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Immunology

Soluble type II interleukin 1 (IL-1) receptor binds and blocks processing of IL-1β precursor and loses affinity for IL-1 receptor antagonist

(cytokine/competitive binding/mutagenesis/inhibition)

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ABSTRACT **Two IL-1 receptors have been identified, termed type I and type II. The extracellular domain of the type II IL-1 receptor is released from certain cells and can function as a specific inhibitor of IL-1β activity. We assessed the ligand-binding properties of the type II membrane-bound and soluble IL-1 receptor (sIL-1R) from the human B cell line Raji by competition. Upon release, the affinity of sIL-1R for IL-1α and IL-1β remained constant, and both soluble and cell surface IL-1 receptors bound to the same regions on the IL-1β molecule as defined by binding of a series of IL-1β mutant molecules. However, the affinity of sIL-1R for the IL-1 receptor antagonist (IL-1ra) decreased by a factor of 2000 when compared with the cell surface receptor. Type II sIL-1R and IL-1ra had an additive effect in inhibiting the binding of IL-1β to cell surface IL-1 receptors. In contrast, the combination of recombinant type I sIL-1R with IL-1ra abrogated the inhibition seen with each of the individual agents alone. The type II cell surface IL-1 receptor failed to bind the biologically inactive IL-1β precursor molecule, but binding to the IL-1β precursor was observed on cellular release of the receptor; this was confirmed with ^{35}S-labeled IL-1β. Binding of IL-1β precursor by sIL-1R inhibited the precursor's ability to be processed to the mature, biologically active 17-kDa species. These observations suggest that the type II sIL-1R inhibits IL-1β at two steps, by preventing processing of propeptide and by blocking the interaction of mature IL-1β with type I IL-1 receptor. In addition, type II sIL-1R does not interfere with inhibition mediated by IL-1ra.**

The human IL-1 gene family comprises IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). All three share limited amino acid identity and bind to the same receptors on different cell types (1). In the case of IL-1α and IL-1β, ligand–receptor interaction activates signaling pathways involved in both immune and inflammatory responses. Binding of IL-1ra to the IL-1 receptor (IL-1R) does not trigger signal transduction, and the actions of IL-1α and IL-1β are blocked by this unique antagonist protein (2).

Both IL-1α and IL-1β are initially synthesized as precursor proteins of 31 kDa and are processed to 17-kDa mature polypeptides upon release. However, release does not seem to be dependent on processing, as significant amounts of IL-1 precursor can be found in the extracellular medium (3). The IL-1α precursor is biologically active but the IL-1β precursor is unable to bind the IL-1R and is biologically inactive (4). The enzyme responsible for processing the IL-1β precursor is a heterodimeric cysteine protease derived from a single proenzyme, possibly by autocatalysis (5, 6). As the processing enzyme possesses no hydrophobic leader sequence, it probably remains within the cytoplasm. The IL-1β precursor can still be processed to an active form after secretion by extracellular proteases such as chymotrypsin, collagenase, elastase, and cathpepsin G, which are present at high levels in some inflammatory fluids (7).

Two distinct IL-1Rs have been identified, termed type I and type II (8, 9). The type I IL-1R is an 80-kDa glycoprotein found predominantly on T cells and fibroblasts, whereas the type II IL-1R is a 60- to 65-kDa molecule that is expressed on activated T cells, B cells, monocytes, and neutrophils (9). Evidence suggests that the type II IL-1R is not signal-transducing (10, 11), and its function on the cell surface is unknown.

The action of IL-1 is modulated not only by IL-1ra but also by the release of a type II soluble IL-1R (sIL-1R) (12). This 47-kDa soluble receptor is present in normal human plasma (13), serum, and synovial inflammatory exudate and in culture supernatants of activated peripheral blood mononuclear cells and neutrophils (14, 15). sIL-1R is also released from the human B-cell Burkitt lymphoma cell line Raji, which expresses only type II IL-1R (16, 17). Here we report the characterization of the ligand-binding properties of sIL-1R in comparison to the cell surface receptor. The results provide evidence that the type II sIL-1R may have evolved as an efficient regulator of IL-1β activity.

MATERIALS AND METHODS

Cell Culture. The human Burkitt lymphoma line Raji was used as a source of type II IL-1R and sIL-1R. The murine thymoma cell line EL-4 NOB.1 was used as a source of type I IL-1R. Both lines were obtained from the European Cell Culture Collection (Porton, Wilts, U.K.). The human monocytic cell line THP-1 was obtained from R. Solari (Glaxo). All cell lines were maintained at 37°C in RPMI 1640 culture medium containing 5% fetal bovine serum in a 5% CO_2/95% air atmosphere. Raji-derived sIL-1R was affinity purified (12). Recombinant human type I sIL-1R produced from the murine myeloma cell line NS1 was obtained from Genzyme.

Assays for Binding to Soluble and Cell Surface Receptors. Assays were performed with 10 nM ^{125}I-labeled IL-1β (NEN; specific activity, 125 μCi/μg; 1 μCi = 37 kBq) as described (14). Recombinant human IL-1α, IL-1β, and IL-1β precursor were gifts from E. Kawashima (Glaxo Institute of Molecular Biology, Geneva). Recombinant human IL-1ra was a gift from S. Eisenberg (Synergen, Boulder, CO).

Immunoblot Analysis of IL-1β. IL-1β peptides were subjected to SDS/15% PAGE and transferred electrophoretically to nitrocellulose filters (0.45-μm pore size; Bio-Rad). After incubation for 1 hr at room temperature in 5% bovine serum albumin/200 mM Tris·HCl, pH 7.4/0.15 M NaCl/0.2% Tween

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Abbreviations: IL-1, interleukin 1; IL-1R, IL-1 receptor; sIL-1R, soluble IL-1R; IL-1ra, IL-1R antagonist.

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20 to block nonspecific sites, the filter was incubated overnight at room temperature with a 1:500 dilution of polyclonal sheep anti-IL-1β antiserum (gift of S. Poole, National Institute for Biological Standards and Control, U.K.) in blocking buffer. Binding of antibody was subsequently detected by incubation with donkey anti-sheep IgG–alkaline phosphatase conjugate (Sigma) followed by naphthol AS-MX phosphate and fast blue RR salt.

Expression of IL-1β Mutants. Construction and expression of site-specific IL-1β mutants have been described (18). Wild-type and mutant IL-1β proteins were obtained by osmotic shock (19). Purity of the recombinant proteins was assessed to be 90–95% by SDS/PAGE and Coomassie blue staining. Competitive inhibition experiments were performed with ^{125}I-labeled human IL-1β and various dilutions of *Escherichia coli* extracts containing unlabeled wild-type or mutant IL-1β and the IC_{50} value was estimated graphically. Results were expressed as a percentage of the IC_{50} for the mutant molecule compared with the unlabeled wild-type IL-1β.

Construction of SP6 Expression Vectors and *in Vitro* Transcription/Translation. An IL-1β precursor expression plasmid was constructed by excision of an *Eco*RI–*Xba* I digestion fragment containing the coding region of the IL-1β precursor from the mammalian expression vector pRSVIL-1βpre (20) followed by insertion into pGEM-4Z (Promega) cut with *Eco*RI/*Xba* I. The mature IL-1β expression plasmid was constructed by cloning an *Nsi* I–*Bam*HI digestion fragment from the *E. coli* expression vector pMGIL-1β (21) into pGEM-3Z cut with *Pst* I/*Bam*HI. Transcription and translation were performed with an SP6 coupled reticulocyte lysate system (Promega) containing 1 μg of supercoiled plasmid DNA and 40 μCi of [^{35}S]methionine (Amersham; specific activity, 1000 Ci/mmol). After transcription/translation, lysates were centrifuged through Sephadex G-50 columns to remove unincorporated [^{35}S]methionine.

Covalent Crosslinking of sIL-1R with IL-1. Affinity-purified sIL-1R was covalently crosslinked in solution to radiolabeled IL-1 molecules (≈10 ng/ml) with disuccinimidyl suberate (14). Specificity was assessed by inclusion of 100-fold excess nonradioactive IL-1α or IL-1β. Crosslinked complexes were identified by SDS/10% PAGE followed by fluorography.

Assay for Inhibition of IL-1β Precursor Processing. Affinity-purified sIL-1R or an identical preparation that had been depleted of sIL-1R by a second passage over an IL-1β affinity column was incubated at various concentrations with 50 ng of recombinant human pro-IL-1β in a final volume of 100 μl. After the binding reaction mixtures were incubated at 4°C overnight, 10 μl of THP-1 cell extract (prepared by freeze–thawing 10^8 THP-1 cells per ml in 10 mM Tris·HCl, pH 8.1) was added and the temperature was raised to 37°C for 2 hr. IL-1β peptides were resolved by SDS/15% PAGE, transferred electrophoretically to 0.45-μm nitrocellulose filters (Bio-Rad) and detected by immunoblotting using sheep polyclonal anti-IL-1β antiserum.

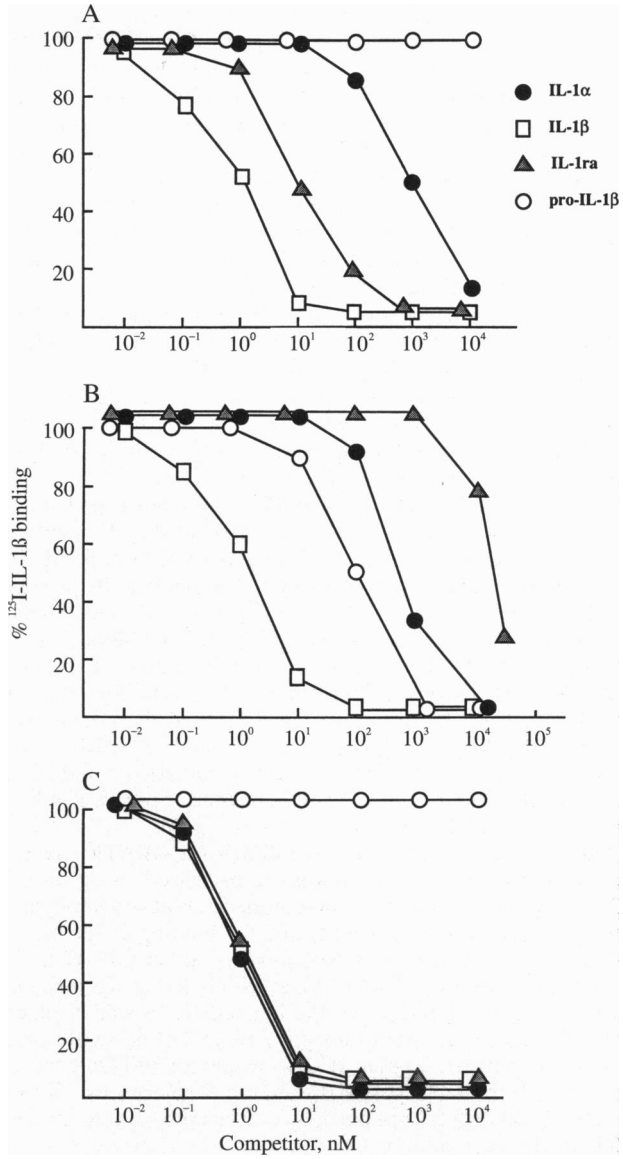

FIG. 1. Cross-competition of ^{125}I-IL-1β binding to IL-1Rs. Iodinated IL-1β (10 nM) was incubated with Raji cells (*A*), Raji sIL-1R (*B*), or EL-4 cells (*C*) together with various concentrations of IL-1α (●), IL-1β (□), IL-1ra (▲), or pro-IL-1β (○) for 4 hr at 8°C. Ligand–receptor complexes were separated from free ligand by centrifugation through phthalate oil mixture (*A* and *C*) or by precipitation with 12% (wt/vol) polyethylene glycol (PEG-8000). (*B*) Binding in the absence of competitor was 4702 ± 242 cpm for Raji cells, 8869 ± 427 cpm for Raji sIL-1R, and 3314 ± 223 cpm for EL-4 cells. Results are representative of at least three experiments.

RESULTS

IL-1 Ligand Binding to sIL-1R and Cell Surface IL-1R. IL-1 ligand binding to the Raji-derived sIL-1R and cell surface type I and type II IL-1Rs was studied by competition binding of unlabeled IL-1 ligands with ^{125}I-labeled IL-1β. Equilibrium dissociation constants (K_d) were estimated from the EC_{50} values by using the Cheng–Prusoff relationship (22). These estimates were based on K_d values for the binding of ^{125}I IL-1β, determined by Scatchard analysis of direct-binding data, of 2.7 nM for the type II sIL-1R and 2.2 nM for the cell surface type II IL-1R (12).

The competitive binding curves (Fig. 1 *A* and *B*) reveal that the various IL-1 ligands bind with different affinities to the type II cell surface IL-1R and sIL-1R. Human IL-1α bound to the sIL-1R and cell surface IL-1R with equally low affinity (K_d values of 1.5 and 1.6 μM, respectively). IL-1ra bound to the cell surface IL-1R with an estimated K_d of 14 nM but had ≈2000-fold lower affinity for the sIL-1R (25 μM). The 31-kDa pro-IL-1β molecule failed to bind the Raji cell surface IL-1R but bound to the sIL-1R with an estimated K_d of 191 nM. The competition assays using the type I IL-1R-bearing murine thymoma cell line EL-4 NOB.1 (Fig. 1*C*) confirmed previously published results (2, 4) in that IL-1α, mature IL-1β, and IL-1ra all bound with approximately equal affinity, whereas pro-IL-1β did not bind at all.

Binding of Mutant IL-1β to IL-1R. Changes in affinity for IL-1ra and pro-IL-1β presumably occur due to conformational changes, loss of key residues, or steric hindrance in the type II IL-1R upon release. To test whether the soluble and cell surface IL-1Rs bound to the same regions on IL-1β, we per-

formed cross-competition receptor binding assays using a series of site-specific mutants of IL-1β. Mutations were generated in three regions of the IL-1β molecule: residues 4–11, on β-strand 1; residues 74–80, a β-turn between strands 6 and 7; and residues 88–97, a large turn between β-strands 7 and 8 (18, 23). Table 1 shows that mutations in the N-terminal region frequently altered binding to the type I IL-1R on EL-4 cells. However, these mutations rarely affected binding to the Raji cell surface IL-1R or sIL-1R. Mutations at residues 76 and 79 had no significant effect on type I IL-1R binding but reduced binding to both cell surface and soluble Raji IL-1R. Mutations between residues 88 and 97, located on the same face of the IL-1β molecule as the N-terminal amino acids, resulted in variable changes in both type I and type II IL-1R binding. No correlation was apparent between type I and type II IL-1R binding to the IL-1β mutants. However, cell surface type II IL-1R and sIL-1R binding was consistently similar with all the mutant IL-1β molecules tested.

Interaction of Type II sIL-1R and Recombinant Type I sIL-1R with IL-1ra. Since the type II sIL-1R produced by Raji cells, in contrast to recombinant type I sIL-1R has low affinity for IL-1ra, it may be more effective in blocking IL-1 activity in the presence of IL-1ra. To test this we titrated Raji sIL-1R, recombinant soluble type I IL-1R, and IL-1ra to give ≈50% inhibition in a Raji cell surface IL-1R binding assay and then mixed IL-1ra with either Raji sIL-1R or recombinant type I sIL-1R (Fig. 2). Combination of Raji sIL-1R and IL-1ra increased the inhibition of ^{125}I-IL-1β binding to Raji cells [86 ± 9% (mean ± SEM, n = 3)]. However, combination of recombinant type I sIL-1R and IL-1ra decreased the inhibition of ^{125}I-IL-1β binding observed with the individual agents (21 ± 3%).

Confirmation That Pro-IL-1β Binds sIL-1R. The 30-kDa and 17-kDa forms of IL-1β were synthesized using an SP6 RNA polymerase transcription-coupled rabbit reticulocyte lysate (Fig. 3*A*). Proteins were tested for binding to the type II sIL-1R by soluble covalent crosslinking. As with 17-kDa ^{125}I-IL-1β (12), crosslinking of 17-kDa ^{35}S-labeled IL-1β formed a 60-kDa complex from which the labeled IL-1β was displaced with 100-fold excess nonradioactive IL-1β but not with excess IL-1α. In contrast, 30-kDa IL-1β formed an 80-kDa complex from which the labeled IL-1β was fully displaced by 100-fold excess 17-kDa IL-1β and partially displaced by 100-fold excess 17-kDa IL-1α (Fig. 3*B*).

Function of Type II sIL-1R Binding to Pro-IL-1β. To assess whether binding of pro-IL-1β to sIL-1R affected the prointerleukin's ability to be processed to lower molecular weight forms, we incubated pro-IL-1β with affinity-purified sIL-1R or an identical preparation that had been depleted of sIL-1R. Neither the sIL-1R preparation nor the THP-1 lysate contained detectable IL-1β (Fig. 4, lanes 1 and 2). Addition of THP-1 lysate to recombinant pro-IL-1β generated an IL-1β

Table 1. Binding of mutant IL-1β molecules to IL-1Rs

Mutation	% competitive binding		
	EL-4 type I	Raji type II	Raji sIL-1R
Arg^4 → Pro	10	130	105
Ser^5 → Gly	51	185	100
Ser^5 → Arg	120	90	86
Thr^9 → Glu	20	100	100
Thr^9 → Gly	95	100	100
Asp^{76} → Val	95	65	40
Thr^{79} → Ala	100	47	35
Lys^{88} → Val	103	52	68
Asn^{89} → Phe	120	64	70
Tyr^{90} → Leu	42	50	40
Lys^{92} → Ser	72	108	80
Lys^{92} → Arg	286	188	150
Lys^{93} → Met	24	285	186
Lys^{93} → Arg	97	350	190
Lys^{94} → Trp	42	85	100
Glu^{96} → Gly	69	85	50

Results are expressed as a percentage of the IC_{50} for the mutant molecule compared with unlabeled wild-type IL-1β.

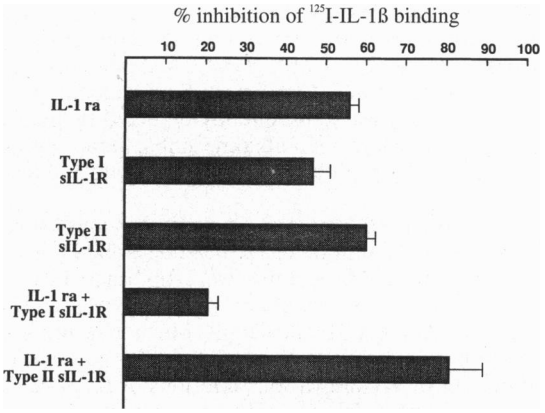

FIG. 2. Interaction of IL-1ra with sIL-1Rs. ^{125}I-IL-1β (10 nM) was incubated with either IL-1ra (100 nM), NS1-derived recombinant human type I sIL-1R (5 μg/ml), Raji-derived type II sIL-1R (1 μg/ml), or combinations of type I sIL-1R or type II sIL-1R with IL-1ra. Inhibitor/IL-1β combinations were then added to Raji cells for 4 hr at 8°C. Ligand–receptor complexes were separated from free ligand by centrifugation through phthalate oil mixture. Specific binding in the absence of competing agent was 4044 ± 628 cpm (mean ± SEM, n = 3).

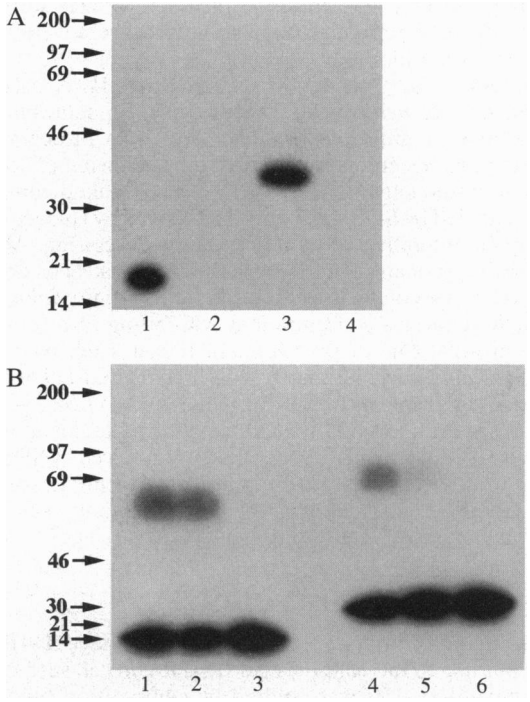

FIG. 3. Crosslinking of ^{35}S-labeled IL-1β peptides to sIL-1R. (*A*) SP6 expression vectors (pGEM-3Z/4Z) containing cDNA encoding mature IL-1β or pro-IL-1β were used to produce ^{35}S-labeled IL-1β molecules by coupled *in vitro* transcription/translation. Peptides were analyzed by SDS/15% PAGE and detected by fluorography. Lane 1, vector containing mature IL-1β cDNA; lane 2, pGEM-3Z vector alone; lane 3, vector containing pro-IL-1β cDNA; lane 4, pGEM-4Z vector alone. (*B*) Labeled 17-kDa IL-1β (lanes 1–3) and 31-kDa IL-1β (lanes 4–6) were incubated with Raji sIL-1R overnight at 4°C and crosslinked with disuccinimidyl suberate. After SDS/10% PAGE, complexes were identified by fluorography. Lanes 1 and 4, no competing agent; lanes 2 and 5, excess IL-1α; lanes 3 and 6, excess IL-1β. Protein size markers are in kilodaltons.

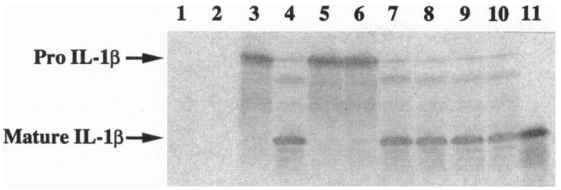

FIG. 4. Binding of pro-IL-1β to sIL-1R inhibits processing. Pro-IL-1β (50 ng) in 100 μl was incubated at 4°C overnight with sIL-1R or identical preparations depleted of sIL-1R by IL-1β affinity chromatography. THP-1 cell lysate (10 μl) was added, the temperature was raised to 37°C, and the incubation continued for 2 hr. IL-1β peptides were analyzed by SDS/15% PAGE and immunoblotting. Lane 1, sIL-1R preparation alone; lane 2, THP-1 lysate alone; lane 3, pro-IL-1β alone; lane 4, pro-IL-1β and THP-1 cell lysate; lanes 5–7, pro-IL-1β and THP-1 cell lysate with 2 μg, 0.5 μg, and 125 ng of sIL-1R, respectively; lanes 8–10, pro-IL-1β and THP-1 cell lysate with preparations equivalent to lanes 5–7 but depleted of sIL-1R; lane 11, recombinant mature IL-1β.

species that comigrated with mature 17-kDa IL-1β (lane 4). Preincubation of pro-IL-1β with 2 μg or 0.5 μg of sIL-1R (as determined by Coomassie blue staining) inhibited the ability of the THP-1 lysate to process pro-IL-1β to the 17-kDa form (lanes 5 and 6); However, 125 ng of sIL-1R failed to inhibit processing of pro-IL-1β (lane 7). The preparation that had been depleted of sIL-1R by IL-1β affinity chromatography with a similar total protein concentration did not inhibit processing at any of the concentrations used (lanes 8–10).

DISCUSSION

This report describes the binding characteristics of the type II sIL-1R and the cell surface type II IL-1R from which it is derived. The Raji cell surface type II IL-1R has greater affinity for IL-1β than for IL-1α. These properties are retained in the sIL-1R, and the affinities for IL-1α and IL-1β seem to be identical to those of the cell surface IL-1R (12). However, shedding of the IL-1R from Raji cells caused significant changes in the affinity for IL-1ra and 31-kDa pro-IL-1β. The affinity of the IL-1R for the IL-1ra fell after shedding, while the shed but not the cell surface type II IL-1R bound pro-IL-1β. The binding of pro-IL-1β by sIL-1R indicates that the N-terminal region of mature IL-1β is unlikely to be important for interaction with the type II IL-1R. Further evidence for this was revealed by the use of single site-specific mutants of IL-1β. Mutations in the N-terminal region of mature IL-1β did not have a deleterious effect on type II IL-1R binding; however, these mutations frequently affected binding to the type I IL-1R. The N terminus has been shown to be important for type I receptor binding and IL-1 bioactivity (24). Our results are in good agreement with previously published data showing that mutation of Arg^4 to Asp but not to other residues reduced IL-1β binding to the murine type II IL-1R (25). However, this mutation also caused loss of binding to the type I IL-1R, possibly indicating that an acidic side chain cannot be tolerated at this position in IL-1β (25). When the N-terminal sequences of the three IL-1 ligands were compared, the only well-conserved residue was Arg^4 (26). We also tested mutants in another region of the IL-1β molecule previously demonstrated to be critical for the binding of the type I IL-1R. Residues 88–97 lie within a large turn between β-strands 7 and 8 and, together with the N-terminal region, form part of a neutralizing epitope on one face of the IL-1β molecule (18). Mutations in this region frequently reduced binding to type I IL-1R and in some cases reduced binding to type II IL-1R. Mutations in the β-turn region between strands 6 and 7 (residues 74–80), a region not important for type I IL-1R binding, significantly reduced binding to both the soluble and cell surface type II IL-1R. These data indicate that type II IL-1R recognizes different epitopes on the IL-1β molecule than type I IL-1R, although it appears that the two receptors are unable simultaneously to bind the same ligand molecules (27), suggesting that some overlap in binding regions occurs.

We found that IL-1ra bound to human cell surface type II IL-1R with high affinity (K_d of 14 nM), in good agreement with published data (28, 29). However, after release from the cell surface the affinity of sIL-1R for IL-1ra fell ≈2000 fold (K_d of 25 μM). If sIL-1R and IL-1ra are to function as effective inhibitors of IL-1 action, it is important that they do not bind and so neutralize each other. The low affinity of the type II sIL-1R for IL-1ra indicates that IL-1ra would be unlikely to compete with IL-1β for binding to sIL-1R. The lack of IL-1ra binding to the shed form of type II IL-1R suggests that regions in type II IL-1R proximal to the transmembrane domain may be important for IL-1ra but not IL-1β binding activity. As yet no published studies are available on the regions of type II IL-1R important for ligand binding.

Type I sIL-1R and IL-1ra are currently undergoing clinical trials for the treatment of allergic and inflammatory diseases, and preliminary results indicate that these agents have considerable therapeutic potential (32). However, use of type II sIL-1R would allow combined therapy with IL-1ra, possibly achieving a greater *in vivo* inhibition of IL-1 action. Conversely, treatment with type II sIL-1R alone would block only IL-1β action, leaving IL-1α function intact; this may be useful in the treatment of chronic inflammatory diseases where sustained complete blockade of IL-1 action may weaken host defense mechanisms.

The binding properties of type II sIL-1R are very similar to those described for the product of the vaccinia virus open reading frame B15R. This protein has 30% amino acid sequence identity to type II IL-1R (9) and exhibits binding properties identical to those of the sIL-1R—i.e., high affinity for IL-1β and low affinity for IL-1α and IL-1ra (30). It is therefore likely that B15R represents a virally acquired extracellular domain of type II IL-1R. The IL-1β precursor is unable to bind the cell surface type I IL-1R and therefore is biologically inactive (4). Here we have demonstrated that pro-IL-1β also fails to bind cell surface type II IL-1R. However, cross-competition data indicated that shed pro-IL-1β was able to bind with an intermediate affinity (K_d of 190 nM) to type II sIL-1R. The sIL-1R may act as a carrier molecule for the precursor in the circulation; however, we showed that sIL-1R would be capable of blocking the processing of the precursor once it has been released from the cell. Presumably, sIL-1R inhibits processing by either masking the cleavage site on pro-IL-1β or altering the conformation of the pro-IL-1β molecule so that IL-1 converting enzyme or other proteolytic enzymes fail to recognize their cleavage sites. Conversion of pro-IL-1β to the mature form is also the target for viral inhibition. Cowpox virus encodes a 38-kDa protein member of the serpin superfamily, CrmA, that inhibits the IL-1 converting enzyme (31). Interestingly, both of the viral mechanisms of IL-1 inhibition so far described are directed specifically against IL-1β whereas sIL-1R found in serum, synovial exudate, and peripheral blood mononuclear cell supernatant binds IL-1β with greater affinity than IL-1α. These findings are consistent with the idea that IL-1β is more important in the inflammatory response and functions as a soluble molecule, whereas IL-1α delivers its signals during cell–cell interaction.

It is clear that IL-1 is unique among cytokines in that its actions are controlled both by a receptor antagonist protein and a soluble receptor. In order that these two regulatory molecules do not neutralize each other, sIL-1R selectively loses affinity for IL-1ra upon release. Additionally, sIL-1R gains affinity for the precursor form of IL-1β and when bound prevents the processing of the inactive precursor to its mature biologically active form. Hence the natural type II sIL-1R can control the bioactivity of IL-1β at the level of receptor binding

and peptide processing and complements the activity of the natural receptor antagonist.

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