The first two N-terminal immunoglobulin-like domains of soluble human IL-1 receptor type II are sufficient to bind and neutralize IL-1β

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Abstract Two forms of soluble human type II interleukin (IL)-1 receptor (shIL-1RII) were generated, one consisting of the complete extracellular three immunoglobulin (Ig)-like domains and one containing only the first two N-terminal Ig-like domains. Both forms bound IL-1β with a dissociation constant (K_d) of 200 pM and neutralized IL-1β in a bioassay. They did not bind or neutralize IL-1α. This demonstrates that the two Ig-like domains of shIL-1RII are sufficient to bind IL-1β with an affinity comparable to full length shIL-1RII. This suggests that this short form of shIL-1RII contributes to the anti-inflammatory effect of soluble IL-1 receptors in vivo. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Interleukin-1; IL-1 receptor; Soluble receptor; Regulation of inflammation

1. Introduction

Interleukin-1 (IL-1) is a potent pro-inflammatory cytokine of the innate and adaptive immune response mediating its biological effects via specific plasma membrane receptors [1]. Whereas the type I IL-1 receptor (IL-1RI) in concert with the co-receptor IL-1R accessory protein (IL-1RAcP) is responsible for signal transduction, the non-signaling type II IL-1 receptor (IL-1RII) is involved in regulating IL-1 responsiveness by several different mechanisms. In its membrane form IL-1RII can either sequester IL-1 and serve as a ligand sink [2] or it can interact with the IL-1RAcP [3,4] and compete for the co-receptor. The membrane form also serves as a substrate for a cytokine-inducible ektoprotease which sheds IL-1RII rapidly from the surface of cells [5] giving rise to a soluble IL-1RII (sIL-1RII) [6,7] consisting of the complete extracellular part of IL-1RII. A smaller IL-1β binding protein was reported, presumably derived from sIL-1RII by further proteolytic processing. However the molecular nature and the biological relevance of this IL-1β binding protein have not been established [8].

Here we report that the two N-terminal immunoglobulin (Ig)-like domains of shIL-1RII are sufficient to bind IL-1β with an affinity comparable to the full length 54 kDa sIL-1RII containing all three Ig-like domains. This short sIL-1RII neutralizes IL-1β in bioassay.

2. Materials and methods

2.1. Cloning and expression of epitope-tagged versions of human IL-1RII

Expression plasmids for the epitope-tagged versions of shIL-1RII were generated by PCR-cloning. The vector pFLAG-CMV-hemagglutinin (HA) was derived from the cloning vector pFLAG-CMV-1 (Kodak, Rochester, NY, USA) by insertion of a HA-tag (YPYDVPDYA) followed by a stop codon between the *Xba*I and *Bam*HI sites. For pFLAG-sRII-HA the cDNA encoding amino acids Phe1–Phe335 of hIL-1RII (three extracellular Ig-like domains) or for pFLAG-RII-Ig1+2-HA the cDNA encoded Phe1–Glu220 (two extracellular Ig-like domains) were inserted between the FLAG- and the HA-tag. Different versions of hIL-1RII were expressed in 293 cells after transient transfection using a standard calcium phosphate precipitation protocol. 40 h after transfection cells or supernatants were either subjected to direct cross-linking with IL-1 or soluble versions of hIL-1RII were purified.

2.2. Cross-linking of IL-1 to hIL-1RII on cells or in supernatants

In order to detect IL-1 binding molecules, cells or supernatants were incubated with 3 ng/ml ^{125}I-radiolabeled rhIL-1α or [^{125}I]rhIL-1β (NEN, Cologne, Germany) for 2 h on ice. Protein complexes were cross-linked by addition of the homobifunctional cross-linker BS^3 (Pierce/KMF, Cologne, Germany) 1 h on ice (final concentration of 5 mM in phosphate-buffered saline pH 7.4 (PBS)). Cells were then washed and lysed in PBS containing 150 mM NaCl and 1% Triton X-100 (30 min on ice). Nuclei were removed by centrifugation and cell lysates or cross-linked supernatants analyzed by SDS–PAGE. Radioactive bands were visualized by phosphorimaging.

2.3. Purification of epitope-tagged short and long sIL-1RII

40 h after transfection of 293 cells supernatants were collected and concentrated 10 fold by ultrafiltration (molecular weight cutoff 30 kDa for sIL-1RII (3Ig) or 10 kDa for sIL-1RII (2Ig)). shIL-1RII molecules were purified by affinity chromatography using anti-FLAG M2 affinity gel (Sigma, Deisenhofen, Germany) in PBS at pH 7.4 and subsequently eluted with 35 mM FLAG peptide. Protein concentrations were determined by a Bradford assay (Bio-Rad, Munich, Germany).

2.4. Binding studies with shIL-1RII (2Ig) and shIL-1RII (3Ig)

Binding studies were performed in a 96 well plate immunosorbent assay. The HA-specific monoclonal antibody (mAb) 12CA5 was coated on P96 Maxisorp plates (NUNC, Wiesbaden, Germany) over night, unspecific binding sites were blocked by 5% BSA in PBS for 2 h. The purified receptor molecules were captured via their C-terminal HA-tag at room temperature for 5 h (unspecific binding: PBS instead of shIL-1RII). After three washes with PBS, different [^{125}I]IL-1β concentrations were added and binding allowed for 15 h at 4°C. Aliquots

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Abbreviations: h, human; s, soluble; r, recombinant; R, receptor; IL, interleukin; Ig, immunoglobulin; HA, hemagglutinin; mAB, monoclonal antibody; PBS, phosphate-buffered saline pH 7.4

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were removed and counted in a γ-counter to determine unbound IL-1β. Wells were washed three times with PBS. Bound IL-1β was released by incubation with 0.1 M glycine pH 3.0 for 30 min at 37°C and counted in a γ-counter. All samples were done in duplicates. Specifically bound IL-1β was plotted vs. free ligand. The equilibrium dissociation constant K_d was calculated by least square analysis of the binding data with a one site binding model.

2.5. Bioassay to detect the neutralizing capacity of soluble human IL-1RII forms

Neutralizing capacity of the two recombinant forms of shIL-1RII was measured in a bioassay using murine EL-4 6.1 cells (25 000 cells/well) which were stimulated in the presence of 50 pg/ml rhIL-1α (kindly provided by J. Sims, Immunex, Seattle, WA, USA) or 50 pg/ml rhIL-1β (kindly provided by D. Boraschi, Dompé, L'Aquila, Italy) and 2.5×10^{-7} M A23187 (Sigma, Deisenhofen, Germany). Increasing concentrations of the shIL-1RII preparations were titrated in the wells before cells were added. After 24 h murine IL-2 was measured in the supernatants using a commercial ELISA kit (Diaclone, Besancon, France) according to the manufacturer's instructions.

2.6. Measurement of shIL-1RII by ELISA

Soluble human IL-1RII forms were detected using a commercial kit (R&D Systems, Wiesbaden, Germany) according to the manufacturer's manual.

3. Results

3.1. Characterization of IL-1β binding proteins in 293 cells overexpressing different forms of human IL-1RII

Two forms of IL-1β binding proteins were released into the supernatant by 293 cells overexpressing membrane-bound IL-1RII as detected by crosslinking with [^{125}I]IL-1β: a large one of 54 kDa, and a small one of 36 kDa (Fig. 1, lane 1). If 293 cells were transfected with a construct encoding a modified IL-1RII (uIL-1RII) that cannot be cleaved between the transmembrane region and the juxtamembraneous Ig-like domain [9], only an IL-1 binding molecule of 36 kDa was detected in the supernatant (lane 2). Transfection with a plasmid encoding all three extracellular Ig-like domains of IL-1RII (sIL-1RII (3Ig), lane 3) yielded a band of 54 kDa migrating comparable to full length IL-1RII, while overexpressing the two N-terminal Ig-like domains (sIL-1RII (2Ig), lane 4) resulted in a band of 36 kDa corresponding in size to the small band observed after expression of full length IL-1RII (lane 1) or the modified uIL-1RII (lane 2). Supernatant of empty vector transfected cells and medium served as negative controls (lanes 5 and 6) whereas supernatant of cells transfected with membrane-bound IL-1RII containing 10% FCS was used as a positive control (lane 7).

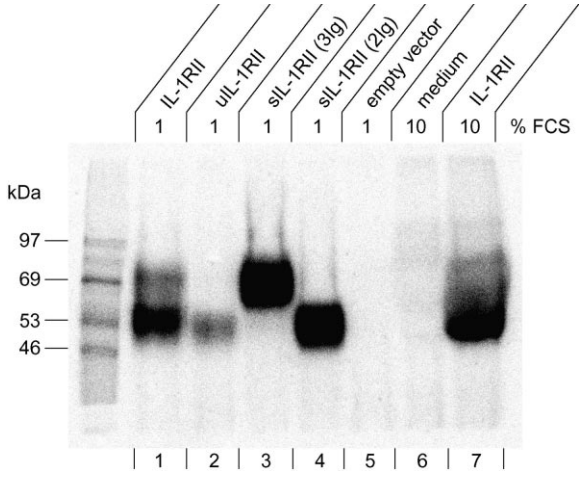

Fig. 1. Cross-linking of supernatants from 293 cells expressing different forms of human IL-1RII with [^{125}I]IL-1β. 293 cells were transiently transfected with expression vectors encoding different forms of hIL-1RII. After 40 h supernatants containing 1 or 10% FCS were collected and incubated with 3 ng/ml [^{125}I]IL-1β for 2 h. By addition of 5 mM BS^3 (1 h) protein complexes were cross-linked and afterwards separated by SDS–PAGE. Radioactive bands were visualized by phosphorimaging.

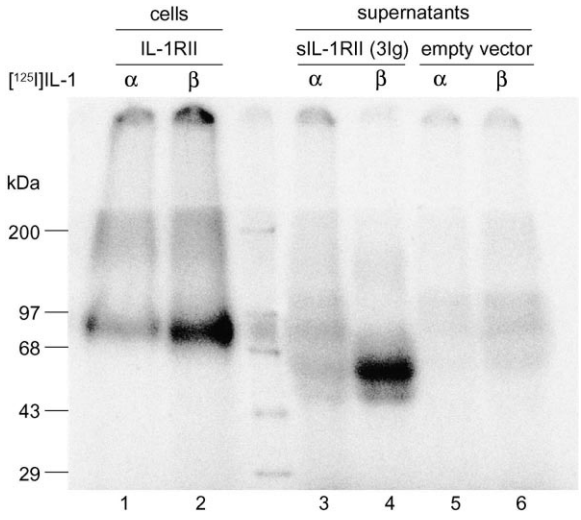

Fig. 2. Cross-linking of membrane and soluble hIL-1RII with [^{125}I]IL-1α or [^{125}I]IL-1β. 293 cells were transfected with expression vectors encoding membrane-bound or soluble human IL-1RII. After 40 h cells and 10 fold concentrated supernatants were incubated with 3 ng/ml [^{125}I]IL-1α or [^{125}I]IL-1β for 2 h. Protein complexes were cross-linked by addition of 5 mM BS^3 (1 h). Proteins were separated by SDS–PAGE and radioactive bands visualized by phosphorimaging.

3.2. Shedding of hIL-1RII from the cell surface results in the loss of IL-1α binding

A band of approximately 80 kDa was identified if radioactive IL-1α was cross-linked to 293 cells overexpressing IL-1RII (65 kDa membrane-anchored IL-1RII covalently coupled to 17 kDa IL-1α) (Fig. 2, lane 1). Cross-linking with IL-1β yielded a much stronger signal of the same size (lane 2). In supernatants of cells transfected with the expression plasmid encoding the complete extracellular part of IL-1RII only a complex of shIL-1RII with IL-1β (lane 4) but not with IL-1α (lane 3) was formed and cross-linked. (The shift in the apparent molecular weight of this complex in comparison to Fig. 1, lane 3, is due to the large amount of FCS in the concentrated supernatants employed for these cross-linking studies).

3.3. The affinities of shIL-1RII (3Ig) and shIL-1RII (2Ig) are comparable in binding assays

An immunosorbent binding assay was performed to compare the equilibrium dissociation constants of the two forms of shIL-1RII. The amount of ligand bound to the respective receptor was measured at different concentrations of radiolabeled IL-1β (Fig. 3A). An equilibrium dissociation constant (K_d) of 186 ± 34 pM was calculated for shIL-1RII (3Ig) and 212 ± 30 pM for shIL-1RII (2Ig) being practically identical

(Fig. 3B) (The difference in saturation binding between the two molecules is due to the different amounts of receptor immobilized on the Maxisorp plate (Fig. 3A).) Under these experimental conditions no binding to either of the two soluble forms of hIL-1RII could be detected with IL-1α as a ligand (data not shown).

3.4. shIL-1RII (3Ig) and shIL-1RII (2Ig) both neutralize IL-1β in a 24 h bioassay

The neutralizing capacity of the two recombinant shIL-1RII molecules was investigated in a 24 h bioassay. The IL-1β-induced IL-2 production by EL-4 6.1 cells was impaired by the addition of increasing amounts shIL-1RII (3Ig) as well as shIL-1RII (2Ig). In this assay full length soluble receptor (3Ig) neutralized at slightly lower concentrations compared to shIL-RII (2Ig) (Fig. 4A). The IL-1α-induced IL-2 production of EL-4 cells was only slightly reduced at a high molar excess of the two forms of shIL-1RII (Fig. 4B).

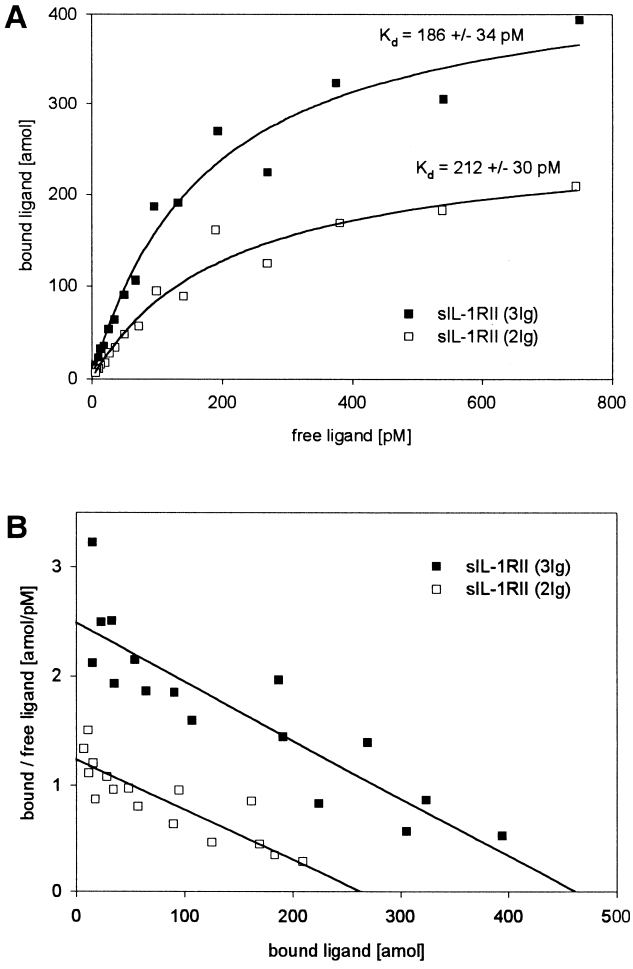

Fig. 3. Binding of [^{125}I]IL-1β to shIL-1RII (3Ig) or shIL-1RII (2Ig). A: The preparations of recombinant sIL-1RII (3Ig) (■) of sIL-1RII (2Ig) (□) were captured on Maxisorp plates via the precoated anti-HA mAb 12CA5. Unspecific binding was determined in absence of shIL-1RII. After 15 h of incubation with different concentrations of [^{125}I]IL-1β free and bound ligand were measured in a γ-counter. K_d was determined by least square analysis with a one site binding model after subtraction of unspecific binding and plotting of bound vs. free ligand. B: Scatchard plot of the binding data.

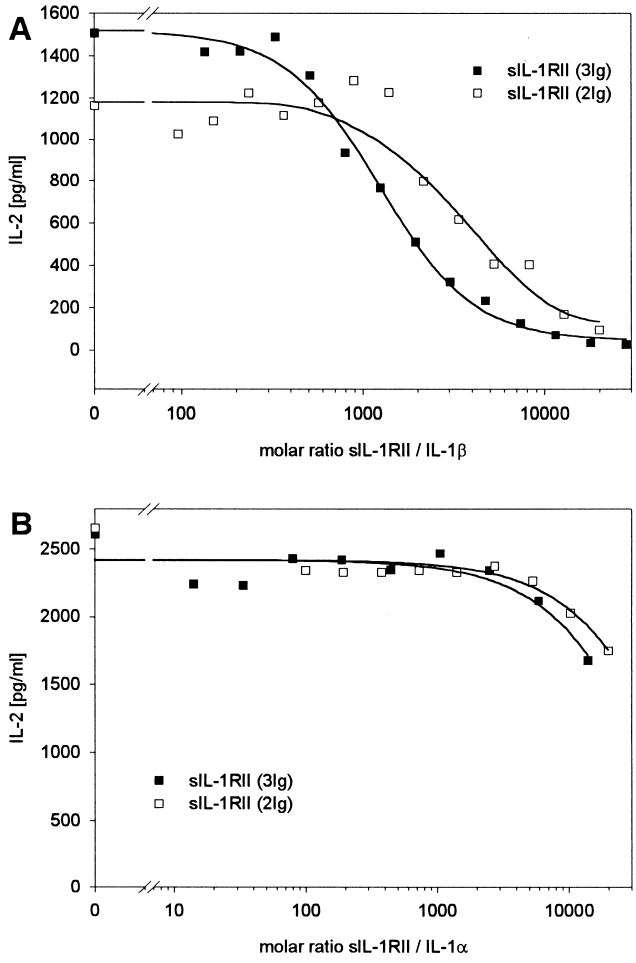

Fig. 4. Neutralizing capacity of shIL-1RII (2Ig) and shIL-1RII (3Ig). EL-4 6.1 cells were stimulated with 50 pg/ml of either rhIL-1β (A) or rhIL-1α (B) and $2{,}5\times10^{-7}$ M A23187 in presence of different concentrations of shIL-1RII (3Ig) (■) or shIL-1RII (2Ig) (□). After 24 h murine IL-2 was measured in the supernatants by ELISA.

3.5. shIL-1RII (2Ig) is detectable by a commercial ELISA kit

Both forms of recombinant shIL-1RII were detected by a commercial ELISA kit. The capture antibody used in this kit recognized the full length shIL-1RII with the three Ig-like domains as well as the form containing only the N-terminal two Ig-like domains (data not shown).

4. Discussion

Membrane-anchored IL-1RII consists of three extracellular Ig-like domains, one transmembrane spanning region, and a short cytoplasmic rest [10]. It can be cleaved between the third Ig-like domain and the transmembrane anchor resulting in a soluble form (sIL-1RII) which binds preferably IL-1β. Soluble IL-1RII was found in cell culture supernatants [6,7,11,12] and in serum from healthy individuals [13] and patients with inflammatory diseases [14–16]. It possesses anti-inflammatory properties by sequestering IL-1β from the circulation.

We confirmed that membrane-anchored IL-1RII bound both forms of IL-1 by cross-linking IL-1α or IL-1β to hIL-1RII. IL-1α showed a weaker cross-linking signal than IL-1β, reflecting the differences in affinity constants previously re-

ported for hIL-1RII [10,17]. 293 cells transfected with full length shIL-1RII released proteins to which only IL-1β could be cross-linked. Thus hIL-1RII lost its ability to bind IL-1α purely by being released from the cell surface. Furthermore, we could not find any evidence that shIL-1RII and IL-1RAcP interacted (Kollewe and Martin, unpublished results).

In addition to the 54 kDa shIL-1RII a smaller form of an IL-1β binding protein had been reported [5,6,18,19]. This molecule was most likely derived from shIL-1RII by proteolytic cleavage in the flexible hinge region between the second and third Ig-like domains. In order to confirm the molecular nature of this smaller shIL-1RII form and elucidate its neutralizing capacity, we generated two recombinant forms of the shIL-1RII, one with all three Ig-like domains and one with the first two N-terminal Ig-like domains. The short form of shIL-1RII showed IL-1β binding and yielded a band in cross-linking experiments of the expected size, proving that the two N-terminal Ig-like domains were sufficient to bind IL-1β. We compared the binding properties of the recombinant shIL-1RII forms in an immunosorbent assay. Both forms of shIL-1RII showed the same dissociation constant K_d of roughly 200 pM which is in good accordance with the values reported by others for shIL-1RII and membrane-anchored hIL-1RII [16,17]. Also both forms neutralized IL-1β but not IL-1α. The need for the relatively high excess of sIL-1RII forms for complete neutralization of IL-1β may be due to uncertainties in the calculation of biologically active recombinant material. Furthermore, sIL-1RII forms have to compete for the ligand with the membrane IL-1RI receptor complex which can be stabilized by IL-1RAcP as reported earlier [20], a form of stabilization not available for sIL-1RII (Kollewe and Martin, unpublished results).

This suggests that membrane-anchored IL-1RII is first cleaved to a 54 kDa soluble receptor containing all three Ig-like domains. Subsequently, a second cleavage in the hinge region between the first two N-terminal and the third Ig-like domains results in a 36 kDa protein which maintains its IL-1β binding and neutralization properties. As the affinity constants are comparable, this means that both forms of shIL-1RII can contribute to IL-1β sequestration and participate in down regulation of inflammation. Although we have not formally proven that the short form of the IL-1 binding protein previously described in serum or cell culture medium is identical to our recombinant molecule, it is highly likely that this sequential processing of hIL-1RII takes place in vivo in a comparable fashion.

Recently, the molecular structure of a soluble hIL-1RI complexed with IL-1β [21], IL-1 receptor antagonist (IL-1Ra) [22] and an antagonistic peptide [23] was resolved by X-ray crystallography. The binding site for IL-1β was mapped to an area between the first two N-terminal Ig-like domains [21,23]. This explains why both our molecules bind IL-1β. From these studies it was further suggested that IL-1β [21,23] possesses a second site of interaction in the third Ig-like domain of IL-1RI. We did not see any difference in binding of the short and long shIL-1RII, strongly suggesting that at least in shIL-1RII the contact of IL-1β to the third Ig-like domain is not essential for binding and does not contribute to the binding constant. This may well be different if the receptor is inserted in the membrane as is strongly suggested by the major differences observed between soluble and membrane-anchored forms with respect to binding of IL-1α and interaction with IL-1RAcP.

As soluble hIL-1RII is regarded as a parameter for the diagnosis of an ongoing inflammation we questioned whether both forms would be recognized by ELISAs. We tested one commercially available kit (from R&D Systems). This kit was capable of measuring both forms of hIL-1RII giving the safety that both biologically relevant forms of soluble human IL-1RII are detected, and the complete IL-1β binding and neutralization capacity of IL-1RII in samples can be monitored.

In summary, we show that the N-terminal two Ig-like domains of soluble human IL-1RII are sufficient to bind and neutralize IL-1β but not IL-1α. We propose that the hinge region between the first two N-terminal Ig-like domains and the third juxtamembraneous Ig-like domain contains a cleavage site which is used to generate a functional small form of the soluble human IL-1RII also in vivo.

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