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The Membrane Form of the Type II IL-1 Receptor Accounts for Inhibitory Function

Detlef Neumann,* Christian Kollewe,* Michael U. Martin,* and Diana Boraschi†

IL-1 signaling is mediated by the type I IL-1R (IL-1RI). The nonsignaling type II receptor has a regulatory function, since it reduces IL-1 effects by scavenging free IL-1 molecules. This regulatory function has been demonstrated only for the soluble form, released from the membrane receptor by action of specific proteases, but is still ill-defined for the membrane receptor itself. To assess the function of membrane IL-1RII, a modified IL-1RII cDNA was constructed, in which the cleavable domain was replaced with the corresponding uncleavable sequence of the epidermal growth factor receptor. The human keratinocyte line HaCaT, which does not express wild-type IL-1RII (wtIL-1RII), was stably transfected with this modified cDNA (unconventionally cleavable IL-1RII (uIL-1RII)). Cells transfected with uIL-1RII expressed the membrane form of IL-1RII, but were unable to produce the 60-kDa soluble receptor. Upon analysis of IL-1 responsiveness, parental HaCaT and vector-transfected cells (E27), expressing IL-1RII and the accessory chain IL-1R accessory protein, were responsive to IL-1. Conversely, cells overexpressing wtIL-1RII (811) or uIL-1RII (9D4) showed comparable reduction in responsiveness to both IL-1α (bound by membrane and soluble receptors) and IL-1β (recognized by the membrane receptor only), suggesting that the membrane form of the IL-1RII is mainly responsible for IL-1 inhibition. In contrast with wtIL-1RII, uIL-1RII did not interact with IL-1R accessory protein. Thus, the membrane form of IL-1RII possesses strong IL-1-inhibitory activity, independent of sequestration of the accessory protein and circumscribed to its ligand sink function. The Journal of Immunology, 2000, 165: 3350–3357.

The pleiotropic cytokine IL-1 is produced in consequence to inflammatory stimuli and regulates development and maintenance of the inflammatory reaction (1). Due to its catabolic properties, prolonged persistence of IL-1 at the site of inflammation can lead to pathological derangements, e.g., in acute and chronic inflammatory diseases such as sepsis and rheumatoid arthritis (1). To avoid the risk of detrimental effects, IL-1 activity in the organism is tightly regulated at multiple levels. The IL-1R antagonist binds to the signaling receptor IL-1RI as effectively as the two agonists IL-1α and IL-1β, but is unable to induce activation, thus inhibiting IL-1 action (2). A second level of regulation, unique to the IL-1 system, is represented by the nonsignaling receptor IL-1RII, which can regulate IL-1 activity by different mechanisms.

IL-1 binding to specific plasma membrane receptors on cells. Two distinct IL-1R molecules have been identified, IL-1RI and IL-1RII, which are able to bind IL-1 specifically (3, 4). A third molecule, the closely related receptor-like chain IL-1R accessory protein (IL-1RAcP),3 does not bind IL-1, but is absolutely required for the formation of the signal transduction complex (5–7). IL-1 signaling is initiated by the formation of a heterodimeric complex of ligated IL-1RI and IL-1RAcP. The generation of a new cytosolic scaffold by the two individual Toll-homology domains in IL-1RI and IL-1RAcP results in the association of the adaptor molecule MyD88, which in turn recruits the protein kinase IL-1R-associated kinase to the IL-1RI/IL-1RAcP complex. Phosphorylation of IL-1R-associated kinase allows interaction with the downstream adapter TNFR-associated factor-6, eventually leading to activation of the transcription factor NF-κB (8–10). The second IL-1R, IL-1RII, lacks the intracellular Toll-homology domain necessary for interaction with MyD88. Thus, although IL-1RII can bind IL-1 and form a heterodimeric complex with the coreceptor IL-1RAcP, it does not induce signaling. Membrane IL-1RII may down-regulate IL-1 activity by acting as a ligand sink that subtracts IL-1 from the signaling IL-1RII (4, 11, 12). In addition, ligated IL-1RII can function as a true decoy receptor by binding IL-1RAcP in a nonsignaling complex, thus depriving IL-1RII of its coreceptor. Most importantly, membrane IL-1RII is the source of soluble IL-1RII (sIL-1RII) molecules, which are released by proteolytic cleavage from many cell types after activation with secondary, IL-1-induced cytokines.

sIL-1RII molecules are found in cell culture supernatants (13–24) and in biological fluids under several physiopathological conditions (13, 25–29). Although it has been reported that the soluble receptor could be generated by an alternatively spliced IL-1RII mRNA (30), sIL-1RII is mainly produced by cleavage of the IL-1RII extracellular domain by a so far unidentified metalloproteinase (31). sIL-1RII is able to bind IL-1β as effectively as the membrane receptor, but it is virtually unable to bind IL-1α and IL-1R antagonist (32). Thus, sIL-1RII captures free agonist IL-1β and may act as negative regulator of IL-1 activity by buffering the systemic action of IL-1 leaking from inflammatory sites (22, 27).

To date, the IL-1-inhibitory function of the IL-1RII has been clearly demonstrated for the soluble form (17, 22, 32) and in systems in which both membrane and sIL-1RII were present (22, 33, 34).
34. It still remains a matter of investigation whether the functional role of membrane IL-1RII is only that of being a source for soluble inhibitory receptor or whether membrane IL-1RII itself contributes to the negative regulation of IL-1 activity by acting as ligand sink, as suggested by indirect data (12, 29).

In this study, we describe the generation of a modified form of human IL-1RII (uIL-1RII) that cannot be cleaved from the cell surface in a conventional manner and that does not interact with the coreceptor IL-1RαCp. Comparison of responsiveness to IL-1α and to IL-1β of cells transfected with either wt or uIL-1RII allows us to demonstrate that the membrane form of IL-1RII does indeed play a role in down-regulating IL-1 responsiveness on target cells by acting as a true ligand sink. This regulatory role of membrane IL-1RII may be especially relevant at very low concentrations of IL-1, e.g., far off the site of inflammation, and may thus represent a protection mechanism of cells not involved in the inflammatory reaction.

Materials and Methods

Plasmids

The human IL-1RII cDNA was modified as shown in Fig. 1. Briefly, the sequence coding for the receptor stalk in the extracellular domain of IL-1RII (5′-CCAGGTGCTTCTCATCTGC-3′) was excised by inverse PCR from the full-length cDNA of human IL-1RII within the expression plasmid pMM38 (22). The excised fragment was replaced with the corresponding PCR-amplified sequence of the human EGFR cDNA (5′-ACACTGCTTGAAGTGGCC-3′). The EGFR cDNA was a kind gift of Dr. Pier Paolo Di Fiore, European Institute of Oncology (Milan, Italy).

PCR amplification of the EGFR sequence was conducted, as described hereafter, with primers containing PspI and SphI restriction sites for directed ligation (Table I). Ligation was performed using T4 DNA ligase (Boehringer Mannheim, Mannheim, Germany), following a standard protocol. The correctness of the resulting construct, uIL-1RII, was checked by DNA sequencing.

Plasmids containing the cDNA coding for IL-1RαCp and its C-terminally truncated form were kindly provided by Dr. Werner Falk (University of Regensburg, Regensburg, Germany).

Cell culture and transfections

The adherent human keratinocyte cell line HaCaT (36), HaCaT-derived clones, and human embryonic kidney (HEK) 293 cells were maintained in DMEM (Life Technologies, Paisley, U.K.), supplemented with 2 mM L-glutamine, 50 μg/ml gentamicin sulfate (Sigma, St. Louis, MO), and 10% heat-inactivated FBS (HyClone, Sterile Systems, Logan, UT). Cells were transfected by the calcium phosphate method, and stable clones were selected by adding G418 (Invitrogen, Carlsbad, CA). HaCaT-derived clones (811, 9D4, and E27) were maintained in culture medium in the presence of an optimal concentration of G418 (selected by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from)

Table I. PCR primer sequences and annealing temperaturesa

<table>
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<tr>
<th>Target</th>
<th>Primer Sequence (5′→3′)</th>
<th>Bases Spanned</th>
<th>Temperature (°C)</th>
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<td>76–111</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>AS: CTTGTTGGGTGTGTTTGTGTTTTCCCTACAGGATAAA</td>
<td>643–675</td>
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<tr>
<td>IL-1RαCp</td>
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<td>771–795</td>
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<tr>
<td></td>
<td>AS: GAATCACCACATGGACGAGCTTTACGGGCC</td>
<td>1096–1120</td>
<td></td>
</tr>
<tr>
<td>IL-1RII</td>
<td>S: CCAAGGAGAGAGAGAGACGGGATG</td>
<td>235–259</td>
<td></td>
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<tr>
<td></td>
<td>AS: CAGGACACAGCAGGTGATATAGCAGGC</td>
<td>604–628</td>
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<td>S: GTTGGATAGGCGACGACCTTTGT</td>
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<tr>
<td></td>
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<td></td>
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<td>963–992</td>
<td>55</td>
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<tr>
<td>EGFR</td>
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<td>1881–1910</td>
<td></td>
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<tr>
<td></td>
<td>AS: ACCATCAAGCTGGTCAGTCTACGGAGGATCTA</td>
<td>1923–1952</td>
<td>72</td>
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</table>

a The 5′→3′ sequences of the sense (S) and antisense (AS) primer pairs and their annealing temperatures are listed. Primers were either synthesized in house or obtained from Eurobio (Milano, Italy). HPRT primer sequences have been published elsewhere (35). Sites for PspI and SphI in the primers used for uIL-1RII cloning are underlined.
gamma inhibition experiments, cells were exposed to mAbs before addition of a fixed dose of \( ^{125}\text{I}-\text{IL-1\beta} \). Preincubation was performed for 2 h at 37°C using Abs against IL-1RI (5 \( \mu \text{g/ml} \); rat IgG2b anti-human IL-1RI; PharMingen, San Diego, CA) or IL-1RII (100 \( \mu \text{g/ml} \); rat IgG2a anti-human IL-1RII; Genzyme, Cambridge, MA) or isotype control (100 \( \mu \text{g/ml} \); rat IgG2a anti-mouse Gr-1, clone RB6-8C5; American Type Culture Collection, Manassas, VA). Ab concentrations used were chosen from preliminary experiments as those with the highest capacity of inhibiting IL-1\beta binding with the minimal nonspecific effect.

Cross-linking of soluble receptors

Cell monolayers (5 \( \times 10^5 \) cells/well of Cluster 24 plates) were incubated for 24 h in 1 ml medium without FBS alone or containing human TNF\( \alpha \) (Sigma, St. Louis, MO) or isotype control (100 \( \mu \text{g/ml} \); rat IgG2a anti-mouse Gr-1, clone RB6-8C5; American Type Culture Collection, Manassas, VA). Ab concentrations used were chosen from preliminary experiments as those with the highest capacity of inhibiting IL-1\beta binding with the minimal nonspecific effect.

Cross-linking of membrane receptors

Cells were seeded in 96-well plates (Cluster 96; Costar) at a density of 1 \( \times 10^4 \) cells/well in culture medium with 10% FBS and incubated for 24 h to allow adherence. Cells were then washed twice in PBS before addition of 5 \( \mu \text{M} \text{BS} \text{3} \) in PBS. After 1 h of incubation, cells were lysed in 300 \( \mu \text{L} \) lysis buffer (150 mM NaCl, 1% Triton X-100), and nuclei and cellular debris were removed by centrifugation (15,000 \( \text{g} \), 30 min at 4°C). Lysates were prepared for SDS-PAGE by heating in Laemmli buffer, and proteins were separated on 7.5% gels. Radioactive complexes were visualized by autoradiography.

IL-6 production

Cells were seeded in 96-well plates (Cluster 96; Costar) at a density of 1 \( \times 10^4 \) cells/well in culture medium with 10% FBS and incubated for 24 h to allow adherence. Cells were then washed twice in PBS and incubated for 24 h in 150 \( \mu \text{L} \) culture medium containing different concentrations of human IL-1\alpha (Immunex, Seattle, WA), IL-1\beta (kindly provided by P. Ruggeri, Dompé, L’Aquila, Italy), TNF\( \alpha \) (BASF-Knoll, Ludwigshaven, Germany), and human IL-8 (1 ng/ml; PeproTech, Rocky Hill, NJ). A total of 20 \( \mu \text{L} \) cell-free supernatants was added to a 1000-fold molar excess of unlabeled IL-1\beta for 3 h. Lysates were prepared for SDS-PAGE by heating in Laemmli buffer, and proteins were separated on 7.5% gels. Radioactive complexes were visualized by autoradiography.

Results

Construction of a mutant cDNA coding for an IL-1RII form resistant to conventional cleavage

To define unambiguously the role of membrane IL-1RII in the regulation of IL-1 responsiveness, a cDNA was generated that coded for a mutant form of human IL-1RII. This mutant form of IL-1RII lacks the cleavage area that in the wtIL-1RII is targeted by specific proteases to generate the 60-kDa sIL-1RII. This mutant receptor should maintain the IL-1-binding capacity, but should become resistant to conventional cleavage by the IL-1RII-processing ectoprotease. As the protease catalyzing the release of the 60-kDa sIL-1RII has not yet been formally identified (31), the precise cleavage site is not known. However, from available data the...
cleavage area could be reasonably restricted to the stretch comprised between the end of the membrane-proximal, third extracellular Ig-like loop, and the beginning of the transmembrane region. Therefore, the cDNA sequence coding for this receptor stalk has been excised by inverse PCR from the full-length cDNA of human IL-1RII contained in the plasmid pMMP38. In its place, the PCR-amplified corresponding sequence of the human EGFR cDNA was ligated, exploiting restriction sites introduced into the primers. The resulting construct pDn031 codes for a IL-1RII/EGFR chimeric receptor consisting of the three Ig-like loops (i.e., the binding domain) of IL-1RII and the stalk derived from the EGFR, followed by the transmembrane region and intracellular domain of IL-1RII (Fig. 1). This mutant IL-1RII (uIL-1RII) should be resistant to conventional processing by proteases and therefore unable to release the 60-kDa sIL-1RII.

**Transfected HaCaT cells express mutant uIL-1RII mRNA**

The human keratinocyte line HaCaT naturally expresses the IL-1R chains IL-1RI and IL-1RACP, as determined both at the mRNA and protein level (22) (Fig. 2A). Expression of IL-1RII mRNA in these cells could only be detected after a very high number of cycles (>45) by RT-PCR, whereas the receptor protein could not be detected either by cytokinofluorometric analysis or in Western blotting or cross-linking experiments (data not shown). HaCaT cells were stably transfected with the cDNA construct pDn031, coding for the uIL-1RII. G418-resistant clones were selected and analyzed for expression of the exogenous gene. Of 18 clones tested, 6 expressed mRNA for the transgene and were further assayed for IL-1β-binding capacity. Two clones (9D3 and 9D4) showed a significantly higher binding capacity for IL-1β when compared with parental HaCaT cells or with cells transfected with the control empty vector. All subsequent studies were performed on the uIL-1RII-transfected clone 9D4 in comparison with the empty vector-transfected control clone E27.

The expression of mRNA for endogenous and transfected IL-1R chains was assessed by RT-PCR analysis in parental HaCaT cells and in stably transfected clones (Fig. 2A). No expression of IL-1RII mRNA could be detected in HaCaT cells under these PCR conditions. The control clone E27 (empty vector) showed the same expression pattern as parental HaCaT cells. With primers amplifying the IL-1RII ectodomain, both the IL-1RII-transfected clone 811 (expressing the transgene coding for wtIL-1RII) (22) and the uIL-1RII-transfected clone 9D4 were positive. On the other hand, by using a pair of primers that amplifies the mutated area (i.e., a 3’ end primer binding to the EGFR stalk sequence and a 5’ end primer binding to the extracellular IL-1RII sequence), mRNA expression for the mutant uIL-1RII could only be detected in 9D4 cells. These results indicate that 9D4 cells express the mutated uIL-1RII at the mRNA level.

**Transfected HaCaT cells express mutant uIL-1RII protein that binds IL-1**

Proof for the expression of the uIL-1RII protein on the cell surface of 9D4 cells was obtained by cytofluorometric analysis (Fig. 2B). The majority of uIL-1RII-transfected 9D4 cells expressed IL-1RII on their membrane, as compared with control E27 cells that were completely negative. In addition, the presence of uIL-1RII protein was demonstrated in the Western blot analysis of 9D4 cell microsomes (Fig. 2C). In fact, a 60-kDa protein was detected by a specific anti-IL-1RII mAb in uIL-1RII-transfected 9D4 cell preparations, which is absent in control E27 cells.

IL-1β binding to the cell surface was assessed by incubating the HaCaT clones with a constant concentration of radiolabeled IL-1β. As shown in Fig. 3A, binding of IL-1β was very low in HaCaT and E27 cells, whereas it was markedly enhanced in the transfected clones 811 (wtIL-1RII) and 9D4 (uIL-1RII). Saturation-binding experiments allowed to calculate over 60,000 IL-1β-binding sites/cell for the 9D4 clone, with a Kd of 1.26 nM. This affinity is comparable with that of both natural and recombinant IL-1RII, as measured in RAJ1 and 811 cells, respectively (Table II). On the other hand, control E27 cells display a very low number of high affinity IL-1β binding sites (226 ± 47, Kd, 0.07 nM), comparable

![FIGURE 3](http://www.jimmunol.org/)

Binding of 125I-IL-1β to HaCaT cells and transfected clones. A. Equal numbers of cells (5 × 10⁵ cells/well) were incubated with 0.2 nM 125I-IL-1β for 4 h at 4°C. Cells were then washed and lysed to determine cell-bound radioactivity. Specific binding was calculated by subtracting the background binding measured in the presence of a 1000-fold excess of unlabeled IL-1β. Results reported are the mean ± SEM of triplicate determinations within a single experiment representative of three performed. B. Inhibition of 125I-IL-1β binding to E27 (empty vector) and 9D4 (uIL-1RII) cells by mAbs against IL-1RI or IL-1RII. Cells were exposed for 2 h at 37°C to medium alone or containing isotype control Ab (rat IgG2a anti-mouse Gr-1), rat anti-human IL-1RI, or rat anti-human IL-1RII, before the addition of 0.3 nM 125I-IL-1β. After 4-h incubation at 4°C, cells were washed and lysed to determine cell-bound radioactivity. Specific binding of 125I-IL-1β in the absence of Abs was taken as 100% binding (E27, 119 ± 12 cpm; 9D4, 8028 ± 421 cpm).

Results are the mean ± SEM of triplicate determinations within one representative experiment of two performed.
with those of parental HaCaT cells (Table II) and possibly due to IL-1β binding to endogenous IL-1RI.

Identification of the IL-1R chains contributing to IL-1β binding was achieved with specific blocking Abs. The weak binding of IL-1β to the control clone E27 was indeed due to endogenous IL-1RI, as it was inhibited by the anti-IL-1RI Ab, but was unaffected by anti-IL-1RII or isotype control Abs. Superimposable results have been obtained on parental HaCaT cells (data not shown) (22). On the other hand, the potent binding of IL-1β to uIL-1RII-overexpressing 9D4 cells was abrogated by anti-IL-1RII Ab, while anti-IL-1RI or isotype control Abs had no measurable effect.

These data indicate that the mutant uIL-1RII is expressed on the cell surface of transfected cells and that it can bind IL-1β with an affinity comparable with that of wtIL-1RII.

**Mutant IL-1RII does not release the 60-kDa sIL-1RII**

Having shown that uIL-1RII is expressed on the cell surface of transfected keratinocytes and maintains the binding characteristics of the wtIL-1RII, additional experiments were designed to assess whether the uIL-1RII receptor was indeed uncleavable, i.e., it could not be proteolytically processed to release the soluble 60-kDa form. To this end, cross-linking experiments were performed with radiolabeled IL-1β on cell supernatants. In culture supernatants from 811 cells (wtIL-1RII), a soluble 60-kDa IL-1β-binding molecule was detected. This appears as a complex of about 80 kDa consisting of 125I-IL-1β (17 kDa) covalently linked to sIL-1RII (60 kDa) (Fig. 4A). In culture supernatants of HaCaT and E27 cells, no IL-1β-binding molecules were detectable, in agreement with data showing the inability of these cells to express measurable levels of IL-1RII. Despite the expression of high levels of membrane uIL-1RII, 9D4 cells did not release detectable amounts of the 60-kDa IL-1β-binding molecule. However, 9D4 cells released low amounts of an IL-1β-binding protein that was smaller compared with the sIL-1RII derived from wtIL-1RII. None of the soluble IL-1β-binding proteins in the culture supernatants could be detected when using 125I-IL-1α in the cross-linking experiments (Fig. 4B). However, 125I-IL-1α could be readily cross-linked to membrane IL-1RII, e.g., on 811 cells or on transiently transfected HEK 293 cells (data not shown).

As shown in Fig. 4C, release of the small sIL-1β-binding protein from 9D4 cells was enhanced by treating 9D4 cells with agents that induce the shedding of wtIL-1RII (data not shown). This suggests that the same protease cleaving wtIL-1RII in the stalk region may process uIL-1RII with less efficiency at a different position and give rise to a smaller sIL-1RII form. Preliminary results indicate that this alternative cleavage may take place in the hinge region between the second and the third N-terminal Ig loops, releasing a truncated sIL-1RII molecule still able to bind IL-1β, but not IL-1α (Kollewe et al., unpublished observations).

It is concluded from these data that the mutant uIL-1RII is not proteolytically cleaved to the 60-kDa sIL-1RII in a conventional fashion, although it could be processed in other positions to release smaller soluble receptor forms.

**Membrane IL-1RII accounts for impaired biological response to IL-1**

Parental HaCaT cells respond to low concentrations of IL-1 by producing IL-6. This and other responses to IL-1 are reduced in 811 cells, which overexpress wtIL-1RII and release large amounts of the 60-kDa sIL-1RII (22, Fig. 5). Upon exposure to increasing concentrations of either IL-1α or IL-1β, the uIL-1RII-overexpressing 9D4 cells released significantly lower levels of IL-6 as compared with parental HaCaT cells or vector-transfected control E27 cells (Fig. 5). No difference was observed by using IL-1α or IL-1β as stimulus. Thus, down-regulation of responsiveness to IL-1 could be similarly achieved by either wtIL-1RII or uIL-1RII at the membrane level. The presence of conventional 60-kDa sIL-1RII (in 811 cells) or small amounts of the lower m.w. soluble product derived from uIL-1RII (in 9D4 cells) did not contribute to this inhibitory effect. In fact, both forms of sIL-1RII can bind IL-1β,

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**FIGURE 4.** Identification of soluble IL-1β-binding proteins in supernatants of HaCaT clones. Cells were incubated for 24 h in medium without FBS. Aliquots of cell-free supernatants were incubated with 125I-IL-1β (A) or 125I-IL-1α (B) and cross-linked with BS3 or DSS, respectively. Cross-linked complexes were run on a 10% SDS-PAGE, and the gel was dried and subjected to autoradiography. Results are from one representative experiment of two performed with superimposable results. Molecular masses (kDa) of marker proteins are indicated at the left. C, 9D4 cells were incubated for 24 h in medium without FBS alone (lanes 1 and 2) or containing TNFα, LPS, DEX, PMA, or IL-8 (lanes 3–7). Aliquots of cell-free supernatants were incubated with 125I-IL-1β, cross-linked with BS3, and run on a 7.5% SDS-PAGE. As a control, an aliquot of the unstimulated cell supernatant was processed in the presence of a 1000-fold molar excess of unlabeled IL-1β (lane 2).
and cross-linked with 125I-IL-1 readily detectable 80-kDa complex consists of wtIL-1RII ligated about 80 kDa and a second complex of roughly 160 kDa. The 110.1 was 18.5 produced by parental HaCaT cells (background IL-6 production was always <5% and are not reported. In the experiments performed, IL-6 production in the absence of stimulation was 84.8 ± 24.2 pg/ml for HaCaT cells, 101.1 ± 3.9 pg/ml for E27 cells, 95.4 ± 4.8 pg/ml for 811 cells, and 94.3 ± 20.7 for 9D4 cells.

but not IL-1α, whereas reduction of IL-1 responsiveness was evident with both IL-1 isoforms and thus conceivably takes place at the level of membrane receptors.

Production of IL-6 in response to other stimuli was not impaired in 9D4 cells. In response to LPS (100 pg/ml), 9D4 cells produced 110.1 ± 1.9 pg/ml of IL-6, as compared with 131.7 ± 25.7 pg/ml produced by parental HaCaT cells (background IL-6 production was 18.5 ± 0.5 and 17.6 ± 0.4 pg/ml for 9D4 and HaCaT, respectively). Accordingly, in response to TNF-α (10 pg/ml), IL-6 production was increased by 4.07 ± 0.01 times in 9D4 cells, as compared with 4.05 ± 0.27 and 4.14 ± 0.16 in HaCaT and E27 cells, respectively.

Mutant uIL-1RII does not interact with IL-1RAcP

Membrane IL-1RII may down-regulate IL-1 responsiveness by competing with the signaling IL-1RI by either subtracting IL-1 (acting as a ligand sink) or, once ligated, by sequestering the coreceptor IL-1RAcP (coreceptor competition). To distinguish between the two mechanisms, experiments were performed to demonstrate direct interaction of IL-1RII and IL-1RAcP in the HaCaT clones.

Interaction between membrane IL-1RII and IL-1RAcP was analyzed by incubation of HaCaT, 811, 9D4, or E27 cells with 125I-IL-1β, subsequent chemical cross-linking, analysis of the proteins by SDS-PAGE, and autoradiography. In HaCaT and vector-transfected control E27 cells, no complexes containing IL-1β were detected, again reflecting the very low endogenous expression of wt IL-1R on these cells.

In 811 cells (overexpressing wtIL-1RII), two complexes with IL-1β were detected, one with an apparent molecular mass of about 80 kDa and a second complex of roughly 160 kDa. The readily detectable 80-kDa complex consists of wtIL-1RII ligated and cross-linked with 125I-IL-1β. The weak larger complex might consist of ligated IL-1RII in a heterodimer with IL-1RAcP. This pattern of cross-linked complexes was also found in 9D4 cells, which express the mutated receptor (data not shown). To identify the components of the larger cross-linked complex, immunoprecipitation with an Ab to human IL-1RAcP was attempted. However, due to the relative weakness of the signals in 811 and 9D4 cells, it was not possible to obtain clear-cut results (data not shown). Thus, interaction between uIL-1RII and IL-1RAcP was studied in a different cellular system that allowed higher expression of the two receptor chains. HEK 293 cells were transiently transfected with plasmids coding either for wtIL-1RII or mutant uIL-1RII alone or in combination with the cDNA coding for the wtIL-1RAcP or a C-terminally truncated form of IL-1RAcP. Results of binding and cross-linking experiments with radiolabeled IL-1 on these cells are shown in Fig. 6. The 80-kDa complex of 125I-IL-1β cross-linked to IL-1RII alone can be found in all cells transfected with either wtIL-1RII or uIL-1RII. When wtIL-1RII was cotransfected with full-length wtIL-1RAcP, an additional complex of about 180 kDa could be detected (Fig. 6, lane 3). Cotransfecting a C-terminally truncated smaller form of IL-1RAcP led to a significant reduction of the apparent m.w. of the larger complex (Fig. 6, lane 5), indicating that this indeed contains the coreceptor IL-1RAcP. However, when mutated uIL-1RII was coexpressed with either full-length wtIL-1RAcP (Fig. 6, lane 4) or truncated IL-1RAcP (Fig. 6, lane 6), no difference in the size of the high m.w. complex was found. This suggests that this complex does not contain the coreceptor IL-1RAcP, but it may rather consist of IL-1RII homodimers.

These results indicate that the alteration in the stalk region in uIL-1RII, while not affecting IL-1 binding, could abrogate interaction with the coreceptor IL-1RAcP. Thus, most likely, the mechanism of coreceptor competition does not contribute to the IL-1-inhibitory effect of uIL-1RII in 9D4 cells.

**Discussion**

IL-1 is a potent inflammatory and immunoregulatory cytokine. Prolongation of IL-1 production, release, and biological effects of

![FIGURE 5](http://www.jimmunol.org/DownloadedFrom/

![FIGURE 6](http://www.jimmunol.org/DownloadedFrom/)
IL-1 may lead to high risk of pathological derangements. Therefore, the IL-1 system needs to be tightly regulated at several levels, including production, availability at the site of inflammation, and biological function via receptor-mediated activation of target cells.

On many cell types, two forms of IL-1R are expressed, which have opposite roles. Both receptors bind IL-1α and IL-1β, but whereas IL-1RI, in concert with the coreceptor IL-1RAcP, is responsible for signal transduction and cell activation, IL-1RII has a different and unique function. Membrane IL-1RII may act as a ligand sink for both IL-1α and IL-1β. This function is particularly important when IL-1RII is abundantly expressed on cells and IL-1R is expressed at low levels, such as in neutrophils or B lymphocytes. At low IL-1 concentrations, the relative excess of IL-1RII vs IL-1RI will capture the few IL-1 molecules available, thus depriving IL-1RI of the ligand required for interaction with the coreceptor IL-1RAcP. By this mechanism, IL-1RII inhibits the formation of signaling complexes and down-regulates IL-1 responsiveness. In addition to its function as a ligand sink, IL-1RII may also compete with IL-1RI for IL-1RAcP binding, by recruiting the coreceptor molecules into nonsignaling complexes in a true decoy fashion.

Beside these two regulatory functions on the membrane, IL-1RII serves as substrate for a yet unidentified metalloprotease (31) in the rapid generation of sIL-1RII molecules. The relative role of membrane vs sIL-1RII in the control of cell activation by IL-1 is not fully understood, as all levels of regulation may take place in parallel. Available data point to a definite inhibitory role for the sIL-1RII, whereas the contribution of membrane IL-1RII as a negative regulator of IL-1 responsiveness is still a matter of discussion.

To address this question, an IL-1RII was generated, modified to prevent cleavage at the traditional site and to release the 60-kDa sIL-1RII. This was achieved by replacing the putative cleavage region in IL-1RII (i.e., the 20-residue stalk sequence VGHLNTLS followed by the beginning of the transmembrane region) with the corresponding region of the EGFR, a membrane protein insensitive to cleavage. Cleavage of sIL-1RII becomes unable to bind IL-1α, whereas on the other hand it sheds the 60-kDa form, which is generated by alterative cleavage and whose soluble form is generated by alternative splicing (41). Although the exact sequence motif recognized by the cleaving protease in IL-1RII is not known, the molecular mass of sIL-1RII (about 60 kDa), structural data, and the presence of three Ig-like loops in the soluble receptor all indicate that the target site for the cleaving metalloproteinase is confined to the stalk region of the receptor molecule (11, 18–24). A construct coding for the mutated receptor uIL-1RII was used to transfect the human keratinocyte line HaCaT (36). One representative clone (9D4) was analyzed in detail. The overexpressed uIL-1RII was the predominant IL-1 binding site in 9D4 cells, as demonstrated with blocking anti-IL-1RI and anti-IL-1RII Abs. The mutant uIL-1RII on the 9D4 cell surface was able to bind IL-1β with an affinity comparable with that of wtIL-1RII, as measured by saturation binding and Scatchard analysis. Thus, it can be concluded that the mutated uIL-1RII has the same ligand-binding characteristics as wtIL-1RII.

Overexpression of the wtIL-1RII in the HaCaT clone 811 did not only lead to enhanced binding capacity on the cell surface, but also to the appearance of readily detectable soluble receptor molecules in cell culture supernatants (Ref. 22; this study). The receptor is shed as a 60-kDa soluble protein by a membrane metalloproteinase, which probably is expressed in all IL-1RII-releasing cells (31).

In 9D4 cells (which overexpress the mutated uIL-1RII), no soluble 60-kDa IL-1β-binding activity was found in the cell supernatant, indicating that removal of the putative cleavage site of the protease was successful. However, a small amount of an IL-1β-binding protein of approximately 34 kDa could be detected in 9D4 cell supernatants and in supernatants from HEK 293 cells transiently transfected with uIL-1RII. At present, the formal identification of this 34-kDa IL-1β-binding protein has not been achieved. As the release of both 60- and 34-kDa IL-1β-binding molecules was stimulated by the same panel of agents in transfected HaCaT cells, it is tempting to speculate that the same protease is responsible for the processing of both wtIL-1RII in the stalk region and uIL-1RII at a different unidentified cleavage site, which becomes available after modification of the primary site. In any case, it is clear that both forms of sIL-1RII, the conventional 60-kDa as well as the short 34-kDa form, can bind IL-1β, but are unable to bind IL-1α.

Parental HaCaT cells and vector-transfected control E27 cells express the type I IL-1R and the IL-1RAcP, which form the signaling receptor complex. These cells respond to stimulation with low IL-1 concentrations by producing cytokines and mediators such as IL-6, IL-8, and PGE2 (Ref. 22; data not shown). Responsiveness to IL-1 could be impaired by transfection and overexpression of the entire IL-1RII (22, 33, 34) or of a recombinant soluble form of IL-1RII encompassing the ectodomain of the receptor (Ref. 33; data not shown). As reported in this study, transfection with the mutated uIL-1RII also led to reduction of the cellular response to both IL-1α and IL-1β. As IL-1α does not bind to any of the sIL-1RII forms, the reduction in IL-1α responsiveness should be solely due to the action of the membrane IL-1RII. Expression of uIL-1RII did not alter cellular responses to other stimulants such as LPS or TNF-α, nor did it hamper expression of the other IL-1R components IL-1RI and IL-1RAcP. This indicates that the observed reduction in the biological response to IL-1 is due to a direct inhibitory effect of membrane IL-1RII on IL-1 responsiveness.

The question arises as to whether this direct inhibitory effect might be only attributed to the ligand-binding capacity of membrane IL-1RII or whether sequestration of the coreceptor IL-1RAcP, necessary for the formation of the signaling complex with IL-1RI, is also involved in the inhibitory mechanism. Whereas the ligand sink effect should contribute to down-regulating responsiveness to low IL-1 concentrations, as discussed above, the coreceptor competition should also be effective at very high concentrations of IL-1.

Although still able to bind IL-1, the mutant uIL-1RII, genetically modified between the transmembrane region and the membrane-proximal Ig-like loop, was unable to interact with IL-1RAcP. This observation emphasizes the notion that membrane IL-1RII can act as a ligand sink, whereas on the other hand it sheds some light on the conformational requirements for an effective interaction between IL-1RII and IL-1RAcP. Results from earlier studies show that wtIL-1RII in its membrane form can bind IL-1α and IL-1β, but not the 31-kDa precursor form of IL-1β (17). Once released from the surface by proteolytic cleavage, the ligand-binding characteristics of IL-1RII change dramatically. The 60-kDa sIL-1RII becomes unable to bind IL-1α, but acquires the capacity of binding the inactive pro-IL-1β. These observations suggest that localization of IL-1RII into the plasma membrane implies some conformational restraints that define its binding specificities and that are lost upon release from the surface. These conformational features may be only partially maintained by the EGFR stalk region in uIL-1RII, which therefore loses its ability to interact with IL-1RAcP. From preliminary indications, it appears that the relative positioning of the membrane-proximal third Ig-like loop to the plasma membrane is critical for effective interaction with IL-1RAcP. Work is in progress to test this hypothesis.
In summary, the observations reported in this work are in support of the different roles of membrane vs sIL-1RII in the control of IL-1 responses. The soluble receptor apparently plays an IL-1-buffering role, to down-regulate and limit the inflammatory response caused by IL-1β released systemically into the body. In fact, sIL-1RII is found abundantly in biological fluids of patients with inflammatory diseases (13, 17, 25–29). The fact that sIL-1RII can also inhibit the maturation of pro-IL-1β further confirms the importance of its anti-inflammatory role in acute and chronic inflammation, a situation frequently implying cell damage and the consequent release of cytoplasmic pro-IL-1β (1). Data presented in this work show that, in addition to the well-characterized role of sIL-1RII, the membrane receptor itself can control IL-1 responses on the surface of target cells by competing with IL-1RI for ligand and coreceptor, acting both as a ligand sink and as a true decoy receptor. These mechanisms may play a role in the protection of cells from premature activation by systemic IL-1α in areas apart from the site of inflammation.

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