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IL-1 Signaling Cascade in Liver Cells and the Involvement of a Soluble Form of the IL-1 Receptor Accessory Protein1,2

Liselotte E. Jensen, Marta Muzio, Alberto Mantovani, and Alexander S. Whitehead

The proinflammatory cytokine IL-1 induces the biosynthesis of a number of immunologically important proteins during infection, tissue damage, and/or stress (reviewed in Ref. 1). A central consequence of cell stimulation by IL-1 is the activation of the transcription factor NF-κB, which mediates the increased transcription of numerous genes and hence increased synthesis of a range of proteins, many of which are involved in immunological responses, e.g., the k light chain of Ig (from which the transcription factor is named (2)) and the major acute phase reactant serum amyloid A protein (reviewed in Ref. 3).

There are two receptors for IL-1. The type I IL-1R (IL-1RI) has an extracellular portion of 319 amino acid residues comprising three Ig domains, a transmembrane region of 20 amino acid residues, and an intracellular domain of 213 residues (4), whereas the three Ig domains, a transmembrane region of 20 amino acid residues comprising an extracellular portion of 319 amino acid residues comprising the major acute phase reactant serum amyloid A protein (reviewed in Ref. 3).

The intracellular signaling cascade involves recruitment of two IL-1R-associated kinases, IRAK1 and IRAK2, and the adapter protein MyD88, events which are dependent on the intracellular domain of membrane-bound IL-1RAcP (mIL-1RAcP). In mouse liver, IL-1RAcP is expressed as a soluble protein (sIL-1RAcP), the function of which is unknown. We have cloned the human sIL-1RAcP and established by sequence analysis that the human sIL-1RAcP mRNA arises from alternative splicing of the IL-1RAcP gene (shown here to encompass 12 exons spanning more than 56 kb). Furthermore, we demonstrate that human HepG2 hepatoma cells express both mIL-1RAcP and sIL-1RAcP and that signal transduction in these cells is mediated through IRAK1, IRAK2, and MyD88. We show that phorbol esters induce a change in the pre-mRNA splice pattern such that sIL-1RAcP mRNA becomes the dominant form. Overexpression of a membrane-anchored fusion protein of sIL-1RAcP and MHC in HepG2 cells inhibits IL-1-mediated NF-κB activation, whereas coexpression of IL-1RI with membrane-anchored sIL-1RAcP restores the capacity of the cells to respond to IL-1. This suggests that sIL-1RAcP may act as an inhibitor of IL-1 by directly interacting with IL-1RI to abolish its capacity to transduce signal. The Journal of Immunology, 2000, 164: 5277–5286.

Interleukin-1 is a pleiotropic cytokine that induces multiple physiological responses after infection, tissue damage, and/or stress (reviewed in Ref. 1). A central consequence of cell stimulation by IL-1 is the activation of the transcription factor NF-κB, which mediates the increased transcription of numerous genes and hence increased synthesis of a range of proteins, many of which are involved in immunological responses, e.g., the k light chain of Ig (from which the transcription factor is named (2)) and the major acute phase reactant serum amyloid A protein (reviewed in Ref. 3).

There are two receptors for IL-1. The type I IL-1R (IL-1RI) has an extracellular portion of 319 amino acid residues comprising three Ig domains, a transmembrane region of 20 amino acid residues, and an intracellular domain of 213 residues (4), whereas the IL-1R type II (IL-1RII) resembles the type I receptor in that it has three extracellular Ig domains (330 amino acid residues) and a transmembrane region of 26 residues, but differs significantly by having only 29 intracellular amino acid residues (5). Both receptors associate with the IL-1R accessory protein (IL-1RAcP) (6–8). IL-1-mediated signaling occurs only via the type I receptor. In contrast, the type II receptor, due to its truncated intracellular domain, acts as a ligand sink (9–11).

The best-described signaling cascade initiated by IL-1 involves a trimeric protein complex of IL-1, IL-1RI, and IL-1RAcP (reviewed in Ref. 12). This complex recruits the adapter protein MyD88 via the intracellular domains of IL-1RI and IL-1RAcP, possibly through protein interactions involving regions of sequence similarity to the C terminus of MyD88 (13). Two kinases, IL-1R-associated kinase (IRAK) 1 and 2, are subsequently recruited, and the signaling cascade further progresses through the TNFR-associated factor 6 (TRAF6), the mitogen-activated protein kinase kinase kinase TGF-β-activated kinase 1, and the NF-κB-inducing kinase (NIK). NIK, and possibly TGF-β-activated kinase 1, is part of the inhibitor of NF-κB (IκB) kinase complex that additionally contains the two IκB kinases IKK-α and IKK-β as well as NF-κB in association with IκB; these may be held together by the scaffold proteins IκK-γ and IκK complex-associated protein (13–21). After the IκB in the IκK complex is phosphorylated, it becomes degraded by the ubiquitin-proteasome pathway, and NF-κB can then translocate to the nucleus where it activates transcription by binding to specific binding sites in the promoters of numerous genes (see Ref. 12 for references). Several kinases are activated by IL-1, and the involvement of a small G protein in IL-1 signaling has been suggested. However, the mechanism whereby these components interact with the above cascade has not been determined (for review, see Ref. 12). Phosphatidylinositol 3-kinase appears to associate with the cytoplasmic region of IL-1RAcP and to initiate a parallel signaling pathway leading to phosphorylation of NF-κB subunits, an event essential for full activation of the transcription factor (22).
The IL-1RAcP is structurally very similar to IL-1RI and IL-1RII and shares ~25% sequence identity with each (6). Its extracellular domain of 340 amino acid residues is divided into three Ig domains and the transmembrane and intracellular domains are 29 and 181 amino acid residues, respectively. IL-1RAcP was identified in 1995 as a component that is needed for the IL-1R to be fully functional (6). Many studies have since confirmed that the IL-1RAcP is indeed essential for IL-1-mediated responses such as activation of IRAK, NF-kB, Jun N-terminal kinase, and acid sphingomyelinase and for receptor/IL-1 complex internalization (23–26). However, the exact mechanism whereby IL-1RAcP contributes to the function of the IL-1 binding complex has remained obscure. There have been conflicting reports of the effect of IL-1RAcP on the binding kinetics and dissociation constants of IL-1α and IL-1β (6, 25, 27, 28). The C terminus of MyD88 has sequence similarity to the intracellular domain of IL-1RAcP and somewhat weaker similarity to that of IL-1RI. When MyD88 is overexpressed, it can be coimmunoprecipitated with IL-1RAcP, but not with IL-1RI (13). Additionally, IRAK1 interacts with IL-1RAcP, whereas IRAK2 preferentially coimmunoprecipitates with IL-1RI; the latter may interact indirectly with the IL-1RAcP via a death domain (protein-protein interaction domain described initially for proteins involved in apoptosis) that is present in the N termini of both IRAK2 and MyD88 (13, 29, 30). The reports documenting the above suggest that the intracellular domain of IL-1RAcP is essential for signaling from the IL-1 binding complex, and indeed a deletion mutant of IL-1RAcP lacking the majority of the intracellular domain fails to recruit IRAK1 to the IL-1 binding complex and activate NF-kB (29).

In mice, the hepatic form of IL-1RAcP mRNA is only ~1.8 kb compared with ~5.3 kb in all other tissues analyzed, a size difference that has been attributed to alternative splicing (6). The mouse hepatic IL-1RAcP lacks the membrane and intracellular domains of the membrane-bound form and is presumably secreted. This soluble IL-1RAcP (sIL-1RAcP) has so far only been described in mouse liver, and its function has not been determined (6). However, it seems unlikely that sIL-1RAcP would be able to participate in signal transduction from the IL-1 binding complex because it lacks the intracellular domain. This raises the question of how signal transduction takes place in hepatocytes. To address this we have cloned the human homologue of the hepatic sIL-1RAcP and investigated its relationship to the IL-1RAcP gene structure, its function in IL-1-mediated NF-kB activation, and its expression during stress responses.

Materials and Methods
Cloning of the sIL-1RAcP
A human acute-phase liver cDNA library (31) was screened using the oligonucleotide 5’-AGTAGATCCTCTGAAAGGCC-3’ (from human membrane-bound IL-1RAcP (mIL-1RAcP); GenBank accession no. AB006537, position 477–460 in Fig. 1A), and a partial cDNA clone encoding the C-terminal 218 amino acid residues and the 767-bp 3’ untranslated region (UTR) of sIL-1RAcP was identified. The remaining sequence corresponding to the 138 N-terminal amino acids of sIL-1RAcP was obtained by RT-PCR using a forward primer (5’-CAAGGGATGACACTTGGCCT-3’) comprising six nucleotides of the Kozak sequence and 15 nucleotides -CAAAGGATGACACTTCTGTGG- of the coding region derived from GenBank accession no. AB006537 and a reverse primer (5’-GACGCGCATCTATTACCTTTC-3’) specific for the soluble form (position 1066–1047 in Fig. 1A). Genomic DNA from HepG2 cells, isolated by conventional methods, and plasmid DNA containing mIL-1RAcP cDNA were used as negative controls to validate the specific amplification of sIL-1RAcP. The PCR product encoding the N-terminal region of sIL-1RAcP was ligated to the sIL-1RAcP-specific C-terminal region of a unique internal Sun I restriction site.

Sequencing of the IL-1RAcP gene
The choice of primers dispersed throughout the cDNA was based on sequence in GenBank accession no. AB006537. Gene fragments were amplified using Expand Long Template, Expand High Fidelity PCR systems (Boehringer Mannheim, Indianapolis, IN) or Human GenomeWalker Kit (Clontech Laboratories, Palo Alto, CA) according to the manufacturer’s instructions. Human genomic DNA (Boehringer Mannheim) or DNA from HepG2 cells were used as templates for long-template PCR. PCR products were gel purified (Qiagen, Santa Clarita, CA) and sequenced. All new sequences reported here and deposited in the GenBank database were sequenced in both directions. Sequence was obtained for the entire available cDNA.

Characterization of sIL-1RAcP
The Rous sarcoma virus (RSV) promoter was amplified by PCR (32) and cloned into the Renilla-luciferase pRL-null vector (Promega, Madison, WI). The deletion mutants IRAK1(1–217) of IRAK1 and TRAF6 (289–529) of TRAF6 were generated by RT-PCR from total RNA isolated from epithelial KB cells and were cloned into the expression vector pCRI-neo (Promega). Primers for IRAK1(1–217) 5’-GGCGCGAGACATGCGCGGGGGG-3’ (forward) and 5’-ATCTTGGACTCTCCCGAGAATGGTGGG-3’ (reverse) were derived from GenBank accession no. L76191. Primers for TRAF6 (289–529) 5’-ATCTCAGAGGTCCCCGAAATTTCC-3’ (forward) and 5’-GTATACCCCTGACTGACACCC-3’ (reverse) were derived from GenBank accession no. U78798. A membrane-bound deletion mutant of mIL-1RAcP (mIL-1RAcP) was generated from an existing construct containing mIL-1RAcP cDNA by PCR using the primer pair 5’-TCAGAAACCGCTGCGGATGACTGG-3’ (forward) and 5’-TCAGACTAATACACATTTCCCTTCC-3’ (reverse) and was cloned into the pFlag-CMV-1 expression vector (Kodak, New Haven, CT). The coding regions for pro-sIL-1RAcP and mature sIL-1RAcP were subcloned into pcI-neo and pFlag-CMV-1, respectively. The fusion protein sIL-1RAcP-MHC was generated by RT-PCR amplification of the conserved transmembrane and intracellular domains (74 C-terminal amino acid residues and stop codon) of MHC class II (GenBank accession no. AF016641) with the forward primer 5’-GGGCTGCGGAAAGCCTCCT-3’ and the reverse primer 5’-TCAAGCTGTGGAGAACACATCAG-3’ and by insertion of the product in frame after the coding region of sIL-1RAcP. A control plasmid was generated by cloning the above MHC product into empty pFlag-CMV-1. The nucleotide sequences of cloned constructs were verified by sequencing. The NF-kB-luciferase reporter construct containing the E-selectin promoter (−730) and the expression constructs specifying Flag-Ag-tagged IL-1RI, Flag-IL-1RAcP, MyD88(152–296), IRAK2(97–590), and NIK(KK429–430AA) are described elsewhere (13).

Cell lines and transfections
Human hepatoma cells (HepG2) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM with 25 mM HEPES and glutamin-1 (L-alanyl-L-glutamine) supplemented with 10% (v/v) FCS, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 50 µg/ml gentamicin (Life Technologies, Grand Island, NY). Cells were grown to ~50% confluence and transfected with 2–3 µg total DNA using the calcium phosphate precipitation method. In experiments in which cells were transfected with varying amounts of expression vectors, the total DNA used in each transfection was held constant by cotransferring appropriate amounts of empty vector. Cells were allowed to recover in fresh medium for 24 h, after which they were treated with medium (control), IL-1β (10 ng/ml; National Cancer Institute, Frederick, MD), or TNF-α (150 ng/ml; Zeneca Pharmaceuticals, Macclesfield, U.K.) for 6 h. Cells were lysed and lysates were assayed for luciferase and Renilla luciferase activity according to the manufacturer’s instructions (Dual-Luciferase Reporter Assay System, Promega). Before RNA extraction, cells were treated with IL-1β, TNF-α, and the phorbol esters PMA and phorbol 12,13-diecanoate (PDD) (Sigma, St. Louis, MO).

Northern blotting and quantitative RT-PCR
Isolation of total RNA, Northern blotting, and RT-PCR were performed as described elsewhere (33). Reverse transcription of 1 µg total RNA was performed at 50°C using avian myeloblastosis virus reverse transcriptase and oligo(dt)15 primer (Promega) and AMV-RTase mRNA inhibitor (Am- bion, Austin, TX). Quantitative PCR was performed as described elsewhere (33). A 357-bp product was amplified from sIL-1RAcP mRNA with the forward primer 5’-GATGGATGCTCTGGCAATGAGG-3’ and the reverse primer QRS2 5’-ACTATGGGTTAGATGCCGGTCTC-3’. A 305-bp
A product was amplified from mIL-1RAcP with the QF primer and the reverse primer QRM (5' - TGAGAATCACCACTAGCAGG - 3'). PCR products were analyzed on SYBR Green I-stained (Molecular Probes, Eugene, Oregon) agarose gels using STORM and ImageQuant technology (Molecular Dynamics, Sunnyvale, CA).

**Results**

**Cloning of sIL-1RaCp**

A human acute-phase liver cDNA library (31) was screened with the oligonucleotide 5' -AGTGATCCTCTGAATGCC-3', which corresponds to antisense sequence in the extracellular domain of the published sequence of mIL-1RaCp. A clone (L1), which shares sequence identity with human mIL-1RaCp (414–1051 in Fig. 1A) at its 5' end but which has a unique 3' end of 806 nucleotides, was identified (from 1052 in Fig. 1A). The 3' end contains a polyadenylation site at position 1791–1796 (Fig. 1A) and also includes 22 residues of the poly(A)-tail, suggesting that this clone represents a uniquely expressed mRNA. A potential alternative splice site is present at position 1050–1054, where the sequence of L1 diverges from that of mIL-1RaCp; this marks the region where the sIL-1RaCp diverges from the membrane-bound form.

A RT-PCR strategy was applied to obtain cDNA sequence corresponding to the N terminus of sIL-1RaCp. A forward primer was designed for the six nucleotides upstream of the translation start site and the first 15 nucleotides of the coding region of the mIL-1RaCp. A reverse primer was designed across the potential alternative splice site at position 1066–1047 in Fig. 1A. A single RT-PCR product of ~1 kb was obtained from HepG2 total RNA.

**FIGURE 1.** Sequence of human sIL-1RaCp and alignment with mouse sIL-1RaCp and human mIL-1RaCp. Nucleotide sequence of human sIL-1RaCp cDNA. The alternative splice site is underlined at position 1050–1053. The polyadenylation site is dotted underlined at position 1791–1796 and the poly(A) tail is double underlined at position 1836–1857. B. The polypeptide sequence of human sIL-1RaCp is aligned with mouse sIL-1RaCp. Vertical lines indicate sequence identity, whereas single or double dots indicate low or high similarity, respectively. The last two and the first 39 aa (including the putative transmembrane domain) after the alternative splice site of human mIL-1RaCp are shown below the sequence of the sIL-1RaCp.
verify that this product could only be derived from sIL-1RACP mRNA, control amplification reactions using either HepG2 genomic DNA or a plasmid containing the mIL-1RACP cDNA were included. These latter PCR reactions did not give any products (not shown). The specific PCR product was fused with the clone isolated from the cDNA library at an internal Sun I restriction site and was cloned into the expression vector pCI-Neo.

The first 350 amino acid residues of the soluble and membrane-bound forms of human IL-1RACP are identical. Clone L1, isolated from the cDNA library, revealed that sIL-1RACP has an additional six residues C-terminal to the common region, giving a full-length polypeptide of 336 amino acid residues. In comparison to this, mIL-1RACP polypeptide of 356 including the signal peptide and a mature protein of 336 amino acid residues. An intron of ~15 kb separates the sIL-1RACP 3′ UTR from the exon encoding the transmembrane domain (exon 10; Fig. 2) and Table I) of mIL-1RACP. The intracellular domain of mIL-1RACP is encoded by two exons (exons 11 and 12; Fig. 2 and Table I), of which the second also encodes the 3′ UTR of mIL-1RACP. All introns follow the GT-AG rule. One sequence discrepancy, an insertion of TA, relative to the cDNA sequence (AB006537) was found at position 2336. The boundaries of exon 5 are in agreement with those previously submitted to GenBank (accession no. AF016261) in connection with mapping of the IL-1RACP gene to chromosome 3 (34).

**Structure of the IL-1RACP gene and alternative splice sites**

It has been suggested that the soluble form of IL-1RACP could arise from alternative splicing (6). To investigate this possibility we designed a forward PCR primer specific for the 3′ end of the common region of the two forms of IL-1RACP and two forward primers specific for the 3′ ends of the 3′ UTR of each. Reverse primers specific for each of the membrane and soluble 5′ ends were designed.

Using human genomic DNA as template, an ~17-kb PCR product (gAcP25) was amplified using the sIL-1RACP 3′ UTR forward primer in combination with the mIL-1RACP 5′ reverse primer. Sequencing of gAcP25 confirmed the presence of additional 5′ mIL-1RACP-specific sequence and revealed the presence of a 1301-bp intron that divides this sequence into two exons (Fig. 2). Intronic sequence (~15 kb) was found upstream of the mIL-1RACP and downstream of the sIL-1RACP-specific sequences. An ~2.5-kb PCR product (gAcP4) was obtained using the common forward primer with a reverse primer specific for the sIL-1RACP 3′ UTR; this yielded the entire sIL-1RACP-specific sequence contiguous with the common region (Fig. 2). An intron of 1.9 kb was identified in the common region amplified in gAcP4.

Primers distributed throughout the full-length mIL-1RACP cDNA sequence (GenBank accession no. AB006537; a complete list of primers may be obtained from the authors upon request) were used to define the remaining exon/intron boundaries. In two regions (5′ UTR and signal peptide-mature protein encoding regions), no products could be amplified using conventional PCR with two gene-specific primers. In these regions, a genome walker strategy from Clontech Laboratories was applied. Sequences covering the entire cDNA sequence were obtained from the resulting PCR products, and 12 exons spanning over 56 kb were identified. The 5′ UTR is specified by exons 1 and 2 (Fig. 2 and Table I), and the signal peptide is encoded by exon 3. The first Ig domain is encoded by a single exon (exon 4; Fig. 2 and Table I). Ig domains 2 and 3 are each encoded by two exons (exons 5 and 6 and exons 8 and 9, respectively; Fig. 2 and Table I) and are separated by a small additional exon (exon 7; Fig. 2 and Table I) of 72 bp. The exon encoding the second half of Ig domain 3 is followed by the exon encoding the C-terminal region and 3′ UTR of sIL-1RACP. An intron of ~15 kb separates the sIL-1RACP 3′ UTR from the exon encoding the transmembrane domain (exon 10; Fig. 2 and Table I) of mIL-1RACP. All introns follow the GT-AG rule. One sequence discrepancy, an insertion of TA, relative to the cDNA sequence (AB006537) was found at position 2336. The boundaries of exon 5 are in agreement with those previously submitted to GenBank (accession no. AF016261) in connection with mapping of the IL-1RACP gene to chromosome 3 (34).

**Engagement of signaling cascade components downstream of the IL-1 binding complex**

To determine which signaling pathway is utilized to transduce signal from the IL-1R and activate NF-κB in human HepG2 cells, we overexpressed dominant-negative mutants of early components in the IL-1 signaling pathway and measured the activity of NF-κB after treatment with IL-1β and TNF-α using a NF-κB-luciferase reporter construct. TNF-α was used as a control to ensure that effects were specific to the IL-1 signaling pathway. The TNF and IL-1 signaling pathways leading to NF-κB activation are initially distinct but converge at NIK, i.e., NIK is activated by both IL-1 and TNF, whereas the earlier components analyzed in this study should be specific to the IL-1 signaling cascade. Cells transfected with empty vector together with the NF-κB-luciferase reporter and RSV-Renilla construct exhibited a strong NF-κB luciferase activity as measured by the NF-κB reporter construct when treated with IL-1β compared with cells treated with medium only (15- to 150-fold induction). Treatment of similarly transfected cells with TNF-α resulted in NF-κB activities of ~50% of those achieved by IL-1β treatment. The significance of results was assessed using standard t test.

Co-transfection of cells with a construct encoding a kinase-inactive NIK, NIK(KK429–430AA), together with the NF-κB reporter and RSV-Renilla constructs resulted in changed IL-1β and TNF-α responses (Fig. 3A) that were both dependent on the concentration of the NIK inactive construct. At the lowest concentrations (0.01 μg/treatment) an increase in IL-1β-induced NF-κB activity was
detected in two of three independent experiments. The significance of this observation is unknown. At the highest concentration (1.25 μg/transfection), the IL-1β- and TNF-α-induced NF-κB activities were reduced to ~35% (p < 0.01) and 30% (p < 0.001), respectively, relative to that in control cells transfected with empty vector.

A dramatic but graded reduction of the IL-1β response from ~60% (p < 0.001) of the full response in control cells (Fig. 3B) to ~25% (p < 0.001) at the lowest and highest concentrations of cotransfected DNA, respectively, was achieved by overexpression of a deletion mutant of TRAF6, TRAF6(289–522), which lacks a RING finger and five potential zinc-finger motifs that are believed to be involved in protein-protein interactions with other members (most probably NIK) of the signaling cascade (14). The TNF-α-induced response remained virtually unchanged (Fig. 3B). This suggests that both NIK and TRAF6 are involved in the IL-1 signaling cascade in HepG2 cells.

Components recruited to the IL-1 binding complex

To investigate whether the IL-1 binding complex functionally requires MyD88 and the IRAK1 and IRAK2 kinases as previously reported, we overexpressed dominant-negative deletion mutants of these proteins. A deletion mutant of MyD88, MyD88(152–296), which lacks the death domain, reduced the IL-1β-induced NF-κB response to ~25% (p < 0.001; at highest concentrations of 1.25 μg/transfection) of that in control cells (Fig. 4A) in a specific and concentration-dependent manner. In contrast, the TNF-α-induced response remained constant. This indicates that MyD88 is involved in IL-1β-mediated signaling leading to NF-κB activation but not in that mediated by TNF-α.

A reduction in readout was achieved by cotransfecting as little as 2 ng/transfection (Fig. 4B) of a deletion mutant of IRAK1 lacking the kinase domain (IRAK1(1–217)). NF-κB activity could be further reduced using higher amounts of this construct, up to 250 ng/transfection, at which the IL-1β-induced response was only ~20% (p < 0.001) of that in control cells. No inhibition of the TNF-α-induced response is apparent (Fig. 4B), suggesting that, as with MyD88, IRAK1 is only involved in the IL-1β-mediated NF-κB activation and not in that mediated by TNF-α. A deletion mutant of IRAK2 (IRAK2(97–590)) lacking the death domain could also specifically, but to a lesser extent (reduction to ~30% at highest concentrations of 1.25 μg/transfection) of that in control cells. No inhibition of the TNF-α-induced response is apparent (Fig. 4B), suggesting that, as with MyD88, IRAK1 is only involved in the IL-1β-mediated NF-κB activation and not in that mediated by TNF-α. A deletion mutant of IRAK2 (IRAK2(97–590)) lacking the death domain could also specifically, but to a lesser extent (reduction to ~30% at highest concentrations of 1.25 μg/transfection) of that in control cells. No inhibition of the TNF-α-induced response is apparent (Fig. 4B), suggesting that, as with MyD88, IRAK1 is only involved in the IL-1β-mediated NF-κB activation and not in that mediated by TNF-α.

Taken together, these results suggest that the “signaling complex” recruited to the IL-1 binding complex is assembled as previously described (13, 15).

Components in the IL-1 binding complex

We further analyzed the effects of overexpressing components in the IL-1 binding complex itself. The cells used in these studies were treated with IL-1β or TNF-α at concentrations that would produce an optimal response (10 ng/ml and 50 ng/ml, respectively) or were treated with a lower concentration of IL-1β or TNF-α, which lacks the death domain, reduced the IL-1β-induced NF-κB response to ~25% (p < 0.001; at highest concentrations of 1.25 μg/transfection) of that in control cells (Fig. 4A) in a specific and concentration-dependent manner. In contrast, the TNF-α-induced response remained constant. This indicates that MyD88 is involved in IL-1β-mediated signaling leading to NF-κB activation but not in that mediated by TNF-α.

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shown). This suggests that it is the number of IL-1RI molecules, and not IL-1RAcP, on the cell membrane that determines the magnitude of the cellular response to IL-1.

Initial experiments using cells transfected with sIL-1RAcP expression constructs did not show any effects that could be attributed to secreted sIL-1RAcP. We speculated that this could be due to dilution of the soluble protein in the tissue culture medium to a low concentration at which its biological activity would be below detectable limits. Therefore, to increase its local concentration at the cell surface we fused the sIL-1RAcP to the transmembrane and intracellular portion of MHC class I, which is conserved in MHC I molecules. When a construct encoding sIL-1RAcP-MHC was cotransfected into cells, a significant concentration-dependent decrease in the response to IL-1β was observed (Fig. 5B). At the highest amounts of transfected sIL-1RAcP-MHC used (1.25 μg DNA/transfection), the responses to suboptimal and optimal amounts of IL-1β were reduced to 5\% (p < 0.001) and 10\% (p < 0.01), respectively, relative to controls. No significant change in the response to TNF-α was observed (Fig. 5B). Cotransfection of a deletion mutant of mIL-1RAcP (IL-1RAcP(1–403)) lacking the entire intracellular domain gave similar results (not shown). Furthermore, cotransfection of a control plasmid, CMV-Flag-MHC, expressing only the MHC domain of the sIL-1RAcP-MHC fusion protein had no such effect (not shown). When cells are cotransfected with both IL-1RI and mIL-1RAcP, a spontaneous activation of NF-κB can be observed in cells treated with medium only (p < 0.001; Fig. 5C). This spontaneous activation has been used in some previous studies of the IL-1 signaling cascade (13) as the model system of IL-1R complex activation. However, such spontaneous activation does not take place when cells are cotransfected with IL-1RI and sIL-1RAcP-MHC (not shown). When cells are cotransfected with a fixed amount of construct encoding sIL-1RAcP-MHC and increasing amounts of IL-1RI construct, a pattern somewhat similar to the one observed
when cells are transfected with the IL-1RI construct alone is seen. In cells transfected with 250 ng of the sIL-1RAcP expression construct, transfection of 2 ng of IL-1RI expression construct is sufficient to bring NF-κB activation after treatment with both suboptimal and optimal amounts of IL-1β back to the level of that observed in control cells transfected with the empty vector (Fig. 5D). However, it is not sufficient to cause the shift of “suboptimal-to-optimal” level of activation seen when the cells are transfected with the IL-1RI construct alone. Transfection of 10 ng IL-1RI construct into cells cotransfected with the sIL-1RAcP-MHC construct results in a 2-fold increase in response to the suboptimal amount of IL-1β compared with control cells transfected with the empty vector (Fig. 5D). This is 100 times more than is needed to cause the same change in the IL-1β response of cells transfected with the IL-1RI construct only. Similar results were obtained when increasing amounts of the mIL-1RAcP construct were cotransfected with a fixed amount of sIL-1RAcP-MHC (not shown).

These results suggest that it is the number of available IL-1RI molecules that determines cellular sensitivity to IL-1β and that sIL-1RAcP may act as an inhibitor of IL-1β by rendering the IL-1RI nonfunctional in a signaling context, although it is probably still able to bind IL-1β.

Expression of membrane and soluble IL-1RAcP mRNA in human hepatoma cells and acute-phase liver

Experiments using conventional Northern blot analysis to determine which form of the IL-1RAcP is expressed in hepatoma cells were not sensitive enough to detect any mRNA. Therefore, we developed a quantitative semicompetitive RT-PCR in which sIL-1RAcP and mIL-1RAcP cDNA products can be amplified using the same forward primer in combination with reverse primers specific for each form; this approach generates 357-bp and 305-bp PCR products, respectively. The first 233 bp of the two PCR products are identical and the relative amounts may be assessed by amplifying both sIL-1RAcP and mIL-1RAcP in the same reaction. To confirm that an accurate determination of the ratio of the two splice variants can be established using this method, plasmids encoding sIL-1RAcP and mIL-1RAcP were mixed in different ratios. Aliquots were removed after different numbers of amplification cycles and were analyzed on SYBR Green-stained 2.5% agarose gels. The ratios of sIL-1RAcP product to mIL-1RAcP product remained unchanged during both the exponential and plateau phases of amplification (not shown).

We then analyzed RNA samples extracted from HepG2 hepatoma cells after treatment with the phorbol esters PDD and PMA for 1–96 h using the above quantitative PCR method. In cells treated with medium only (0 h; Fig. 6), mIL-1RAcP mRNA constituted approximately two-thirds of the total amount of IL-1RAcP mRNA; PDD elicited a gradual change in the ratio of the two alternative splice variants over time: after 12 h of incubation with PDD, mIL-1RAcP and sIL-1RAcP cDNAs were present in equal amounts, whereas after 24 and 36 h, the sIL-1RAcP splice form had become the dominant species (Fig. 6). After 72 h, at which time the cells still appeared healthy, the ratio showed a reversal in

![Figure 5. Effects of overexpressing components in the IL-1 binding complex. HepG2 cells were cotransfected and relative luciferase activity was represented as described in Fig. 3. Transfected cells were treated with medium (control, dotted bars, not visible), 0.1 ng/ml IL-1β (filled bars), 10 ng/ml IL-1β (open bars), or 50 ng/ml TNF-α (grey bars). A, Cells transfected with a full-length and functional IL-1RI showed an increased sensitivity to IL-1β with the suboptimal concentration of 0.1 ng/ml IL-1β after transfection with increasing amounts of IL-1RI construct (filled bars). B, The fusion protein sIL-1RAcP-MHC inhibits the IL-1β-induced NF-κB activation in a concentration-dependent manner. C, Introduction of mIL-1RAcP into cells transfected with 150 ng IL-1RI (bars labeled with “+”) causes concentration-dependent spontaneous activation of NF-κB in cells treated with medium only (dotted bars). D, Introduction of IL-1RI into cells transfected with 250 ng sIL-1RAcP-MHC (bars labeled with “+”) reestablishes the sensitivity of the cells to IL-1β in a concentration-dependent manner.](http://www.jimmunol.org/content/159/11/5283/F5.large.jpg)
Increasing the cell surface expression of IL-1RI would shift the level of membrane expression of IL-1RI or of IL-1RAcP that is necessary for IL-1-induced signaling from the IL-1RI leading to activation of NF-κB, but the mechanism whereby it contributes to this process is still largely unknown. In addition to the membrane-bound form of IL-1RAcP, a soluble form of the protein has also been identified (6). The sIL-1RAcP has so far only been identified in mouse liver (6), mouse T cell lymphomas (25), and rat brain (35), and its function has remained obscure. We have cloned the gene encoding mouse sIL-1RAcP together causes a "spontaneous activation" of the IL-1 receptor signal transduction cascade. In the first model, IL-1RI and IL-1RAcP become associated only after IL-1 binds to IL-1RI and may involve a permissive conformational change that facilitates recruitment of IL-1RAcP to the IL-1RI. We have demonstrated that overexpression of a membrane-anchored form of sIL-1RAcP that lacks the entire intracellular domain can also inhibit IL-1-mediated signaling. This establishes that extracellular interactions between IL-1RAcP and IL-1RI are sufficient to mediate the association of the two proteins, leading to measurable biological effect. The possibility that there are additional interactions between the transmembrane and intracellular domains is not formally excluded; however, such interactions, if they do occur, are not necessary for the biological effects reported here.

There are currently two models for the interaction among IL-1RI, IL-1RAcP, and IL-1. In the first model, IL-1RI and IL-1RAcP are already associated and the subsequent binding of IL-1 results in a conformational change in IL-1RI that initiates the downstream signaling cascade. In the second model, IL-1RI and IL-1RAcP become associated only after IL-1 binds to IL-1RI and may involve a permissive conformational change that facilitates recruitment of IL-1RAcP or downstream signaling components. However, the observation that overexpression of IL-1RI and mIL-1RAcP together causes a "spontaneous activation" of the IL-1 signaling cascade (reported in this paper and elsewhere (13)) suggests that such an IL-1-mediated conformational change is not essential for activation of the signaling cascade and provides good evidence in favor of the second model. Under this model, IL-1RI and mIL-1RAcP will only rarely come together by chance because they are normally expressed at low levels and spontaneous activation, therefore, does not occur; however, when both proteins are overexpressed, random association and spontaneous activation becomes more likely. In nontransfected cells with normal levels of membrane components, IL-1 may promote an increased affinity between IL-1RI and mIL-1RAcP to produce a complex that is sufficiently stable to allow the recruitment of additional intracellular signaling components.

Only a limited number of studies of IL-1RAcP expression has been published. Bacterial LPS causes a moderate up-regulation of mIL-1RAcP mRNA in human monocytes (38), and induction of balance toward more IL-1/IL-1RI complexes and would result in a higher level of NF-κB activation, which is in accordance with our observations. Because the increased expression of mIL-1RAcP reported here had no influence on the efficiency of signal transduction, it is likely that the level of IL-1RI is the variable that controls the response to IL-1. Our observations that expression of a membrane-anchored form of sIL-1RAcP results in almost 100% inhibition of the NF-κB activation that can be induced by IL-1 at physiological concentrations and that this inhibition can be reversed/prevented by coexpression of IL-1RI suggest that IL-1RI is regulated at the membrane by sIL-1RAcP such that, although all of the IL-1RI molecules are capable of binding IL-1, only a limited number are actually able to mediate signal transduction. This interaction constitutes an additional layer of receptor competition. Given that we have only been able to achieve an effect on the level of NF-κB activation with the membrane-anchored form of sIL-1RAcP, we cannot exclude the possibility that the native protein may have an additional function. A component downstream of the IL-1 binding complex might be an additional limiting factor because NF-κB activation is clearly saturated at optimal concentrations of IL-1. However, we do not believe that this is physiologically significant because the optimal concentration of IL-1 in cell culture experiments is probably much higher than that achieved in vivo.

It is not known precisely how IL-1RAcP interacts with the IL-1RI. We have demonstrated that overexpression of a membrane-anchored form of sIL-1RAcP in which both the transmembrane and small intracellular domains are unrelated to the equivalent domains in mIL-1RAcP results in specific inhibition of the IL-1 signaling cascade. We have also observed that a deletion mutant of IL-1RAcP that lacks the entire intracellular domain can also inhibit IL-1-mediated signaling. This establishes that extracellular interactions between IL-1RAcP and IL-1RI are sufficient to mediate the association of the two proteins, leading to measurable biological effect. The possibility that there are additional interactions between the transmembrane and intracellular domains is not formally excluded; however, such interactions, if they do occur, are not necessary for the biological effects reported here.

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**Discussion**

mIL-1RAcP is an accessory protein to the IL-1R that has recently been identified (6). It has been established that mIL-1RAcP is necessary for IL-1-induced signaling from the IL-1RI leading to activation of NF-κB, but the mechanism whereby it contributes to this process is still largely unknown. In addition to the membrane-bound form of IL-1RAcP, a soluble form of the protein has also been identified (6). The sIL-1RAcP has so far only been identified in mouse liver (6), mouse T cell lymphomas (25), and rat brain (35), and its function has remained obscure. We have cloned the human sIL-1RAcP and demonstrated that it arises from alternative splicing. The relative proportion of alternatively spliced forms of IL-1RAcP mRNA in HepG2 cells changes in response to stress and/or acute-phase induction and becomes biased toward sIL-1RAcP such that, although all of the IL-1RI molecules are capable of binding IL-1, only a limited number are actually able to mediate signal transduction. This interaction constitutes an additional layer of receptor competition. Given that we have only been able to achieve an effect on the level of NF-κB activation with the membrane-anchored form of sIL-1RAcP, we cannot exclude the possibility that the native protein may have an additional function. A component downstream of the IL-1 binding complex might be an additional limiting factor because NF-κB activation is clearly saturated at optimal concentrations of IL-1. However, we do not believe that this is physiologically significant because the optimal concentration of IL-1 in cell culture experiments is probably much higher than that achieved in vivo.

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![FIGURE 6. Expression of mIL-1RAcP and sIL-1RAcP mRNA in response to phorbol esters. After reverse transcription with oligo(dT), the relative abundance of mIL-1RAcP and sIL-1RAcP cDNAs was determined using a semicompetitive quantitative approach in which mIL-1RAcP and sIL-1RAcP cDNAs were coamplified using common forward and isoform-specific reverse primers. The PCR products generated from sIL-1RAcP and mIL-1RAcP cDNAs are 357 bp and 305 bp, respectively. HepG2 cells were treated with 50 ng/ml of the phorbol ester PDD, and RNA was harvested after various time points between 1 and 72 h. Top panel, SYBR Green-stained agarose gel of duplicate RNA samples for each time point. Lane marked “M” represents size markers of 300 and 400 bp, respectively. Lane marked “N” represents negative control for reverse transcription and PCR. Time points are indicated above the respective duplicate lanes. Graph shows the relative abundance of the two IL-1RAcP mRNA species as determined using STORM and ImageQuant technology (Molecular Dynamics). sIL-1RAcP and mIL-1RAcP are shown with filled symbols and dashed line and open symbols and solid line, respectively.](http://www.jimmunol.org/)
mIL-1RαCp mRNA has been demonstrated in lung, spleen, and thymus tissue from mice injected with IL-1 (6). High levels of constitutive mIL-1RαCp expression have been demonstrated in mouse brain tissue (6); however, another group has reported that in rats, IL-1 causes an up-regulation in the expression of both sIL-1RαCp and mIL-1RαCp in brain tissue (see Ref. 35 and its references). In this latter study, no significant change in the ratio of mIL-1RαCp to sIL-1RαCp mRNA was reported. Expression of mouse liver sIL-1RαCp mRNA has previously been shown to be constitutive (6). Our results establish that both mIL-1RαCp and mouse liver sIL-1RαCp mRNA has previously been shown to be constitutive mIL-1RαCp expression have been demonstrated in brain tissue (see Ref. 35 and its references). In this latter study, no significant change in the ratio of mIL-1RαCp to sIL-1RαCp mRNA was reported. Expression of mouse liver sIL-1RαCp mRNA has previously been shown to be constitutive (6). Our results establish that both mIL-1RαCp and mouse liver sIL-1RαCp mRNAs, generated by alternative splicing, are expressed in human HepG2 cells; however, in untreated cells, the ratio of mIL-1RαCp mRNA to sIL-1RαCp mRNA is ~2:1. The ratio of these alternatively spliced mRNAs changes significantly after treatment with inflammatory mediators. The phorbol esters PMA and PDD, wide spectrum inducers of nitric oxide synthase, and protein kinase C activation (39, 40) induce an early response (3–36 h) in which the ratio becomes biased toward sIL-1RαCp mRNA. Factors that control tissue and developmentally specific alternative splicing have previously been identified (see Ref. 41 for references), and such a factor may direct the alternative splicing of IL-1RαCp pre-mRNA in liver cells; if so, the activity of the factor could be controlled by extracellular stimuli. Another mechanism whereby the ratio of the two IL-1RαCp mRNA species may be altered is differential stability. The 3′ UTRs of the sIL-1RαCp and mIL-1RαCp mRNAs, respectively, contain two and 14 AUUUA sequence motifs (L. E. Jensen, unpublished observation) that are common in mRNAs of components of the inflammatory response and have previously been shown to confer instability (42, 43). Therefore, the mIL-1RαCp mRNA may be significantly less stable than sIL-1RαCp mRNA during the acute-phase response; rapid degradation of mIL-1RαCp mRNA would yield an IL-1RαCp mRNA profile that is dominated by sIL-1RαCp mRNA. IL-1 is a proinflammatory cytokine involved in the early stages of inflammation and the acute-phase response (1). Extracellular signals received by liver cells during the acute-phase response, analogous to those we generated by the phorbol esters PMA and PDD, may change the bias in alternative splicing of the IL-1RαCp pre-mRNA to permit the sIL-1RαCp mRNA to become predominant, thereby limiting the cellular response to IL-1.

The IL-1RI and IL-1RII genes are believed to be derived from a common ancestor because the positions of introns relative to sequence encoding the extracellular regions are almost identical (44). The IL-1RαCp gene structure resembles that of IL-1RI in that (1) two exons specify the 5′ UTR, 2) the signal peptide is encoded by one exon, and 3) the extracellular, transmembrane, and intracellular regions are encoded by six, one, and two exons, respectively. In addition, the second intracellular exon also encodes the 3′ UTR. Introns are positioned at similar positions within the two genes. The genomic arrangement of the IL-1RII gene is consequently very similar but distinguishes itself by not having an intron between the 5′ UTR and signal peptide-encoding regions (44). The IL-1RI gene distinguishes itself by having a shorter exon 4 encoding the N terminus of the mature protein than the equivalent exons 4 and 3, respectively, in the IL-1RαCp and IL-1RII genes do. Unlike the IL-1RI and IL-1RII genes, which are both located on chromosome (45), the IL-1RαCp gene maps to chromosome 3 (34). All of the above suggest that the IL-1RI, IL-1RII, and IL-1RαCp genes have evolved from a common ancestor and have adopted features required to mediate different functions.

From the IL-1RαCp gene structure we have been able to determine that the two forms of IL-1RαCp arise from alternative splicing and that the sIL-1RαCp mRNA is derived from the first nine exons, whereas mIL-1RαCp mRNA arises from splicing of exon 10 to exon 9 at an internal splice site in exon 9 (Table 1). This internal splice site is seven nucleotides further downstream of the one suggested by Greenfeder et al. (Ref. 6 and Fig. 1B). A splice site similar to the one identified by us in humans is also present in the mouse mRNA sequence and complete amino acid sequence have never been published, and it is therefore not possible to determine whether different splice sites for the mIL-1RαCp are used in mice and humans.

In this paper we have reported the cloning of the human sIL-1RαCp and have characterized the IL-1RαCp gene that encodes the two alternatively spliced forms (sIL-1RαCp and mIL-1RαCp). In addition, we have shown that a membrane-anchored form of sIL-1RαCp inhibits IL-1-mediated cell activation and that the splicing pattern of the IL-1RαCp pre-mRNA changes in response to stress. This suggests that IL-1RαCp is a very important factor in controlling the IL-1 response. Future work on the biological activity of the native sIL-1RαCp and characterization of the tissue-specific expression of mIL-1RαCp and sIL-1RαCp and the mechanism whereby the above switch is triggered during the acute-phase response will enhance our understanding of IL-1-mediated signaling and may contribute to our overall understanding of the biochemical basis of cytokine and hormone actions in general.

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