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Characterization of a Low Molecular Weight Isoform of IL-1 Receptor Antagonist

Mark Malyak,* Joel M. Guthridge,* Kenneth R. Hance,* Steven K. Dower,2‡ John H. Freed,1§ and William P. Arend3‡

IL-1R antagonist (IL-1Ra) exists in two well-characterized forms, 17-kDa secretory IL-1Ra (sIL-1Ra) and 18-kDa intracellular IL-1Ra (icIL-1Ra), that arise by alternative transcription of the same IL-1Ra gene. A third, lower molecular mass form (∼16 kDa) was detected by immunoblot within lysates of a variety of cells, including human monocytes and myelomonocytic cell lines. The 16-kDa isoform was designated icIL-1RaII, and the previously established 18-kDa form was designated icIL-1RaI. Intracellular IL-1RaII bound type I IL-1R up to fivefold less avidly than did sIL-1Ra and icIL-1RaI. Microsequencing of cyanogen bromide fragments of purified icIL-1RaII provided evidence consistent with initiation of protein translation at the second start site in either 16-kDa mRNA. The results of site-directed mutation experiments established that icIL-1RaII could be derived by alternative translation initiation. In vitro transcription and translation of intact sIL-1Ra cDNA in rabbit reticulocyte lysates led to both pro-sIL-1Ra and icIL-1RaI proteins, whereas transcription and translation of icIL-1RaI cDNA produced both icIL-1RaI and icIL-1RaII proteins. Mutation of the first 5′ ATG in sIL-1Ra cDNA led to translation of only icIL-1RaII, while only sIL-1Ra was observed after mutation of the second ATG. These results indicate that icIL-1RaII is a third member of the IL-1Ra family and is a 16-kDa, 143-amino acid intracellular protein derived by alternative translation initiation from either sIL-1Ra mRNA or icIL-1Ra mRNA. The role in biology of either intracellular form of IL-1Ra remains unknown. The Journal of Immunology, 1998, 161: 1997–2003.

Interleukin-1α and IL-1β probably play important proinflammatory roles in the pathogenesis of many acute and chronic disorders through engagement of specific receptors on cell surfaces (1, 2). IL-1R antagonist (IL-1Ra) is a naturally occurring cytokine that competitively inhibits binding of IL-1α and IL-1β to IL-1Rs without exhibiting detectable agonist activity (3, 4). An anti-inflammatory role for IL-1Ra in diseases is supported by the presence of IL-1Ra in various acute and chronic inflammatory human disorders and its ability to abrogate the effects of IL-1 in various in vivo animal models of inflammation (5–12).

IL-1Ra exists as two well-characterized forms (3, 13, 14). Secretory IL-1Ra (sIL-1Ra) and intracellular IL-1Ra (icIL-1Ra) are distinct peptide products of the same IL-1Ra gene, resulting from different first exons and alternative RNA splicing. Thus, sIL-1Ra and icIL-1Ra have different mRNAs with unique transcriptional regulatory regions (15–17). sIL-1Ra is translated with a leader sequence, promptly processed to a 17-kDa peptide, glycosylated, and secreted by cells as a 22- to 25-kDa species (13). The sIL-1Ra is produced by monocytes, macrophages, neutrophils, fibroblasts, and hepatocytes. The icIL-1Ra is an 18-kDa peptide that lacks a leader sequence, is not glycosylated, and remains within the intracellular space in all systems studied to date (14). The icIL-1Ra is constitutively produced by human keratinocytes and other epithelial cells, and is a delayed synthetic product in monocytes.

A third, 16-kDa IL-1Ra isoform has been observed by Western blot analysis within a variety of human cells, including keratinocytes (18), corneal epithelial and stromal cells (19), the hepatoma cell line HepG2 (20), neutrophils (21), monocytes (21), and the myelomonocytic cell lines U937 (19) and THP-1. The objectives of the present studies were to characterize the 16-kDa isoform and to determine its mechanism of origin. To keep order among the growing list of IL-1Ra isoforms, we propose to name the 16-kDa species icIL-1RaII, and the previously established 18-kDa isoform, icIL-1RaI. The name of the secertory isoform, sIL-1Ra, is unchanged.

Materials and Methods

Purification of icIL-1RaII

Intracellular IL-1RaII initially was partially purified from U937 cells in preparation for IL-1R binding studies. U937 cells (CRL 1593, American Type Culture Collection, Rockville, MD), a human diffuse histiocytic lymphoma cell line, produce all three isoforms of IL-1Ra upon PMA differentiation and LPS stimulation. U937 cells were cultured in RPMI 1640, 1 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, and 10% heat-inactivated, low endotoxin FCS at 37°C in 5% CO2. PMA (100 ng/ml) was added for 72 h when the cells attained a concentration of 1 × 106/ml to induce terminal differentiation into a monocyte-like cell, followed by stimulation with 100 ng/ml LPS for an additional 24 h. Cells were then isolated and lysed using 0.5% Nonidet-P40 in 20 mM Tris, pH 7.5, and the following proteinase inhibitors: 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF, and 1 mM EDTA, followed by centrifugation and isolation of the supernatant. The supernatant was diluted in buffer containing 20 mM Tris (pH 7.6), 1 mM EDTA, and 1 mM PMSF, followed by application...
onto a 20-ml bed volume Q-Sepharose fast flow column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the identical buffer. The column was then eluted with a gradient of 0 to 100% 1 M NaCl in the above buffer. The icIL-1RαII, as determined by ELISA and Western blot, eluted at approximately 300 mM NaCl, whereas sIL-1Ra and icIL-1RαI eluted at approxi- mately 160 mM NaCl. The icIL-1RαII fraction was diluted in 10 mM NaCl, 50 mM NaF, 1% Tween 20, and 0.1% Tween 80 in preparation for affinity chromatography. The affinity column was constructed through covalent linkage of three anti-IL-1Rα mAb to protein G-Sepharose 4B Fast Flow beads (Sigma, St. Louis, MO) using the dimethylimidelinate technique (22). Anti-IL-1Rα mAb were developed as previously described (23). The icIL-1RαII fraction was precleared with protein G-Sepharose 4B beads containing covalently bound human IgG (Sigma) followed by application onto the affinity column. After washing, the column was eluted with 3.5 M Mgl2, followed by dialysis of the eluent against Dulbecco’s PBS solution and concentration. Using sensitive and specific ELISAs, there was no contaminating IL-1α in the icIL-1RαI sample, whereas IL-1β was detectable in trace amounts compared with icIL-1RαII (w/w, <0.008). Identical procedures were followed to purify icIL-1RαII from LPS-stimulated THP-1 cells (TB 202, American Type Culture Collection) and unstimulated HepG2 cells (HB 8065, American Type Culture Collection). These two cells contain primarily icIL-1RαII and were the sources of the semipurified and purified icIL-1RαII preparations used for the final experiments, as described below.

Depending on the cells and the culture technique, i.e., in T-175 flasks or roller bottles, 0.3 to 3.0 ng total IL-1Rα protein was produced per 106 cells. The numbers of cells used for each purification varied from 0.6 to 15.6 x 106. The amount of IL-1Rα protein (all isoforms) per total protein in the culture supernatants varied between 1:40,000 and 1:173,000. Ion exchange chromatography on Q-Sepharose yielded about 20-fold purification, and after affinity chromatography the preparation represented approximately 1,500-fold purification of possible contaminating proteins. The two-dimensional gel with the preparation sample of icIL-1RαII from ion exchange and affinity chromatography was transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue. The spot where icIL-1RαII was expected to be located was subjected to amino-terminal microsequencing using pulsed liquid Edman chemistry on an Applied Biosystems Procise 492 sequencer. The results obtained indicated that the amino terminus was blocked. The icIL-1RαII was then subjected to treatment on the polyvinylidene difluoride membrane with cyanogen bromide/TFCA vapor, and the resulting mixture of fragments was resequenced.

Preparation of recombinant icIL-1RαII

Recombinant icIL-1RαII was prepared both as a fusion protein, with three extra amino-terminal residues, and as a protein with the native structure. To prepare recombinant icIL-1RαII with the native structure, the sIL-1Rα cDNA was inserted into the pcDNA-3 vector (Invitrogen, Carlsbad, CA) and was amplified by PCR using VENT polymerase (New England Biolabs, Beverly, MA) and the following primers: upstream primer (5’ to 3’), G CCT GAG CAT ATG CAA GCC TTC AGA ATC; and downstream primer (3’ to 5’), ATG AGA GTC CTC CAG ATC AGG ATA G. The icIL-1RαII PCR product was digested with NdeI and BamHI, then was ligated into the pRSETa-5 vector (Invitrogen). Escherichia coli BL-21 was transformed with the plasmid containing the icIL-1RαII cDNA, then was cultured as recently described (24). The lysate was centrifuged at 750 x g for 10 min, then the proteins were precipitated with ammonium sulfate at 50 and 90% concentrations. The majority of the IL-1Rα protein was found by ELISA in the 90% fraction and was resuspended in 20 mM Tris (pH 7.0), 1 mM PMSF, and 1 μg/ml leupeptin, then dialyzed extensively before application onto a Q-Sepharose fast protein liquid chromatography ion exchange column (Pharmacia) equilibrated with the same buffer. The column was eluted with a continuous gradient of 20 mM to 1 M NaCl in the same buffer, and the icIL-1RαII was eluted at 200 mM NaCl. This protein was further purified by chromatofocusing on Mono-P PPLC (Pharmacia), and gel filtration over Sephadex G-75 (Pharmacia). The resulting recombinant protein preparation was >90% pure by SDS-PAGE and silver staining, and the expected amino-terminal structure was confirmed by sequencing. Recombinant icIL-1RαII was also prepared as a glutathione-S-transferase fusion protein, as recently described (24). The sIL-1Rα cDNA in the pcDNA-3 vector was amplified by PCR, as described above, using the following primers: upstream primer (5’ to 3’), ACT GGG ATG CAA ATG CAA GCC TTC AGA ATC AGG ATA G, and downstream primer (3’ to 5’), TG AGA GTC CTC CAG ATC GCT A.

The icIL-1RαII PCR product was digested with BamHI and XhoI, then was cloned into the vector for expression in Escherichia coli BL-21 (Stratagene). The resulting recombinant icIL-1RαI gene fusion protein was further prepared as recently described (24). This preparation was >95% pure, and the expected amino-terminal structure was confirmed by sequencing, including the three extra residues Gly, Ile, and Gln.

IL-1Ra ELISA

IL-1Ra protein concentrations were measured using a modification of a previously described sandwich ELISA in which the secondary Ab was horseradish peroxidase-conjugated rabbit anti-IL-1Rα (25). The primary and secondary rabbit antibody anti-IL-1Rα Abs recognized epitopes on all three isoforms (sIL-1Ra, icIL-1RαII, and icIL-1RαI); therefore, the ELISA detected all three IL-1Ra isoforms and could not distinguish among them. The standard curve for the ELISA was generated with human recombinant 17-kDa sIL-1Ra. The ELISA accurately measured both recombinant sIL-1Ra and recombinant icIL-1RαII with a sensitivity of 78 pg/ml.

Western blot analysis

Western blot analyses were performed using an anti-sIL-1Ra mAb, as recently described (20). Bound secondary Ab that was peroxidase conjugated was detected using a photon-emitting peroxidase substrate following the manufacturer’s instructions (ECL Western Blotting System, Amersham, Arlington Heights, IL). Photodetection was performed immediately.

Amino-terminal microsequencing of native icIL-1RαII

Partially purified icIL-1RαII obtained from THP-1 cells by ion exchange and affinity chromatography, as described above, was further purified on a two-dimensional gel. A standard isoelectric focusing gel, using ampholytes in the pH range of 3 to 10, was focused overnight at 800 V. A 15% SAGE-PAGE was performed as the second dimension. A parallel two-di- mensional gel was run for analysis of IL-1Ra protein by Western blot. The immunoreactive material was present as a single spot near the middle of the pH 5 region and by silver staining was clearly separated from other spots of possible contaminating proteins. The two-dimensional gel with the preparation sample of icIL-1RαII from ion exchange and affinity chromatography was transferred to a polyvinylidene difluoride membrane and stained with Coomassie blue. The spot where icIL-1RαII was expected to be located was subjected to amino-terminal microsequencing using pulsed liquid Edman chemistry on an Applied Biosystems Procise 492 sequencer. The results obtained indicated that the amino terminus was blocked. The icIL-1RαII was then subjected to treatment on the polyvinylidene difluoride membrane with cyanogen bromide/TFCA vapor, and the resulting mixture of fragments was resequenced.

IL-1R binding studies

The kinetics of binding of icIL-1RαII partially purified from U937 cells, recombinant icIL-1RαI, and recombinant sIL-1Ra to soluble types I and II IL-1R (sIL-1Rα and sIL-1RαII, respectively) were characterized using the BIAcore instrument (Pharmacia Biosensor, Piscataway, NJ), as recently described (23). Native icIL-1RαII was semipurified, as described above, using ion exchange and affinity chromatography. The sIL-1Rα and sIL-1RαII were directly immobilized onto the sensor chip at densities of 63.8 and 164 mno/mm2, respectively. Association data were obtained by measurement of ligand binding to immobilized sIL-1Rα or sIL-1RαII. Ligand (50 nM) was injected at a flow rate of 3 μl/min for 10 min, with collection of data points every 10 s. The ligand solution was then replaced with BIAcore buffer running at the same flow rate; dissociation data were compiled for an additional 60 min, with collection of data points every 20 s.

Bioassy of icIL-1RαII

The bioactivity of recombinant icIL-1RαII, either as a fusion protein with three extra amino-terminal residues or with the native structure, was ex- amined by its ability to inhibit IL-1β-augmented proliferation of PHA-stimulated murine C3H/HeJ thymocytes, as previously described (18).

Cloning of sIL-1Ra cDNA

Secretory IL-1α cDNA and two site-directed mutants (either first (mut1) or second (mut2) 5’ ATG mutated to TTG, encoding a leucine) were manufac- tured in preparation for determining IL-1Rα isotype protein expression using the rabbit reticuloocyte lystate method. All three amplified sIL-1Ra cDNA contained a sequence encoding a nine-amino-acid influenza hemagglutinin epitope tag at the carboxyl terminus. The hemagglutinin tag was employed to permit Western blot analyses with specific Abs to this epitope and to allow differentiation from endogenous proteins by size in other studies. The following primers were used to amplify sIL-1Ra cDNA from template cDNA prepared by RT of total cellular RNA from PBMC: up- stream primer (5’ to 3’), G CAT GGA TCC TGC AGT CAC AGA GTA GAA ATC; and downstream primer (3’ to 5’), AAG ATG AGA GTC CTC CTG CTC ATG GAT ATG CAT CGG GGT CTG CTC CTG AGT GAT ATG CAT CGG GGT CTG CTC ATG CAG TAC G.
The upstream primer contained 12 nucleotides corresponding to the 5’ untranslated region directly upstream of the start codon and nine nucleotides of the coding region of sIL-1Ra along with a sequence encoding a BamHI site. The downstream primer contained a stop codon, an EcoRI site, and a 27-nucleotide region encoding the hemagglutinin tag. The sIL-1Ra cDNA was amplified as previously described (16), then gel purified and digested with BamHI and EcoRI followed by ligation into pGEM-3 previously digested with the same enzymes. Transformation of competent DH5α cells was performed, followed by isolation of cDNA.

Site-directed mutagenesis of sIL-1Ra cDNA

Site-directed mutation of the first 5’ ATG of sIL-1Ra cDNA (mut1) was performed to eliminate the start codon for sIL-1Ra protein. The upstream primer exchanged a TTG for the ATG, but was otherwise identical with the upstream primer described above. The downstream primer was identical with that used to amplify sIL-1Ra cDNA. Since the second 5’ ATG existed relatively deep within the sIL-1Ra cDNA, it was elected not to use a very large upstream primer encoding this mutated site (mut2); rather, a two-stage approach was employed. Two fragments of mut2 were manufactured, followed by ligation, extension, and amplification. The upstream fragment of mut2 was manufactured using the following primers: upstream primer (5’→3’), GCT GGA TCC TCG CAC AGA ATG GAA ATC; and downstream primer (3’→5’), GGG AGA CCC TCT TTC AGG TTC CGG TTC AAC GTT. The downstream fragment of mut2 was manufactured using the following upstream primer (5’→3’): TCC AGC AAG TTG CAA GCC TTC. The downstream primer for the downstream fragment of mut2 was identical to that used to amplify native sIL-1Ra cDNA. The method used to amplify the fragments was identical with that described above. The fragments were Klenow treated and gel purified. The two fragments of mut2 were then annealed and extended followed by amplification with primers identical with those used for amplification of native sIL-1Ra cDNA. Mut2 was then gel purified and digested with BamHI and EcoRI followed by insertion and ligation into pGEM-3.

In vitro transcription and translation of sIL-1Ra and site-directed mutants

The effects of site-directed mutations of sIL-1Ra cDNA on translation of IL-1Ra isotypes were examined using the rabbit reticulocyte lysate system. In vitro transcription of the sIL-1Ra and icIL-1Ra cDNA and of the two site-directed sIL-1Ra mutants was performed with the Stratagene kit (Stratagene Cloning Systems, La Jolla, CA). Briefly, purified plasmid was digested with EcoRI and BamHI and a 27-nucleotide region encoding the hemagglutinin tag. The sIL-1Ra and icIL-1Ra cDNAs were then amplified by using the Stratagene Cloning System kit (Stratagene Cloning Systems, La Jolla, CA) and the primers identical with those used to amplify sIL-1Ra cDNA. Since the second 5’ ATG existed relatively deep within the sIL-1Ra cDNA, it was elected not to use a very large upstream primer encoding this mutated site (mut2); rather, a two-stage approach was employed. Two fragments of mut2 were manufactured, followed by ligation, extension, and amplification. The upstream fragment of mut2 was manufactured using the following primers: upstream primer (5’→3’), GCT GGA TCC TCG CAC AGA ATG GAA ATC; and downstream primer (3’→5’), GGG AGA CCC TCT TTC AGG TTC CGG TTC AAC GTT. The downstream fragment of mut2 was manufactured using the following upstream primer (5’→3’): TCC AGC AAG TTG CAA GCC TTC. The downstream primer for the downstream fragment of mut2 was identical to that used to amplify native sIL-1Ra cDNA. The method used to amplify the fragments was identical with that described above. The fragments were Klenow treated and gel purified. The two fragments of mut2 were then annealed and extended followed by amplification with primers identical with those used for amplification of native sIL-1Ra cDNA. Mut2 was then gel purified and digested with BamHI and EcoRI followed by insertion and ligation into pGEM-3.

Results

Partial purification of icIL-1RaII

The 16-kDa isoform of IL-1Ra was partially purified from the lysates of all three cell lines by ion exchange and affinity chromatography, as outlined in Materials and Methods. IL-1Ras in these semipurified preparations, as determined by ELISA, represented about 0.5 to 1.0% of the total protein present. Western blot analysis of a preparation from HepG2 cells revealed that only icIL-1RaII was present; there was no detectable presence of the larger molecular mass isoforms sIL-1Ra or icIL-1RaI (Fig. 1). Identical results were obtained with icIL-1RaII obtained from U937 or THP-1 cells. Further purification on a two-dimensional gel was conducted in preparation for amino-terminal sequencing, as described below.

Binding to sIL-1RI and sIL-1RII

The binding characteristics of partially purified native icIL-1RaII from U937 cells, recombinant icIL-1Ra, and recombinant sIL-1Ra to immobilized sIL-1RI and sIL-1RII as examined by the BIAcore instrument are shown in Figure 2. Rate constants obtained from SUVs of sIL-1Ra, recombinant sIL-1RI (lane 2), and native sIL-1Ra partially purified from HepG2 cells. One nanogram of material was applied to each lane, followed by an mAb that recognizes all the isoforms of IL-1Ra. Identical patterns were observed with lysates of THP-1 cells and U937 cells.

FIGURE 2. Western blot analysis of recombinant icIL-1Ra (lane 1), recombinant sIL-1RI (lane 2), and native sIL-1Ra partially purified from HepG2 cells. One nanogram of material was applied to each lane, followed by an mAb that recognizes all the isoforms of IL-1Ra. Identical patterns were observed with lysates of THP-1 cells and U937 cells.

mobilization of sIL-1RI resulted in minimal loss of ligand binding capacity. In this study, 64.8 fmol/mm² of sIL-1RII were immobilized on the sensor chip, and this receptor bound 47.5, 55.5, and 58.0 fmol/mm² of icIL-1RaI, icIL-1Ra, and sIL-1Ra, respectively, at equilibrium. These values were about 90% of the predicted receptor occupancy as determined by analysis of affinity constants, suggesting that direct binding of sIL-1RI to the sensor chip led to an approximately 10% loss in binding capacity. As shown in Figure 2, upper panel, and Table I, sIL-1Ra and icIL-1RaI demonstrated almost identical binding characteristics to sIL-1RI. However, icIL-1RaII bound with a four- to fivefold lower affinity constant, secondary to both slower association and more rapid dissociation.

Direct immobilization of sIL-1RII resulted in more substantial loss of ligand binding capacity. In this study, 164 fmol/mm² of sIL-1RII were immobilized on the sensor chip, and this receptor bound 10.0, 16.7, and 16.7 fmol/mm² of icIL-1RaII, icIL-1Ra, and sIL-1Ra, respectively, at equilibrium. These values were about 30% of the predicted receptor occupancy, suggesting an approximately 70% loss in sIL-1RII binding capacity upon direct binding.
of receptors to the sensor chip. Despite these limitations, icIL-1RaII interacted with sIL-1RII with characteristics similar to those of sIL-1Ra and icIL-1Ra1 (Table I). The low calculated affinity binding of all three IL-1Ra ligands to immobilized sIL-1RII resulted from relatively fast dissociation rates.

**Biologic activity of icIL-1RaII**

The abilities of the three isoforms of IL-1Ra to inhibit the biologic effects of IL-1 were examined in the murine thymocyte assay. Serial dilutions of recombinant preparations of all three isoforms of IL-1Ra were added to PHA-induced thymocytes in the presence of 50 pg/ml recombinant human IL-1β. Both sIL-1Ra and icIL-1Ra showed equivalent abilities to inhibit the stimulatory effects of IL-1β; 50% inhibition was observed with 1.4 ng/ml sIL-1Ra and 1.2 ng/ml icIL-1Ra, amounts 24- to 28-fold greater than that of the IL-1 present (Fig. 3Ab). However, icIL-1RaII was 2- to 4-fold less active than the other two isoforms of IL-1Ra, exhibiting 50% inhibition at 3.0 ng/ml (pGEX recombinant protein) and 4.2 ng/ml (pGEX recombinant gene fusion protein), a 60- to 84-fold molar excess over the IL-1β present (Fig. 3Bb). None of the preparations of rIL-1Ra exhibited any direct stimulation or inhibition of thymocyte proliferation in the absence of added IL-1β.

**Amino-terminal sequence of icIL-1RaII**

IL-1Ra from THP-1 cells, semipurified by ion exchange and affinity chromatography, was further purified on a two-dimensional gel before sequencing. The amino terminus of the icIL-1RaII was found to be blocked. The polyvinylidene difluoride membrane containing the icIL-1RaII that failed to produce amino-terminal sequence was treated with cyanogen bromide to produce a mixture of peptides by cleavage on the carboxyl-terminal side of methionines in the protein. Sequence analysis of this mixture revealed two predominant sequences: one corresponded to the peptide produced by cleavage after the methionine encoded by the second AUG in the sIL-1Ra mRNA and the other from cleavage after the methionine encoded by the fifth AUG (Table II). Two weaker sequences corresponded to the products produced by cleavage after the methionines encoded by the third and fourth AUG triplets.

Because the sequence of the last cyanogen bromide fragment extends to within five residues of the predicted carboxyl terminus of sIL-1Ra, it is highly likely that icIL-1RaII shares its carboxyl terminus with other members of the IL-1Ra family. The sequencing of a cyanogen bromide fragment derived from cleavage after the methionine encoded by the second AUG is consistent with initiation of translation of icIL-1RaII at this site. However, the data cannot formally exclude the less likely possibility that icIL-1RaII is derived from exoprotease processing to the amino-terminal end of sIL-1Ra to produce a molecule with one or two residues amino terminal to the methionine.

**FIGURE 3.** Inhibition of IL-1β augmentation of mitogen-induced proliferation of murine thymocytes. A (upper) depicts results with recombinant sIL-1Ra (○) and recombinant icIL-1Ra1 (■). B (lower) shows inhibition by two different recombinant icIL-1RaII preparations, either as a fusion protein (pGEX) or with the native structure (pRSET). The cells were cultured for 48 h with 50 pg of human IL-1B and increasing concentrations of the three isoforms of IL-1Ra. The data are presented as log/log plots, with the percentage of control vs the amount of IL-1Ra added through 10 ng/ml, a 200-fold molar excess over the amount of IL-1β present. Computer-based linear regression analysis yielded the amounts of proteins giving 50% inhibition of the stimulatory effects of IL-1β. The data points represent the mean ± SD of three replicate wells from one experiment; identical results were obtained in two additional experiments. If no error bars are visible, they fall within the size of the symbol.

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**Table I. Binding characteristics of sIL-1Ra, icIL-1RaI, and icIL-1RaII with types I and II IL-1R**

<table>
<thead>
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<th></th>
<th>sIL-1RI</th>
<th>sIL-1RII</th>
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</thead>
<tbody>
<tr>
<td><strong>Association rate constants</strong></td>
<td>$k_a = 2.4 \times 10^{9} M^{-1}s^{-1}$</td>
<td>$k_a = 1.9 \times 10^{8} M^{-1}s^{-1}$</td>
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<tr>
<td><strong>Dissociation rate constants</strong></td>
<td>$k_d = 1.9 \times 10^{-4} s^{-1}$</td>
<td>$k_d = 5.1 \times 10^{-3} s^{-1}$</td>
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<tr>
<td><strong>Affinity constants</strong></td>
<td>$K_d = 1.2 \times 10^{15} M^{-1}$</td>
<td>$K_d = 3.8 \times 10^{15} M^{-1}$</td>
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<tr>
<td><strong>Association rate constants</strong></td>
<td>$k_a = 2.2 \times 10^{9} M^{-1}s^{-1}$</td>
<td>$k_a = 1.7 \times 10^{8} M^{-1}s^{-1}$</td>
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<tr>
<td><strong>Dissociation rate constants</strong></td>
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<tr>
<td><strong>Affinity constants</strong></td>
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<td>$K_d = 3.3 \times 10^{15} M^{-1}$</td>
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<tr>
<td><strong>Association rate constants</strong></td>
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<td><strong>Dissociation rate constants</strong></td>
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<td><strong>Affinity constants</strong></td>
<td>$K_d = 3.0 \times 10^{15} M^{-1}$</td>
<td>$K_d = 2.9 \times 10^{15} M^{-1}$</td>
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</table>

* Association rate and dissociation rate constants were estimated from association kinetics using the Biosensor instrument. The calculated affinity constants represent the association rate constant divided by the dissociation rate constant. The data are representative results from one of three experiments performed.
In vitro transcription and translation of intact and mutated sIL-1Ra cDNA

To further explore the mechanism of origin of icIL-1RaII, in vitro transcription and translation in rabbit reticulocyte lysates were studied with intact sIL-1Ra or icIL-1RaI cDNA, and with sIL-1Ra cDNA mutated in either the first or second 5′ ATG (Fig. 4). The relative sizes of the translation products were determined by 15% SDS-PAGE and autoradiography. Two translation products were observed with the intact sIL-1Ra cDNA, corresponding to the pro-sIL-1Ra and icIL-1RaII proteins (Fig. 5). In a control experiment, incubation of recombinant sIL-1Ra with the rabbit reticulocyte lysates yielded no band suggestive of icIL-1RaII. The intact icIL-1RaI cDNA produced two proteins that corresponded to icIL-1RaI and icIL-1RaII. However, mutation of the first 5′ ATG in the sIL-1Ra cDNA led to the appearance of only the icIL-1RaII protein, and a cDNA mutated in the second 5′ ATG gave origin only to the pro-sIL-1Ra protein. These results establish that icIL-1RaII is a product of transcription and translation of both sIL-1Ra and icIL-1RaI cDNA, with the most likely mechanism being alternative translation initiation from the second 5′ ATG.

Discussion

The data reported herein indicate the presence of a third species of IL-1Ra, a 16-kDa intracellular molecule that is derived from both sIL-1Ra and icIL-1RaI mRNA by alternative translation initiation from the second 5′ ATG. Previous studies have detected this 16-kDa IL-1Ra species within the lysates of a variety of cells. This isoform is the only intracellular IL-1Ra species in neutrophils (21) and HepG2 cells (20), is a major product of monocytes (21), is found in small but variable amounts in keratinocytes (18), and is a major intracellular constituent of U937 cells as well as of corneal epithelial and stromal cells (20). A 16-kDa species of IL-1Ra isolated from the lysates of rabbit peritoneal exudate neutrophils has an identical amino-terminal structure as the isoform described in our studies (26, 27). Furthermore, we have observed a similar 16-kDa isoform of IL-1Ra in lysates of the murine macrophage line RAW 264.7 (24). To distinguish this form from the well-characterized 18-kDa icIL-1RA and 17-kDa sIL-1Ra species, we proposed to name the 16-kDa species icIL-1RaII and the 18-kDa intracellular species icIL-1RaI.

Table II. Nucleotide sequences of sIL-1Ra cDNA 3′ to each ATG, the corresponding protein sequences predicted, and the sequences found after cyanogen bromide cleavage of icIL-1RaII

<table>
<thead>
<tr>
<th>Sequence</th>
<th>First ATG</th>
<th>Predicted</th>
<th>Found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>32-ATG</td>
<td>GAA ATC TGC AGA GCC CTC</td>
<td>Met- Glu- Ile- Cys- Arg- Gly- Leu</td>
</tr>
<tr>
<td></td>
<td>124-ATG</td>
<td>CAA GCC TTT AGA ATC TGG</td>
<td>Met- Gin- Ala- Phe- Arg- Ile- Trp</td>
</tr>
<tr>
<td></td>
<td>299-ATG</td>
<td>TGC CTG TCC TGT GTC AAG</td>
<td>Met- Cys- Leu- Ser- Cys- Val- Lys</td>
</tr>
<tr>
<td></td>
<td>479-ATG</td>
<td>GAA GCT GAC CAG CCC GTC</td>
<td>Met- Glu- Ala- Asp- Gin- Pro- Val</td>
</tr>
<tr>
<td></td>
<td>530-ATG</td>
<td>GTC ACC AAA TTC TAC TTC</td>
<td>Met- Val- Thr- Lys- Phe- Tyr- Phe</td>
</tr>
</tbody>
</table>

a The nucleotide sequences 3′ to the five ATGs in the sIL-1Ra cDNA and the corresponding protein sequences predicted are derived from published data (15). The first ATG is the start site for pro-sIL-1Ra, and the second ATG represents the potential start site for icIL-1RaII. The “__” indicates that the predicted amino acid was not detectable in that sequence cycle. A residue in parentheses indicates an amino acid found in very low yield. The sequence arising from cleavage after the methionine encoded by the fourth ATG was present in low yield.

FIGURE 4. The nucleotide and amino acid sequence of the 5′ region of the sIL-1Ra cDNA. The first potential start site for translation, used to produce pro-sIL-1Ra, was mutated (mut1). The second potential start site, located nine codons 3′ of the nucleotide encoding the amino-terminal Arg in the 17-kDa mature sIL-1Ra, was mutated to create mut2. Initiation of translation at this second AUG in the mRNA would produce a 16-kDa protein with the predicted amino-terminal sequence of Met-Gln-Phe-Arg-Ile, as described in Table II.
An origin of the 16-kDa IL-1Ra isofrom by proteolysis was considered. However, since a wide range of proteinase inhibitors was added to cells before lysis, inadvertent proteolysis of sIL-1Ra or icIL-1RaI to a 16-kDa peptide during cell handling was unlikely. Furthermore, since icIL-1RaII is found only within the cytoplasmic compartment of neutrophils and monocytes (21), in vivo proteolysis of sIL-1Ra to icIL-1RaII would be highly unlikely, as sIL-1Ra is translated with a leader sequence and then immediately enters the membrane-bound secretory pathway. The icIL-1RaI is also a cytoplasmic peptide and hypothetically might undergo in vivo proteolysis to yield a 16-kDa species. Small amounts of icIL-1RaII are present within keratinocytes that do not transcribe sIL-1Ra (18), and we have shown that icIL-1RaII can be transcribed and translated from icIL-1RaI mRNA. However, since we have not identified any icIL-1RaI mRNA or protein in neutrophils (21), and icIL-1RaII appears within monocytes before detection of icIL-1RaI mRNA or protein (21), proteolysis of icIL-1RaI cannot be the major source of icIL-1RaII in these cells.

Other potential mechanisms of origin of icIL-1RaII include a unique mRNA arising from the IL-1Ra gene or alternative translation initiation from sIL-1Ra mRNA. A smaller mRNA of the appropriate size for icIL-1RaII has never been observed by our laboratory. Initiation of sIL-1Ra mRNA translation at the next downstream AUG from the conventional start site would produce a 16-kDa, in-frame, IL-1Ra species that would remain within the cytoplasmic compartment of neutrophils and monocytes (21), in vivo proteolysis to yield a 16-kDa species. Small amounts of icIL-1RaII are present within keratinocytes that do not transcribe sIL-1Ra (18), and we have shown that icIL-1RaII can be transcribed and translated from icIL-1RaI mRNA. However, since we have not identified any icIL-1RaI mRNA or protein in neutrophils (21), and icIL-1RaII appears within monocytes before detection of icIL-1RaI mRNA or protein (21), proteolysis of icIL-1RaI cannot be the major source of icIL-1RaII in these cells.

Alternative translation initiation from either sIL-1Ra or icIL-1RaI mRNA. However, this finding does not prove that this mechanism is operative in vivo in intact cells. In ongoing studies, we have observed 16-kDa IL-1Ra in the lysates of CHO cells stably transfected with the sIL-1Ra cDNA, although the levels of this isoform vary considerably between lines (M. Malyak and W. P. Arend, unpublished observations). Alternative translation initiation has been described as a mechanism of origin of other cytoplasmic proteins (28–37). In most of these examples, the two or more intracellular isoforms of the same protein exhibited differences in degrees of biologic activity, similar to our observations. The mechanisms of alternative translation initiation have been recently reviewed (38–41).

A cDNA for a larger intracellular isoform of IL-1Ra from human neutrophils was reported by other investigators, and the mRNA for this isoform was also identified in activated fibroblasts, in keratinocytes, and at low levels in monocytes (42). This cDNA contained an in-frame 63-bp insert between the first and second exons of icIL-1RaI, coding for an additional 21 residues in the amino-terminal region. This cDNA was expressed in COS cells, and the recombinant 25-kDa protein in cell lysates was equivalent to icIL-1RaI in inhibition of biologic activity of IL-1. However, these investigators did not describe a 25-kDa isoform of IL-1Ra to be present in any resting or stimulated cell or cell line. In Western blots performed by our laboratory over the past 5 yr on a variety of human and murine cells, cell lines, and organs, a naturally occurring protein corresponding to this purported larger molecular mass species of icIL-1RaI has never been observed.

Cellular responses to IL-1 are mediated by IL-1RI, whereas the IL-1RII appears to be biologically inactive. Intracellular IL-1RaII exhibited 2- to 5-fold less biologic activity than either icIL-1RaI or sIL-1Ra, both in direct binding studies with IL-1RI and in inhibition of IL-1 effects on murine thymocytes. These findings may have implications for understanding the role in biology of the IL-1Ra proteins. Five residues in IL-1Ra are critical for binding to type I IL-1RI: Trp16, Gln20, Tyr34, Gln36, and Tyr147 (43). The possibility exists that the loss of nine amino-terminal residues between sIL-1Ra and icIL-1RaII creates conformational changes involving one or more of these critical residues, leading to a decrease in the avidity of binding to IL-1RI. Up to a 50-fold molar excess of sIL-1Ra over IL-1 is necessary to inhibit the biologic responses of murine thymocytes to IL-1 (44). This requirement is because full responses are exhibited with occupancy of five or fewer IL-1RI per cell, and most cells possess large numbers of receptors. The reduced affinity of icIL-1RaII for IL-1RI would necessitate an even higher molar excess over IL-1 than required for sIL-1Ra and icIL-1RaI to observe inhibitory effects toward IL-1-induced biologic responses.

Since neither isoform of icIL-1RaI is found in the extracellular space, their role in biology may not be to compete with IL-1 for binding to cell surface receptors. Intracellular IL-1α has been demonstrated to promote senescence in human endothelial cells after transport to the nucleus (45, 46). Conceivably, both isoforms of icIL-1Ra may play a role counter-regulatory to the purported intracellular effects of IL-1α. In addition, icIL-1RaI may play an intracellular role similar to that described for icIL-1RaI, decreasing the production of particular gene products presumably through destabilization of mRNAs (47).

Acknowledgments
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References

FIGURE 5. Autoradiography of a SDS-PAGE depicting the three species of IL-1Ra generated by in vitro transcription and translation of cDNA in rabbit reticulocyte lysates in the presence of [35S]methionine. The cDNAs examined were: lane 1, intact sIL-1Ra; lane 2, sIL-1Ra mutated in the first ATG (mut1); lane 3, sIL-1Ra mutated in the second ATG (mut2); and lane 4, icIL-1RaI. The three species of IL-1Ra protein are identified as pro-sIL-1Ra (the precursor sIL-1Ra, before processing), icIL-1RaI, and icIL-1RaII.


