (Fig. 3A, right panel). The weaker band strength was not due to unequal expression of IL-6R-PC and IL-6R-HA in the double transfected COS-7 cells, since a Western blot on the lysates from the double transfected cells with either the anti-PC or the anti-HA antibody demonstrated roughly equal levels of IL-6R-PC and IL-6R-HA (Fig. 2A). Addition of IL-6 (50 ng/ml) to the cells prior to lysis did not affect coprecipitation of the alternatively tagged IL-6R. Since the possibility remained that only a proportion of the IL-6Rs at the cell surface had bound IL-6, we increased the IL-6 concentration up to 1 μg/ml. However, dimer formation of the differently tagged IL-6R mutants remained unaffected by IL-6 (Fig. 3B, lanes 3–6), the control lanes (1 and 2) clearly showed that the signal in lanes 3–6 represented coprecipitated IL-6R. Although the signal seemed to become stronger upon the addition of IL-6 (lanes 4 and 5), in several other experiments (see Fig. 3A) we did not observe any difference in the coprecipitation in the absence and presence of IL-6. We next asked whether dimerization of the IL-6R would also occur in solution. Therefore lysates from cells only expressing IL-6R-PC or IL-6R-HA respectively were mixed and subjected to either precipitation with the anti-PC

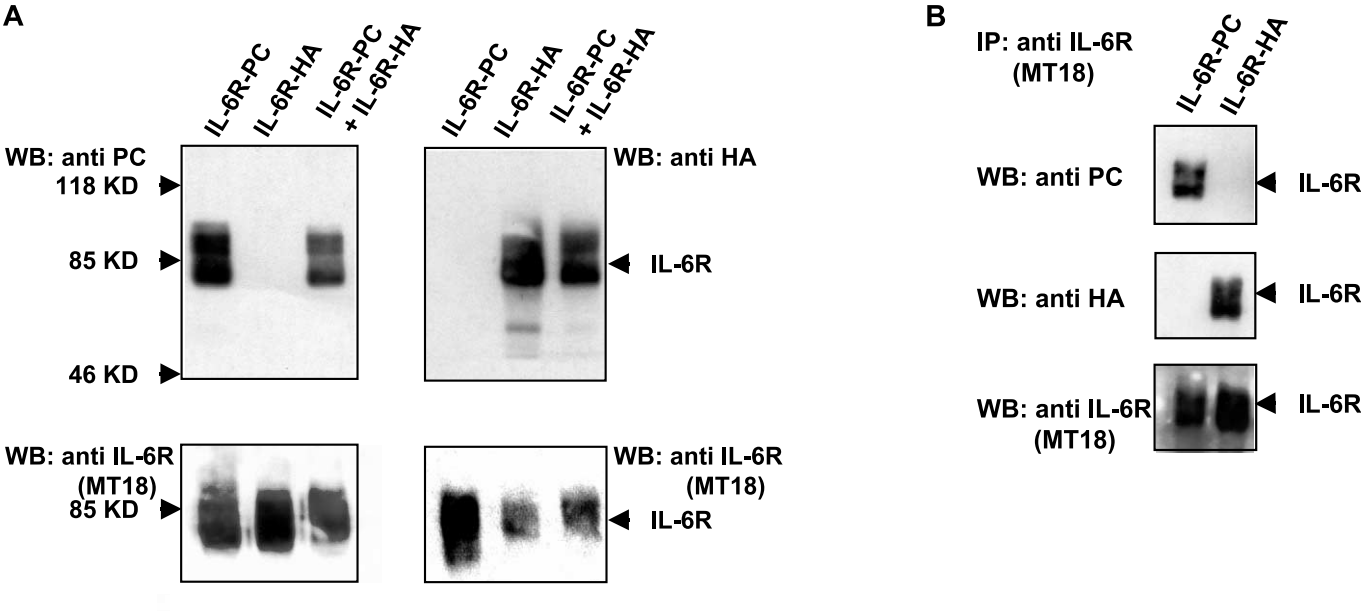

Fig. 2. A: COS-7 cells transfected with IL-6R-PC, IL-6R-HA or both were lysed in 1 ml lysis buffer. 5 μl of the lysate was subjected to SDS–PAGE and blotted with the anti-PC antibody HPC4 (left panel) or with the anti-HA antibody sc805 (right panel) and as a loading control with the anti-IL-6R antibody MT-18. B: COS-7 cells transfected with IL-6R-PC or IL-6R-HA were lysed and immunoprecipitated with the anti-IL-6R antibody MT-18 before subjecting to SDS–PAGE. The receptors were detected with the anti-PC antibody HPC4 or with the anti-HA antibody sc805 and as control with the anti-IL-6R antibody MT-18.

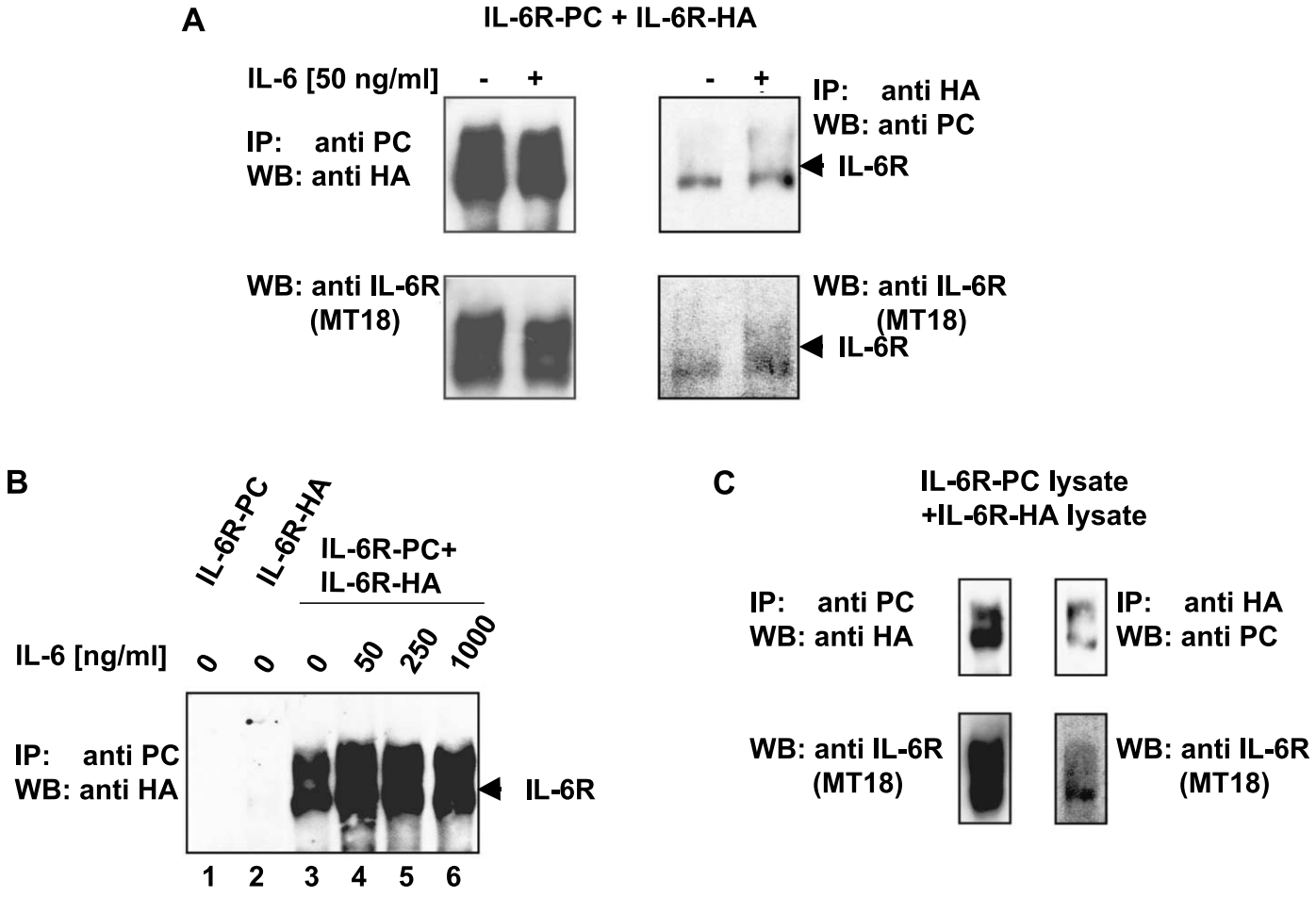

Fig. 3. A: COS-7 cells double transfected with IL-6R-PC and IL-6R-HA were lysed in the absence or presence of IL-6 and subsequently immunoprecipitated with HPC4 and blotted with the anti-HA antibody sc805 (left panel) or precipitated with the anti-HA antibody 12CA5 and blotted with the anti-PC antibody HPC4 (right panel). Loading was controlled by Western blot with the anti-IL-6R antibody MT-18 after stripping. B: In lanes 1 and 2, COS-7 cells were single transfected with IL-6R-PC or IL-6R-HA, the lysates were immunoprecipitated with HPC4 and blotted with the anti-HA antibody sc805. The double transfected cells (lanes 3–6) were preincubated with increasing concentrations of IL-6 prior to lysis. C: Lysates (500 μl) of single transfected COS-7 cells (IL-6R-PC or IL-6R-HA) were mixed, immunoprecipitated with HPC4 and blotted with sc805 (left panel) or precipitated with the 12CA5 and blotted with the HPC4 antibody (right panel). Loading was controlled by Western blot with the anti-IL-6R antibody MT-18 after stripping.

or the anti-HA antibody. Coprecipitation of the alternatively tagged IL-6R mutant was observed under both conditions (Fig. 3C), confirming that dimerization of the IL-6R also occurred in solution.

4. Discussion

An increasing body of evidence suggests that cytokine receptors in the plasma membrane are preassembled rather than associated by ligand binding. This has been conclusively demonstrated for the receptors for erythropoietin and growth hormone [17–19] and more recently for the interferon-γ and IL-10R [20,21]. Consequently, receptor dimerization per se is not sufficient for receptor activation. Rather, ligand binding induces changes in the spatial orientation of the receptor chains that cause triggering of intracellular signalling cascades or even inactivate receptor dimers [22,23]. However, in contrast to the above mentioned receptors the α-receptors of the IL-6 family including the IL-6R are not involved in signal transduction and have so far been presumed to exist as monomers.

The recent discovery of a large dimerization interface in the crystal structure of the human IL-6R raised the possibility that the IL-6R could dimerize in the two-dimensional surface of the plasma membrane [12]. Our results clearly demonstrate that this is indeed the case, but further illustrate that dimerization of the IL-6R also occurs in solution. Furthermore, IL-6 does not affect IL-6R dimerization (Fig. 3B), although IL-6 possesses only one binding epitope to the IL-6R (site I). This excludes the possibility that IL-6R dimerization occurs via the ligand binding epitope as in the erythropoietin receptor dimer (Fig. 4A, [17]). Consequently, it appears very likely that the IL-6R dimerization observed in our experiments occurs via the dimerization interface observed in the crystal structure [12]. Dimerization along the long axis of the domains constituting the IL-6R CBM as suggested by Varghese et al. would not prevent binding of IL-6 to the IL-6R in the dimer (Fig. 4B). In line with this notion, published mutations in the dimerization interface did not affect ligand binding, but slightly impaired signalling [12].

A somewhat speculative model for the hexameric IL-6R complex was therefore suggested in which the two IL-6R molecules are still dimerized and which would therefore explain the effect of these mutations as a consequence of disturbed dimerization [12]. The model is, however, difficult to reconcile

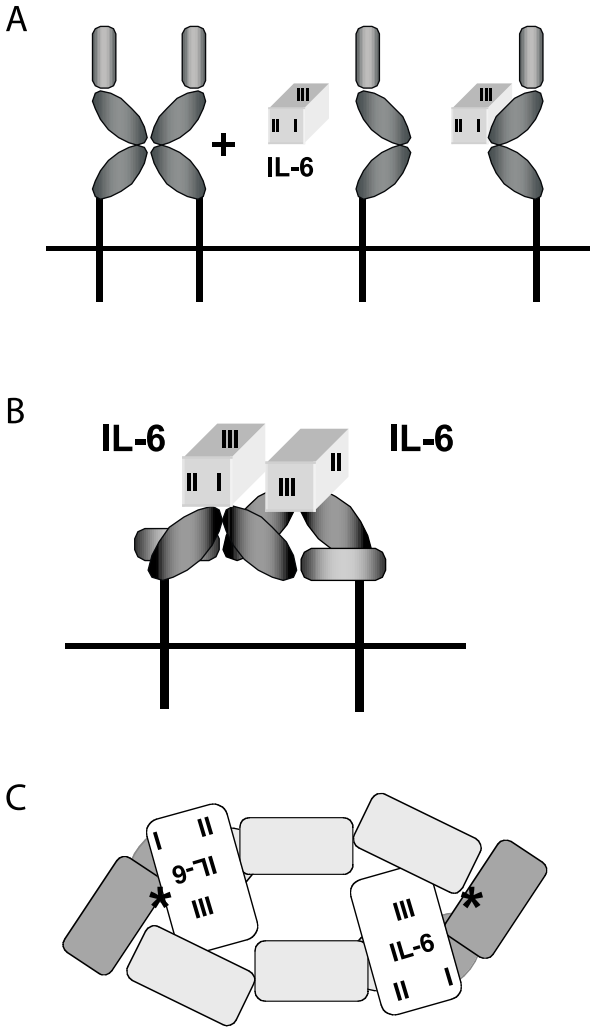

Fig. 4. A: A possible IL-6R dimer based on the crystal structure of the erythropoietin receptor would be disrupted by binding of IL-6. B: Schematic representation of the IL-6R dimer observed in the crystal structure. C: Model of the hexameric IL-6R complex derived from the tetrameric vIL-6/gp130 complex (modified from [15,26]). The asterisk denotes the dimer interface of the IL-6R in the crystal structure. The IL-6R CBM is depicted in dark gray, the three NH_2-terminal domains of gp130 in light gray. The Ig-like domain of gp130 contacts site III of IL-6, the CBM of gp130 site II as in the vIL-6.

with the geometry of the solved X-ray structure of the tetrameric vIL-6/gp130 complex which is considered an excellent template for the hexameric IL-6R complex, since the epitopes on vIL-6 that contact gp130 are identical to the gp130 binding epitopes on IL-6 [9,24]. Taking into account a model for the binding of IL-6 to the IL-6R [25], we and others have recently derived a different model for the hexameric IL-6R complex based on the vIL6/gp130 complex ([15,26], Fig. 4C). Here, the interface of the IL-6R dimer (marked by an asterisk) faces gp130 and mutations may well affect signalling without any effect on ligand binding. Importantly, there is no space to accommodate the second IL-6R molecule of the dimer which implies that the IL-6R dimer dissolves upon formation of the receptor complex. If one supposed that the dimer remained, the IL-6R complex would be at least octameric and furthermore prone to form large aggregates via the IL-6R dimer. Multimer or aggregate formation of the IL-6R alone is very unlikely, since in contrast to the dimerization interface observed in the crystal structure, all other epitopes of the IL-6R (apart from the ligand binding epitope) are prone to glycosylation, thus disabling them as potential dimerization sites.

Our results clearly show the existence of IL-6R dimers both in the cell membrane and in solution. However, the function of these dimers is unknown at present as and it is unclear how the gp130 dimer in the active IL-6R complexes affects the preformed IL-6R dimer. Our ongoing work is concerned to clarify these questions.

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