Cloning and Characterization of a New Isoform of the Interleukin 1 Receptor Antagonist

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Summary

By reverse transcriptase polymerase chain reaction on messenger RNA from human polymorphonuclear cells, we have isolated a sequence identical to the cDNA coding for intracellular interleukin 1 receptor antagonist (icIL-1ra), but containing an additional in-frame 63-bp sequence located three codons downstream of the translation start of icIL-1ra. This additional sequence is inserted between the first and second exon of the intracellular form, the latter of which is colinear with part of the first exon of the secreted form of IL-1ra. The additional sequence is coded by an extra exon located 2 kb downstream the first icIL-1ra–specific exon. The complementary DNA sequence of the alternatively spliced form of icIL-1ra shows that the predicted protein differs from classical icIL-1ra in the NH_2 terminus by insertion of a leaderless sequence of 21 amino acids rich in glycine and glutamic acid residues. Transcripts coding for this new form of icIL-1ra were detected in activated fibroblasts, keratinocytes, and at low levels in myelomonocytic cells. The recombinant protein expressed in COS cells had an apparent molecular mass in sodium dodecyl sulfate polyacrylamide gel electrophoresis of 25 kD compared to 22 kD of classical icIL-1ra, and was mostly intracellular. The ability of this new form of icIL-1ra to inhibit IL-1 activity, in terms of induction of E-selectin and human immunodeficiency virus replication, was comparable to that of classical icIL-1ra. We propose to refer to this new form of icIL-1ra as icIL-1ra type II.

IL-1α and IL-1β are pleiotropic cytokines exerting a variety of effects on different tissues (1). The potent inflammatory effects of IL-1 are under strict physiological control. IL-1 synthesis is inhibited by antiinflammatory cytokines, prostaglandins, and glucocorticoids (1). IL-1 receptor type II (IL-1RII) is a nonsignaling, IL-1–binding molecule that acts as a regulated decoy target for IL-1 (2–4). Finally, IL-1 is the only cytokine for which a polypeptide receptor antagonist (IL-1ra) has been described (5–7). The existence of multiple levels of inhibition of IL-1 points to the necessity for a tight control of this mediator.

IL-1ra is a polypeptide molecule that binds IL-1R type I (IL-1RI) and, less avidly, IL-1RII without any agonistic activity (8). IL-1ra is induced in different cell types, including mononuclear phagocytes, polymorphonuclear cells, and fibroblasts by IgG, cytokines, and bacterial products (8). As an IL-1 competitor, IL-1ra may represent a potentially useful pharmacological tool in inflammatory diseases (1).

Two molecular forms of IL-1ra have been identified and cloned. Secreted IL-1ra (sIL-1ra) contains a classical 25–amino acid leader peptide giving a mature protein of 152 amino acids (6, 7). Intracellular IL-1ra (icIL-1ra) has no leader sequence, thus predicting that this protein remains intracellular (9). sIL-1ra and icIL-1ra are generated from the same gene. icIL-1ra transcripts originate from an alternative start site and splicing of an alternative first exon into an internal splice acceptor site located in the first exon of sIL-1ra (9). The predicted proteins are thus identical except in their NH_2 end, in which the first 21 amino acids of sIL-1ra are substituted by four amino acids in icIL-1ra. Expression of transcripts coding for sIL-1ra and icIL-1ra is differentially regulated (9). sIL-1ra and icIL-1a have similar capacity to inhibit IL-1 activity (10). The biological significance of icIL-1ra is still unclear.

Here, we report the identification, cloning, and functional characterization of a new molecular form of icIL-1ra generated by the expression of a new 63-bp exon located 2 kb downstream the first intracellular specific exon. We propose to refer to this new form of icIL-1ra as icIL-1ra type II (icIL-1raII).

Materials and Methods

Cells. Human circulating PMN, monocytes, and endothelial cells (EC) were obtained and cultured as described in previous reports (11). Chronically infected U1 cells were cultured as described (12). COS cells were cultivated in DMEM medium (Gibco, Glasgow, Scotland) with 10% FCS (Hyclone Laboratories, Logan, UT) and

8387 fibroblast cells in RPMI 1640 medium (Gibco) with 10% FCS (Hyclone). Human keratinocyte cultures were kindly provided by Prof. M. L. Tenchini (Dept. of Biology and Genetics for Medical Sciences, University of Milano, Milano, Italy).

Stimuli. Human recombinant (hr) IL-1β (3 × 10^7 U/mg) was from Dompè (L 'Aquila, Italy). hrTNF-α (6.6 × 10^7 U/mg) was from BASF (Knoll, Germany). LPS (LPS B from *Escherichia coli* 055:B5) was purchased from Difco Laboratories (Detroit, MI). PMA was from Sigma hrIL-1α and hrIL-4 (5 × 10^7 U/mg) were from Immunex (Seattle, WA). hrIL-10 (10^7 U/mg) was from DNAX (Palo Alto, CA). HrIL-13 was from Sanofi Elf Bio Recherches (Labege, France). IgG for clinical use was from Sandoz (Basel, Switzerland) and was aggregated by heating at 63°C for 30 min.

Reverse Transcriptase PCR. Total RNA was extracted by the guanidinium thiocyanate method (13). RT-PCR was performed as described (14). Oligonucleotides were obtained from Duotech (Milano, Italy). The sequences of oligos used to selectively amplify icIL-1ra were identical to those previously described (9). In particular, we used oligos GM397 (indicated here as IRA 1) and GM368 (IRA 4). For icIL-1raII amplification, we used IRA 4 and IRA 5 (5' CTGACTTGTATGAAGAAGGAGGTGG 3'), which specifically recognizes the extra exon described here included in the icIL-1raII sequence (Fig. 1). The oligonucleotides for β-actin amplification were 5' GCGCTCGTCGTCGACAACGG 3' and 5' GATAGACAA-CGTACATGGCTG 3'. Amplified products were run through a 1% ethidium bromide–stained agarose gel and transferred onto a nitrocellulose filter (Stratagene, La Jolla, CA); hybridization was carried out according to standard protocols (13), with a previously labeled oligonucleotide specific for icIL-1raII (5' GACTCAAAG-GAGACGATCTGCCG 3') (13). Amplification products were subcloned (TA cloning system; InVitrogen, San Diego, CA) and sequenced by the chain termination method. Analysis of icIL-1raII sequence was performed with the Proexplore software package (Oxford Molecular Ltd., Oxford, UK).

PCR with Genomic DNA. Genomic DNA was extracted following standard protocols (13) from human circulating monocytes. 0.5 μg of genomic DNA was amplified at 94°C for 1 min, 60°C for 1 min, and 70°C for 4 min. We used IRA 1 (see above), IRA 9 (5 TTTGAGTCAGCATTGTCTTCA 3), IRA 7 (5 CTGACT-TGTATGAAGAAGGAGGTGG 3), and IRA 10, which is identical to B(BS1.1) (15).

Expression of icIL-1ra Products in COS Cells. The cDNAs containing 32 bp of the 5' untranslated region, the complete open reading frame, and 6 bp (including the stop codon) of the 3'-untranslated region of both the icIL-1raI and icIL-1raII were obtained by RT-PCR with oligonucleotides IRA 4 and IRA 5 as detailed above, ligated back into the pSG5 expression vector, and transfected into COS cells. After 2 d, culture-concentrated supernatants and sonicated cell lysates were examined by ELISA or immunoblotting as detailed below.

Identification of Immunoreactive IL-1ra. A commercial ELISA assay (Amersham, Buckingamshire, UK), which identifies both sIL-1ra and icIL-1ra, was used. For Western blot analysis two polyclonal antisera (rabbit, generous gift from Dr. C. Dinarello, Tufts University, Boston, MA; and goat, Amersham) were used. COS cells lysates samples and supernatants were run on 12.5% SDS-PAGE and then blotted onto a nitrocellulose filter (Stratagene). Immunoreactive protein bands were revealed by a chemiluminescence-based procedure (ECL detection; Amersham) according to the manufacturer's instructions.

IL1 Responses. Confluent EC cultivated in 96-well plates (Falcon Labware, Oxnard, CA) were incubated for 30 min with an amount of transfected COS cell lysate (see above) corresponding to 25–100

ng of recombinant IL-1ra (either icIL-1raI or icIL-1raII), as assessed by a specific ELISA assay (Amersham). As a control, an equal amount of COS lysate obtained from mock-transfected cells was used in parallel. Next, 0.1–1 ng/ml human recombinant IL-1β was added to the cultures for 6 h. The detection of E-selectin expression was made with an ELISA assay on adherent EC with the anti–E-selectin mAb BB1G-E2 (courtesy of Dr. A. Gearing, British Biotechnology, Oxford, UK) as primary Ab and a rabbit anti–mouse Ig antiserum conjugated with horseradish peroxidase as a secondary Ab. Optical density quantification was determined with a spectrophotometer (Flow, Milano, Italy) at a 405-nm wavelength.

Chronically infected U1 cells (2 × 10^5 cells/ml in 24-well plates; Falcon) were stimulated with IL-1β (0.01–10 ng/ml) in the presence or absence of 100-fold excess of icIL-1raI or icIL-1raII or an equal amount of COS lysate obtained from mock-transfected cells. Cell culture supernatants were harvested 72 and 144 h after stimulation and tested for the presence of Mg^{++}-dependent RT activity as a parameter of virion production (12).

Results

Identification of a New Isoform of icIL-1ra. Specific oligonucleotide primers were designed (Fig. 1, *IRA1* and *IRA4*) to amplify the entire coding sequence of icIL-1ra (Fig. 1) by RT-PCR. Amplified products from human polymorphonuclear cells were subcloned and sequenced. In addition to the previously known sequence of icIL-1ra, we isolated a number of clones whose sequences were identical to the published icIL-1ra coding sequence, with the notable exception of an extra 63-bp sequence between nucleotides 132 and 133 of the icIL-1ra sequence. Given the described exon–intron boundaries of icIL-1ra (8), the extra sequence is inserted between the first leaderless exon of icIL-1ra and its second exon, which corresponds to the 3' half of the first exon of sIL-1ra (Fig. 1). The predicted amino acid sequence is shown in Fig. 1. The putative new protein (thereafter referred to as icIL-1raII) has the first three amino acids at the NH$_2$ end in common with the classical icIL-1ra (therefore referred to as icIL-1raI), followed by a new sequence of 21 amino acids. The rest of the two proteins are identical. The complete icIL-1raII consists of 180 amino acids. The most striking characteristic of the inserted extra sequence is the presence of seven glycine residues, six of which are consecutive. Glycine residues are flanked on both sides by glutamic acid residues. Computer analysis of icIL-1raII–specific sequence was performed, and a number of genes showed a similarity to the icIL-1raII specific exon (not shown). The overall hydrophilic pattern of icIL-1raII is similar to that of icIL-1raI, lacking a hydrophobic leader peptide at the NH$_2$ terminus (not shown).

Genomic Organization of IL-1ra Gene. The icIL-1raI first exon had been previously localized ~9.6 kb upstream of the sIL-1ra first exon (reference 8 and Fig. 2), but sequence information was available for a 6-kb region located immediately upstream of the sIL-1ra first exon (15, 16). This intronic sequence did not contain our putative extra exon. We hypothesized, therefore, that the putative extra exon might be localized in the remaining 3.5-kb genomic sequence not yet available (15, 16). We therefore designed the IRA 10 oligonucleotide, which is colinear with the most 5' sequence of

Secreted IL-1ra

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GAATTCCGGGCTGCAGTCACAGAATGGAAATCTGCAGAGGCCTCCGCAGTCACCTAATCACTCTCCTCCTCTTCCTGTTCCATTCAG
                MetGluIleCysArgGlyLeuArgSerHisLeuIleThrLeuLeuLeuPheLeuPheHisSer
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Intracellular IL-1ra type I

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        IRA 1
──────────────────────►
CAGAAGACCTCCTGTCCTATGAGGCCCTCCCCATGGCTTTAG
                          MetAlaLeu
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Intracellular IL-1ra type II

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        IRA 1                              IRA 5
──────────────────►                ──────────────────────►
CAGAAGACCTCCTGTCCTATGAGGCCCTCCCCATGGCTTTAGCTGACTTGTATGAAGAAGGAGGTGGAGGAGGAGGAGAAGGTGAAGACAATGCTGACTCAAAGG
                          MetAlaLeuAlaAspLeuTyrGluGluGlyGlyGlyGlyGlyGlyGluGlyGluAspAsnAlaAspSerLys
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Common IL-1ra sequence

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 AGACGATCTGCCGACCCTCTGGGAGAAAATCCAGCAAGATGCAAGCCTTCAGAATCTGGGATGTTAACCAGAAGACCTTCTATCTGAGGAACAACCAACTAGTTGCTGGATACTTGCAA
 GluThrIleCysArgProSerGlyArgLysSerSerLysMetGlnAlaPheArgIleTrpAspValAsnGlnLysThrPheTyrLeuArgAsnAsnGlnLeuValAlaGlyTyrLeuGln

 GGACCAAATGTCAATTTAGAAGAAAAGATAGATGTGGTACCCATTGAGCCTCATGCTCTGTTCTTGGGAATCCATGGAGGGAAGATGTGCCTGTCCTGTGTCAAGTCTGGTGATGAGACC
 GlyProAsnValAsnLeuGluGluLysIleAspValValProIleGluProHisAlaLeuPheLeuGlyIleHisGlyGlyLysMetCysLeuSerCysValLysSerGlyAspGluThr

 AGACTCCAGCTGGAGGCAGTTAACATCACTGACCTGAGCGAGAACAGAAAGCAGGACAAGCGCTTCGCCTTCATCCGCTCAGACAGTGGCCCCACCACCAGTTTTGAGTCTGCCGCCTGC
 ArgLeuGlnLeuGluAlaValAsnIleThrAspLeuSerGluAsnArgLysGlnAspLysArgPheAlaPheIleArgSerAspSerGlyProThrThrSerPheGluSerAlaAlaCys

 CCCGGTTGGTTCCTCTGCACAGCGATGGAAGCTGACCAGCCCGTCAGCCTCACCAATATGCCTGACGAAGGCGTCATGGTCACCAAATTCTACTTCCAGGAGGACGAGTAGTAC
 ProGlyTrpPheLeuCysThrAlaMetGluAlaAspGlnProValSerLeuThrAsnMetProAspGluGlyValMetValThrLysPheTyrPheGlnGluAspGlu*
                                                                            ◄────────────────
                                                                                  IRA 4
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Figure 1. cDNA sequence and predicted protein sequence of icIL-1raII compared to classical icIL1ra (icIL-1raI) and sIL-1ra. The upper part of the figure shows the cDNA and protein sequences specific for sIL-1ra, icIL-1raI, and icIL-1raII. The lower part of the figure shows the sequence in common among the three forms of IL-1ra. For clarity, the cDNA sequence of icIL-1ra starts from nucleotide 91 of the published 5′ untranslated sequence, and only 6 bp of the 3′ untranslated sequence are reported. The common IL-1ra sequence starts with the internal acceptor site located in the first exon of sIL-1ra, corresponding to nucleotide 133 of the complete icIL-1raI sequence and to nucleotide 88 of the complete sIL-1ra sequence. The PCR primers (see text) are indicated with arrows. The asterisk indicates the stop codon. The sequence is available from EMBL/Gene Bank/DDBJ under accession number X84348.
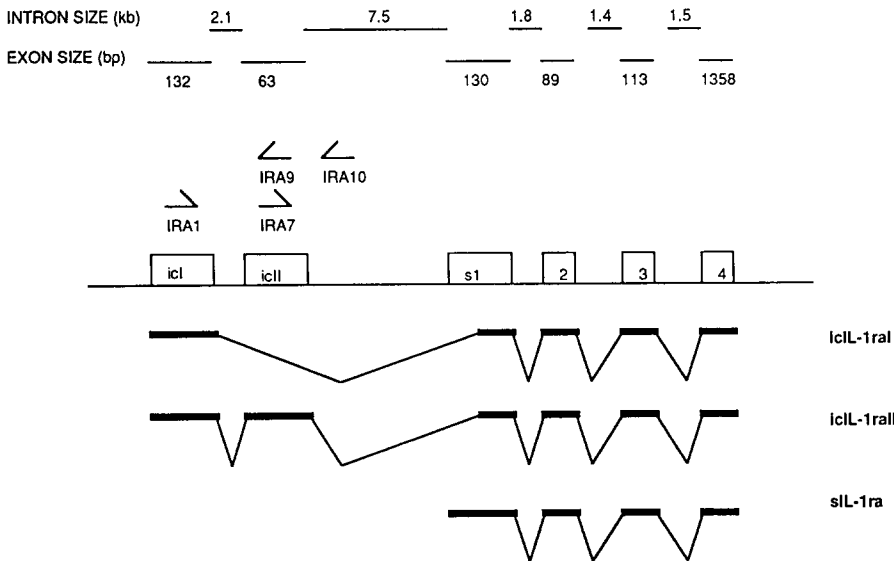

Figure 2. Organization of the human IL-1ra gene. The boxes represent the exons (the size is not representative of the actual length in basepairs). *icI*, the first exon of the intracellular form; *icII*, the new exon described in this paper; *s1*, the first exon of the soluble form; *2, 3*, and *4*, the 3′ exons. The characterized isoforms described so far are schematically represented in the lower part. The lengths of the exons and introns are indicated above. The PCR primers (see text) are indicated with arrows.
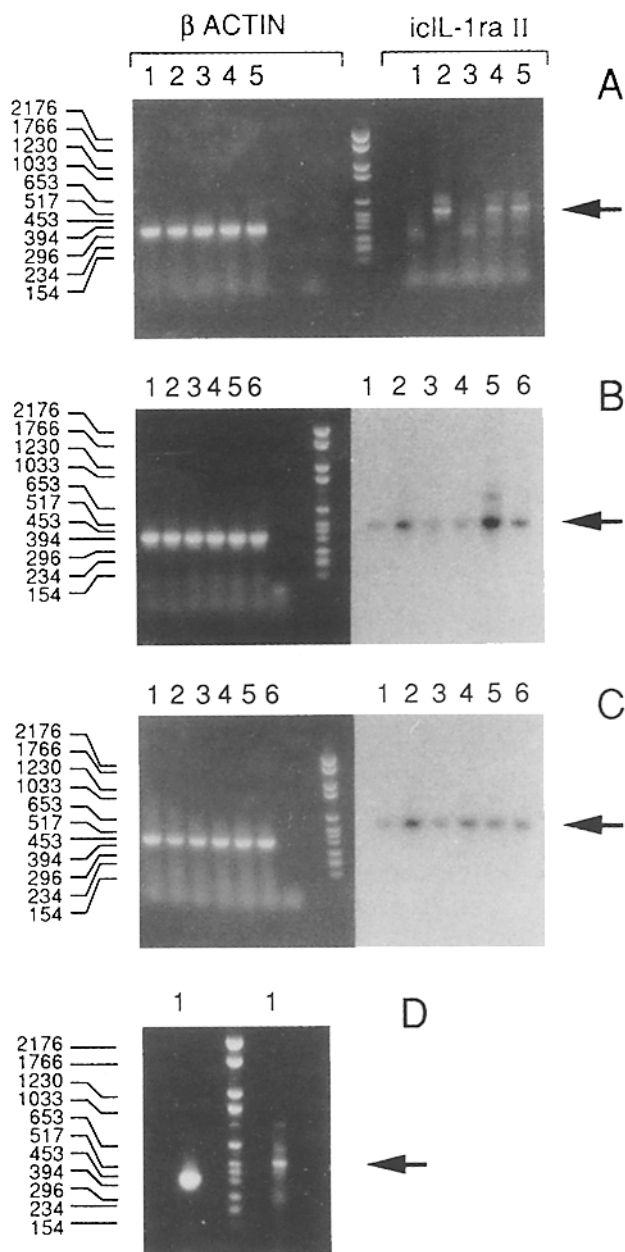
β ACTIN   icIL-1ra II

Figure 3. RT-PCR analysis of icIL-1raII expression in different cell types. RNAs from 8387 fibroblasts (*A*), monocytes (*B*), polymorphonuclear cells (*C*), and keratinocytes (*D*) treated with different stimuli as indicated below were reverse-transcribed. Each cDNA synthesis reaction was then divided in two aliquots, one of which was amplified with oligonucleotides IRA 5 (forward) and IRA 4 (backward) (see Fig. 1) for detection of icIL-1raII transcripts, and the other with β-actin–specific oligonucleotides. Amplified products were then examined through an ethidium bromide–stained agarose gel and the signals from monocytes and polymorphonuclear cells were amplified by Southern blotting (*B* and *C*). Amplified products corresponding to β-actin are on the left part of each panel and amplified products corresponding to icIL-1raII (on the right) panel are indicated by an arrow. (*A*) 8387 fibroblasts were incubated with medium (lane *1*), 10 ng/ml PMA (lane *2*), 50 ng/ml LPS (lane *3*), 500 U/ml TNF-α (lane *4*), and 10 ng/ml IL-1α (lane *5*). (*B*) Monocytes were incubated with medium (lane *1*), 10 ng/ml IL-4 (lane *2*), 10 ng/ml IL-10 (lane *3*), 10 ng/ml IL-13 (lane *4*), 100 ng/ml LPS (lane *5*), and 200 μg/ml aggregated IgG (lane *6*). (*C*) Polymorphonuclear cells were incubated with medium (lane *1*), 10 ng/ml IL-4 (lane *2*), 10 ng/ml IL-10 (lane *3*), 10 ng/ml IL-13 (lane *4*), 100 ng/ml LPS (lane *5*), and 200 μg/ml aggregated IgG (lane *6*). Keratinocytes, (panel *D*) were unstimulated (1).

the known intronic sequence (16), and IRA 1 oligonucleotide, whose sequence is contained within the first icIL-1ra exon. The genomic 3.5-kb fragment was amplified, cloned, and sequenced. As expected, the corresponding 63-nucleotide sequence was found at a distance of 2.1 kb downstream relative to the first icIL-1raI exon and was flanked by the exon–intron junction consensus AG and GT nucleotides. To further verify the exact localization of the extra exon, other oligonucleotides were constructed (IRA 9 and IRA 7; see Fig. 2), and the size of the amplified fragments was consistent with the indicated localization (data not shown).

Expression of icIL-ra in Different Cell Types. To identify icIL-1raII transcripts, RT-PCR analysis was performed with IRA 4 and IRA 5 oligonucleotides (Fig. 1). As shown in Fig. 3 *A*, a signal specific for icIL-1raII is not present in quiescent (lane *1*) or LPS-stimulated (lane *3*) 8387 fibroblasts, but was clearly induced upon exposure to PMA (lane *2*), TNF-α (lane *4*), and IL-1α (lane *5*). In parallel experiments, these same stimuli were equally effective for the induction of the icIL-1raI expression (data not shown and reference 17). When expression in monocytes was studied (Fig. 3 *B*), a distinct band for icIL-1raII was evident after hybridization with a specific oligonucleotide and was induced by LPS treatment (lane *5*). In parallel experiments, IL-4, IL-10, IL-13, LPS, and aggregated IgG were equally effective in inducing icIL-1raI expression (data not shown and reference 14). Expression of icIL-1raII was also studied in polymorphonuclear cells, either unstimulated or exposed to several stimuli including IL-4, IL-10, IL-13, LPS, and aggregated IgG. A band was present after hybridization with a specific oligonucleotide and was not modified much by different stimuli (Fig. 3 *C*). Under identical conditions, we had reported the induction of icIL-1raI upon exposure of polymorphonuclear cells to IL-13 (14). Finally, keratinocytes, which express icIL-1raI (9), showed high levels of icIL-1raII mRNA. The specificity of amplified products from PMA-treated fibroblasts and LPS-stimulated monocytes was confirmed by subcloning and sequencing (not shown). All in all, these results indicate that icIL-1raII is expressed in a variety of cell types, with a pattern of induction that is to some extent different from that of icIL-1raI and sIL-1ra.

Expression of Recombinant icIL-1raII and Biological Activity. COS cells were transfected with the cDNA sequences coding for icIL-1raII or icIL-1raI, and cell lysates and supernatants were examined in Western blots. The rabbit polyclonal antisera used in these experiments recognized equally well icIL-1raII and icIL-1raI, which were found mostly in cell lysate (data not shown). Recombinant icIL-1raI migrated as a major 22-kD band, whereas icIL-1raII showed a mass of ∼25 kD (Fig. 4).

Recombinant icIL-1raI and icIL-1raII were examined for their ability to inhibit IL-1–induced E-selectin expression on endothelial cells. Lysates from mock-transfected COS cells did not significantly reduce IL-1 activity. icIL-1raII had no agonistic activity. As shown in Fig. 5 (representative of three experiments), recombinant icIL-1raII inhibited E-selectin induction in a dose-dependent fashion. The inhibitory activity of icIL-1raII was similar to that exerted by icIL-1raI expressed
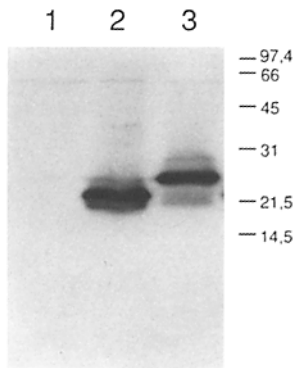
Figure 4. Western blot analysis of recombinant icIL-1raII. Cell lysates from COS cells transfected with the cDNAs coding for icIL-1raI (lane 2), icIL-1raII (lane 3), or with the empty vector (lane 1) were examined by immunoblotting with anti–IL-1ra rabbit polyclonal antiserum. Molecular weight standards are indicated.

under the same experimental conditions. Recombinant icIL-1raI and icIL-1raII were also tested for their ability to inhibit IL-1–induced HIV expression in U1 cells. Induction of HIV expression from U1 cells was not observed at any tested concentration (up to 1 μg/ml) of icIL-1raII. icIL-1raII suppressed IL-1β–dependent triggering of HIV expression in U1 cells (data not shown).

Discussion

In this report, we describe a new molecular form of IL-1ra, termed icIL-1raII. icIL-1raII is identical to icIL-1raI, except for an additional stretch of 21 amino acids located within the NH$_2$-terminal portion of the molecule. This icIL-1raII sequence is encoded by a novel exon located within the 9.6-kb region between the first and second exon of icIL-1raI. Thus, by differential splicing, three isoforms of IL-1ra are generated and their expression is, to some extent, differentially regulated in different cell types and/or in response to different stimuli.

icIL-1raII is biologically active, in that the molecule expressed in COS cells inhibit IL-1β activity with efficiency comparable to that of icIL-1raI. It has previously been reported that icIL-1raI and sIL-1ra have similar capacity to inhibit IL-1 (10). The actual biological significance of the intracellular isoforms of IL-1ra and of their differential expression remains unclear. They may represent reservoirs of IL-1ra, to be released upon cell death or to inhibit putative intracellular actions of IL-1.
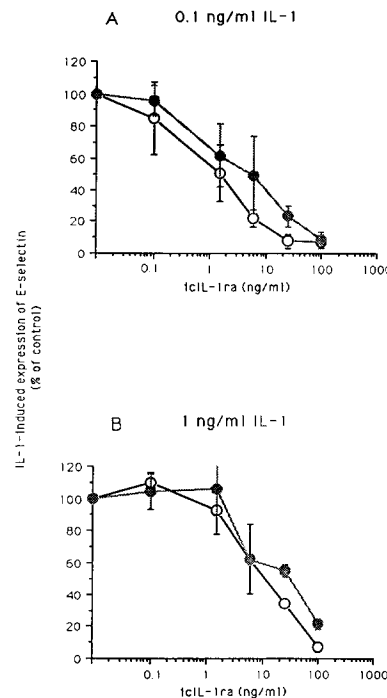

Figure 5. Effects of recombinant icIL-1raI and icIL-1raII on IL-1–induced expression of E-selectin on ECs. ECs were treated with 0.1 (A) or 1 (B) ng/ml human IL-1β in the presence or absence of 0.1–100 ng/ml recombinant icIL-1raI, icIL-1raII, or equivalent amounts of COS lysates obtained from cells transfected with an empty vector (mock). After a 6-h incubation, endothelial cells were examined for E-selectin expression by an ELISA assay performed on adherent cells. Data are expressed as percentage of IL-1–induced expression of E-selectin with respect to mock (mean of triplicates). –O–, icIL-1raI; –O–, icIL-1aII.

The IL-1 system shows an extraordinary level of complexity, consisting of two agonists, two receptors (one of which is an inhibitor of IL-1 [2–4]), and a receptor antagonist (of which at least three different molecular forms exist). Although the biological significance of the intracellular forms of IL-1ra remains to be clearly established, our data indicate that two different forms of icIL-1ra with different NH$_2$ termini are generated by alternative splicing in response to selected external stimuli. The existence of multiple levels of control of IL-1 points to the requirement for a tight physiological control of the inflammatory potential of this cytokine.

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