Insertion of a Structural Domain of Interleukin (IL)-1β Confers Agonist Activity to the IL-1 Receptor Antagonist

IMPLICATIONS FOR IL-1 BIOACTIVITY*

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Scott A. Greenfeder§, Tracey Varnell$, Gordon Power‡, Kathleen Lombard-Gillooly‡, David Shustert‡, Kim W. McIntyre‡, Dene E. Ryan†, Wayne Levint†, Vincent Madison†, and Grace J. ut | From the Departments of iInflammation/ Autoimmune Diseases and iPhysical Chemistry, Roche Research Center, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

We showed previously that replacement of Lys-145 in the IL-1 receptor antagonist (IL-1ra) with Asp resulted in an analog (IL-1ra K145D) with partial agonist activity. To identify additional amino acids that affect IL-1 bioactivity, we created second site mutations in IL-1ra K145D. Substitutions of single amino acids surrounding position 145 were made; none of these substitutions increased the bioactivity of IL-1ra K145D. However, the insertion of the β-bulge (QGEESN) of IL-1β at the corresponding region of IL-1ra K145D resulted in a 3–4-fold augmentation of bioactivity. An additional increase in agonist activity was observed when the β-bulge was coexpressed with a second substitution (HIS-S4 → Pro) in IL-1ra K145D. We also show that the bioactivity of both IL-1ra K145D and the triple mutant IL-1ra K145D/H54P/QGEESN is dependent on interaction with the newly cloned IL-1 receptor accessory protein.

The interleukin 1 (IL-1) family of cytokines includes two potent mediators of inflammatory and immune responses, IL-1α and IL-1β (1). The third member of this family is naturally occurring inhibitor of IL-1 responses termed the IL-1 receptor antagonist (IL-1ra) (2, 3). IL-1α and IL-1β mediate cellular signals by binding to an 80-kDa cell surface receptor, the Type I IL-1 receptor (Type I IL-1R), which is found mainly, but not exclusively, on T cells and fibroblasts (4–6). IL-1α and IL-1β also bind to a 68-kDa cell surface receptor found predominantly on B cells and neutrophils (the Type II IL-1 receptor) (5–7). Recent studies indicate that only the Type I IL-1R is capable of transducing an IL-1 signal, whereas the Type II IL-1R is dispensable for signaling and may act as a decoy receptor (8–10). IL-1ra binds to the Type I IL-1R with an affinity equivalent to the IL-1 agonists and inhibits the binding of both IL-1α and IL-1β (2, 3, 11, 12). In contrast to IL-1α and IL-1β, IL-1ra elicits no discernible IL-1-associated responses (2, 11, 13, 14); therefore, IL-1ra acts as a pure receptor antagonist.

The mechanism by which IL-1ra can interact with the Type I IL-1R but not trigger a biological response is still unclear. The amino acid sequence of IL-1ra is 19% identical to that of IL-1α and 26% identical to that of IL-1β (3). Despite this relatively low sequence conservation, all three ligands share a common tertiary structure as determined by x-ray crystallography and NMR (15–20). Each of these proteins is comprised of 12 β-strands, which form a β-barrel structure closed at one end. Thus, amino acid sequence alignments and structural comparisons among these ligands have not shed light on the basis for their functional differences.

Because the Type I IL-1R appears to be essential for IL-1-induced biological responses, the molecular interactions of the IL-1 ligands with this receptor have been well characterized. Extensive mutagenesis studies of both IL-1α and IL-1β have identified the amino acid residues important for binding of these ligands to the Type I IL-1R (21–26). In these studies, all of the residues involved in binding to the Type I IL-1R, although not contiguous, are located on one face of the IL-1 structure (the open end of the β-barrel).

Mutagenesis studies have indicated that residues essential for IL-1 biological activity could be distinguished from the residues required for receptor binding. Substitution of Thr-9 (numbering of the mature protein) (27), Arg-11 (28), and Asp-145 (29) in IL-1β each resulted in significantly reduced bioactivity with little effect on binding to the receptor. These amino acid residues are located in regions that are distinct and at a distance from the Type I IL-1R binding site. To date, the molecular mechanisms by which these residues contribute to IL-1 agonist activity have not been elucidated.

In addition to the residues described above, a region of charged amino acids (a β-bulge) positioned between β-strands 4 and 5 has been implicated in the ability of IL-1β to transduce a signal through the IL-1R (30). The β-bulge residues (Gln-48 to Asn-53) form a protrusion on the edge of the open end of the β-barrel adjacent to the receptor binding epitope. Direct evidence for the importance of these amino acids comes from mutagenesis studies in which deletions or substitutions of residues in this region reduced IL-1β agonist activity without affecting receptor binding (31, 32). In addition, one group has suggested that a synthetic peptide containing the β-bulge residues has immunostimulatory activity (33–36), although independent confirmation of these results is not available. A similar β-bulge is present at the homologous position in IL-1α, but absent in IL-1ra.

Previously, we provided evidence for a role for Asp-145 in IL-1β agonist activity (29). Whereas the substitution of Lys for Asp-145 of IL-1β greatly reduced agonist activity, we showed that the corresponding substitution in IL-1ra (Lys-145 → Asp) conferred partial agonist activity to the receptor antagonist. In

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$ Present address: Dept. of Allergy, Schering-Plough Research Institute, Schering-Plough Corp., Kenilworth, NJ 07033.

† To whom correspondence should be addressed. Tel.: 201-235-5621; Fax: 201-235-5046.

§ The abbreviations: IL-1, interleukin 1; IL-1α, interleukin 1α; IL-1β, interleukin 1β; IL-1ra, interleukin 1 receptor antagonist; IL-1R, interleukin 1 receptor; IL-1R AcP, interleukin 1 receptor accessory protein; hu, human; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; mAb, monoclonal antibody.
In the present study, we extend our examination of the regions of IL-1 important for agonist activity by creating second site mutations in IL-1ra K145D that further increase agonist activity. In addition, we present evidence to suggest that the lack of agonist activity of IL-1ra is a result of its inability to interact with the newly cloned and characterized IL-1 receptor accessory protein (IL-1R AcP) (37).

**MATERIALS AND METHODS**

**Bacterial Strains and Plasmids**—The human IL-1ra coding sequence was obtained by polymerase chain reaction (PCR)-directed amplification from a human lung cDNA library (Stratagene), using primers based on the published sequence (3). The Escherichia coli plasmid used for IL-1ra and IL-1ra analog production was pDS56-RBSII (38), which contains the isopropyl-1-thio-β-D-galactopyranoside-inducible icl promoter promoter P<sub>lac</sub>..<sub>68</sub>. All IL-1ra expression plasmids were propagated in E. coli strain MC1061 transformed with the compatible plasmid pDM1, which encodes the lac repressor (39). Mutations were constructed using PCR-directed mutagenesis (40). Primers encoding each mutation also contained a new restrictionendonuclease cleavage site so that incorporation of the oligonucleotide at the correct location could be monitored. Mutations of interest were confirmed by nucleotide sequence analysis as described previously (29).

**Preparation of Crude Extracts of IL-1ra Analogs**—E. coli transformed with a plasmid encoding IL-1ra expression plasmids were grown at 37°C to mid-logarithmic phase (A<sub>600</sub> = 0.4) in M9 media. Isopropyl-1-thio-β-D-galactopyranoside was added to 1 mM to induce protein production, and the cells were grown to A<sub>600</sub> = 1.0. Aliquots (1.5 ml) were pelleted at 13,000 x g for 20 min at 4°C, and supernatants were removed. The pellets were either stored at -20°C or processed immediately for immunoprecipitation. For each mutation, crude extracts of at least three independent isolates were prepared. Each series of extracts of mutant analogs were prepared in parallel with extracts of cultures induced to produce either IL-1ra or IL-1ra K145D. In this way, extracts of IL-1ra analogs could be directly compared to extracts of IL-1ra K145D.

**Purification of IL-1ra Analog Proteins**—Human IL-1ra analog proteins were purified as follows. Ten grams of E. coli cells (stored at -90°C) induced to express analog protein were thawed and gently suspended in 100 ml of cold 50 mM Tris-HCl, pH 8, containing 20% sucrose, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 2 μg of aprotinin/ml. The cell suspension was centrifuged at 100,000 x g for 10 min, and pellets were gently resuspended in 100 ml of cold H<sub>2</sub>O containing 1 mM EDTA and incubated at 4°C for 1 h. Intact cells and debris were removed by centrifugation. Supernatants from both low speed centrifugations were pooled, and the sample was centrifuged at 100,000 x g for 1 h. The supernatant, containing soluble IL-1ra analog protein, was applied to a Sephacryl S-200 column (1.6 x 88 cm) equilibrated with 20 mM Tris-HCl, pH 7.5. Bound proteins were eluted with a 400-ml linear gradient of 0–0.5 M NaCl in 20 mM Tris-HCl, pH 7.5. The fractions containing IL-1ra analog protein were pooled, concentrated, and applied to a Sephacryl S-200 column (1.6 x 88 cm) equilibrated with 20 mM Tris-HCl containing 200 mM NaCl, pH 7.5. Each IL-1ra analog protein eluted from the sizing column as a monomer (mass ~19–20 kDa). The fractions containing the IL-1ra analog protein were pooled, concentrated, and dialyzed into phosphate-buffered saline. The analog proteins were purified to homogeneity as determined by SDS-PAGE and NH<sub>2</sub>-terminal sequence analysis.

**Purified Human IL-1ra (huIL-1<sub>ra</sub>)** was supplied by Synergen Inc. Purified wild-type huIL-1<sub>ra</sub> (huIL-1<sub>rp</sub>) was the gift of S. Roy, Hoffmann-La Roche Inc.

**IL-1 Receptor Binding Assays**—Binding of IL-1ra analogs to IL-1 receptors on EL-4 cell membranes was assessed by competitive inhibition plots. The 125I-huIL-1<sub>ra</sub> analog and both substitutions at Cys-122 had little or no effect on either the binding or bioactivity of IL-1ra K145D. These results indicate that the identity of these residues is not critical to the structure or activity of the protein. These data also suggest that Cys-122 is not required for disulfide bond formation. The substitution of Thr-108 with Lys resulted in a 2-fold increase in receptor binding activity compared to wild-type IL-1<sub>ra</sub> (29).

**RESULTS**

In a previous study, we showed that the substitution of a single amino acid (Asp-145 → Lys) in huIL-1<sub>ra</sub> reduced bioactivity by ~90%, while retaining 100% binding to the Type I IL-1<sub>R</sub> (29). We noted that this analog of IL-1<sub>ra</sub>, designated IL-1<sub>ra</sub> D145K, had a phenotype similar to IL-1α, i.e. binding to the Type I IL-1R, no binding to the Type II IL-1R, and reduced biological activity compared to wild-type IL-1<sub>β</sub> (29). Alignment of the amino acid residues of IL-1<sub>α</sub> and IL-1<sub>ra</sub> indicated that Asp-145 of IL-1<sub>ra</sub> aligned with Lys-145 of IL-1<sub>α</sub>. The corresponding mutation (K145D) was made in IL-1<sub>ra</sub>, this mutant analog (IL-1<sub>ra</sub> K145D) maintained receptor binding and gained partial agonist activity (29). These observations implicated Asp-145 in IL-1<sub>ra</sub> agonist activity and suggested that relatively minor alterations of the protein could have marked effects on biological activity. In this study, we extend our examination of regions important for IL-1 agonist activity by constructing second site mutations in the IL-1ra K145D analog using PCR-based site-directed mutagenesis. Each analog was examined for Type I IL-1<sub>R</sub> binding on EL-4 cell membranes (as a measure of conformational integrity) and for agonist activity in the D10 proliferation assay (29). We were interested in those analogs that gained significant agonist activity (>2-fold increase) while maintaining receptor binding.

**Construction and Analysis of IL-1ra Analogs**—Five amino acid residues in IL-1ra were initially targeted for mutagenesis. Mutations were made at Val-18, Thr-108, Cys-116, Cys-122, and Tyr-147 because the side chains of these residues appear to be in close proximity to Lys-145 in IL-1<sub>ra</sub>, based on its crystal structure (20). These five residues in IL-1ra were also chosen because they differ in IL-1α and IL-1<sub>β</sub> when the amino acid sequences were aligned and compared (3). Substitutions were made at each of these five positions in IL-1ra K145D, usually by replacement with the corresponding amino acid of IL-1<sub>β</sub> at each position. Extraction containing each substitution analog were analyzed for the relative binding to murine EL-4 membrane bioactivity (Table I). Activities are compared to extracts of IL-1ra K145D prepared from cultures induced in parallel (see "Materials and Methods").

As shown in Table I, none of the substitutions at the five positions listed above resulted in the desired effect. The mutation at Cys-116 had little or no effect on either the binding or bioactivity of IL-1ra K145D. These results indicate that the identity of these residues is not critical to the structure or activity of the protein. These data also suggest that Cys-122 is not required for disulfide bond formation. The substitution of Thr-108 with Lys resulted in a >2-fold increase in receptor binding activity compared to wild-type IL-1<sub>ra</sub> (29).

**D10 Proliferation Assay**—The activity of IL-1ra analogs was determined using D10.N4.M proliferation as described previously (42).

Briefly, samples of crude extracts or purified proteins were serially diluted in RPMI 1640 medium (Life Technologies, Inc.) containing 5% fetal bovine serum, 0.5 μg/ml mercaptoethanol, 8 μg/ml gentamicin (Life Technologies, Inc.), 3 mg/ml concanavalin A (Sigma), and 40 units/ml recombinant human IL-2 (provided by M. Gately and P. Bailon, Hoffmann-La Roche Inc.). Each sample (100 μl) was added to the wells of a 96-well plastic plate along with 5 x 10<sup>5</sup> D10 cell line cells suspended in the above medium. After 72 h at 37°C (5% CO<sub>2</sub>) the sample was added and incubation continued another 6 h. The amount of radioactivity incorporated during this incubation period was then determined. The effective dose (ED<sub>50</sub>) was defined as the dilution or concentration of sample that resulted in 50% maximal [3H]thymidine incorporation. The ability of various mAbs to inhibit the agonist activity of purified proteins was examined by performing the above assay with a constant concentration (2–3-fold above the ED<sub>50</sub>) of IL-1ra analogs or huL-1β and a titration of each of the various mAbs. The percent activity was determined as a ratio of the activity of the IL-1ra analog or IL-1β in the presence of each mAb versus the activity in the absence of the mAb.

2 M. Hatada, unpublished results.
Relative binding and bioactivity of crude extracts of IL-1ra analogs

Crude extracts of IL-1ra analogs were prepared and analyzed in the EL-4 membrane and D10 proliferation assays as described under “Materials and Methods.” The relative binding to EL-4 membranes was calculated as the ratio of the IC50 of each analog to that of IL-1ra. The relative bioactivity in the D10 proliferation assay was calculated as the ratio of the ED50 of each analog to that of IL-1ra K145D. All mutations were made in the IL-1ra K145D analog. QGEESN refers to the β-bulge region from IL-1β.

| Mutation       | Aligned residue in IL-1β | Bindinga | Bioactivityb |
|----------------|-------------------------|----------|--------------|
| IL-1ra K145D   | Asp                     | 1.0      | 1.0          |
| V185           | Ser                     | 1.0      | 1.0          |
| T308K          | Lys                     | 0.7      | 0.3          |
| C116F          | Phe                     | 1.0      | <0.04        |
| C122S          | Ser                     | 1.0      | 1.0          |
| C122A          |                       | 1.0      | 1.7          |
| Y147T          | Thr                     | <0.1     | <0.04        |
| Y147G          | Ser                     | 1.0      | <0.04        |
| H54P           | Ile                     | 1.0      | 2.0          |
| H54I           |                       | 1.0      | <0.04        |
| Insertion      |                         |          |              |
| QGEESN (Ile-51) |                         | 1.0      | 3.6          |
| QGEESN (Pro-53) |                         | 1.0      | 3.1          |
| QGEESN/H54P    | (Ile-51)                | 1.0      | 8.0          |

a EL-4 membrane assay.
b D10 proliferation assay.

Table I

The complete loss of bioactivity seen with the Cys-116 replacement in IL-1ra analogs indicates that there are no gross alterations in the overall structure of the C116F analog. These data also indicate that the overall structure of the protein or that Lys is not tolerated in this position.

More significant effects were seen when substitutions were made at Cys-116 in IL-1ra K145D. Replacement of Cys-116 with Phe resulted in the complete loss of bioactivity, whereas full receptor binding activity was maintained. The observation that receptor binding activity is preserved indicates that there are no gross alterations in the overall structure of the C116F analog. The complete loss of bioactivity seen with the Cys-116 → Phe substitution is difficult to understand. Cys-116 in IL-1ra overlaps Phe-117 in IL-1β, and it appears that the phenyl ring in the C116F analog could be accommodated in the IL-1ra structure.

Varying results were obtained depending on whether Tyr-147 was substituted with Thr or with Gly in the IL-1ra K145D analog. The Y147T analog lost all detectable activity (both binding and bioactivity), whereas the Y147G analog lost all detectable binding but retained 100% bioactivity. These data suggest that Tyr-147 is important for bioactivity of IL-1ra K145D, either by direct interaction with signaling proteins for the local conformation. These data also indicate that the overall structure of IL-1ra K145D will not tolerate Thr at position 147.

Since none of the single amino acid substitutions discussed above resulted in increased bioactivity, the sequence alignments of IL-1ra and IL-1β (3) were examined for regions in which short segments of IL-1β residues were absent from IL-1ra. One such region includes the six amino acids comprising the loop between the fourth and fifth β-strands of IL-1β (30, 43). These six amino acids (Gln-48 to Asn-53) have been termed a β-bulge and have been implicated in early IL-1 signaling events and the immunostimulatory properties of IL-1 (30, 33–36). These six amino acids or similar β-bulge-like structure are absent from IL-1ra. Therefore, the β-bulge region from hIL-1β was inserted into IL-1ra K145D either after Ile-51 or after Pro-53; the position of these two amino acids in IL-1ra aligns with the location of the β-bulge in IL-1β. Two sites were chosen for insertion due to ambiguities in the structural alignment of IL-1ra and IL-1β in this region (3). The insertion of the β-bulge (QGEESN) after either position 51 or 53 of IL-1ra K145D resulted in analogs that retained full Type I IL-1R binding and increased bioactivity 3–4-fold (Table I).

We next tested the ability of the QGEESN insertion to impart agonist activity to IL-1ra in the absence of the K145D mutation. The β-bulge was inserted either after amino acid 51 or 53 of IL-1ra. None of the plasmid clones with the insertion at position 51 of IL-1ra produced the appropriate protein, suggesting that this analog is extremely unstable in E. coli. We were able to isolate clones with the insertion at position 53, which produced protein; however, these analogs retained only 10–20% of the Type I IL-1R binding activity (data not shown). These data indicate that the insertion of QGEESN into IL-1ra at either position 51 or 53 results in gross distortion of the structure. This structural alteration was not seen in the IL-1ra K145D/QGEESN analogs.

Further analysis of the QGEESN insertion analogs revealed a surprising variant. In the case of the QGEESN insertion after Ile-51 of IL-1ra K145D, five individual isolates were analyzed. Four isolates were similar; each yielded increases in bioactivity in the range of 3–4-fold (Table I). The fifth isolate had an 8-fold increase in bioactivity. The plasmid DNA encoding this fifth isolate was isolated and the nucleotide sequence of the mutated region determined. The DNA sequence revealed that, in addition to the QGEESN insertion, the His-54 codon was mutated to encode Pro in this clone, probably as a result of the PCR amplification used to create this analog. We then analyzed the contribution of the H54P substitution by incorporating this substitution into the IL-1ra K145D analog. As shown in Table I, altering His-54 → Pro in IL-1ra K145D results in a modest 2-fold increase in bioactivity while maintaining receptor binding. This result indicates that the increased bioactivity of the triple mutant IL-1ra K145D/H54P/QGEESN is a result of the additive effects of the individual mutations. Additionally, when the IL-1ra K145D/H54I analog (replacing His-54 with the aligned IL-1β amino acid Ile) was made and analyzed, all bioactivity was lost (Table I). These data indicate that the 2-fold increase in activity is due to the introduction of Pro and not the removal of His at position 54 in IL-1ra K145D.

Analysis of Purified IL-1ra Analog Proteins—To characterize in more detail the properties of IL-1ra K145D/H54P/QGEESN, this analog and IL-1ra K145D were purified to homogeneity as described under “Materials and Methods.” The binding of these purified proteins to the murine Type I IL-1R was assessed by competitive binding with 125I-IL-1α (23) and compared to pu-
We have recently shown by cross-linking analysis that this protein (the murine IL-1R accessory protein (muIL-1R AcP)) forms a complex on the cell surface with the Type I IL-1R and either IL-1α or IL-1β (37). Interestingly, IL-1ra does not form a similar cross-linked receptor complex but only interacts with the Type I IL-1R. The observation that only the IL-1 ligands that have agonist activity associate with the IL-1R AcP suggests that interaction with the IL-1R AcP is required for IL-1 signal transduction. In light of these findings, we tested the ability of mAb 4C5 (anti-muIL-1R AcP) to inhibit the bioactivity of IL-1β, IL-1ra K145D, and IL-1ra K145D/H54P/QGEESN proteins.

Fig. 3 shows the results of inhibition of the bioactivity of IL-1β and the two IL-1 analogs by mAb 4C5 (anti-muIL-1R AcP), mAb 35F5 (anti-muType I IL-1R) (5), or a control mAb 7B2 (anti-IL-12) (44). As described under “Materials and Methods,” the amounts of IL-1β and the IL-1ra analog proteins used in this assay were chosen to induce equivalent amounts of proliferation (2-3-fold above ED50). Panel A shows that mAb 35F5 inhibits the bioactivity of IL-1β in a dose-dependent manner, whereas the control mAb 7B2 has no inhibitory activity. The mAb 4C5 (anti-muLL-1R AcP) also is capable of blocking completely the activity of IL-1β, although it appears to be less potent than mAb 35F5. The same pattern of inhibition by these mAbs is seen for both IL-1ra K145D and IL-1ra K145D/H54P/QGEESN (Fig. 3, B and C, respectively). These data indicate that, like IL-1β, the bioactivity of IL-1ra K145D and IL-1ra K145D/H54P/QGEESN requires interaction with both the Type I IL-1R and IL-1R AcP on D10 cells. One interesting difference between IL-1β and the IL-1ra analogs was noted. There was an ~25-fold increase in the amount of mAb 4C5 needed to neutralize the activity of IL-1β (IC50 ~2.5 μg/ml), compared to the amount of mAb 4C5 that inhibited the IL-1ra analogs (IC50 ~0.1 μg/ml).

**DISCUSSION**

Amino acid changes that confer agonist activity to IL-1ra have yielded insight into the regions of the IL-1 ligands that are important for bioactivity. We have generated second site mutations in the partial agonist IL-1ra K145D (29) in an attempt to increase agonist activity while retaining IL-1R binding. We have increased the bioactivity of IL-1ra K145D 3-fold by the insertion of the IL-1β bulge into IL-1ra K145D. This activity is further augmented 2-fold by the substitution of His-54 with Pro. Our data suggest that the agonist activity of IL-1ra K145D and IL-1ra K145D/H54P/QGEESN is likely due to the ability of these analogs to interact with the newly doned IL-1 receptor accessory protein (37).

Structural alignment of IL-1β, IL-1α, and IL-1ra reveals that both IL-1β and IL-1α have a β-bulge structure between the fourth and fifth β-strands, whereas no such structure is found in IL-1ra. We therefore inserted the six amino acids comprising the IL-1β bulge into IL-1ra K145D in an attempt to augment agonist activity. There have been suggestions from previous studies that the β-bulge region of IL-1β (QGEESN, residues 48–53) may be important for bioactivity. Simoncits et al. (32) have recently shown that deletion of amino acids 52–54 (SND) in IL-1β reduces Type I IL-1R binding by 10-fold and biological activity by 1000-fold. In addition, the substitution of the β-bulge with loops from various protease inhibitors led to a reduction in bioactivity without a significant effect on binding to EL-4 cells (31). One group has also suggested that a synthetic peptide derived from IL-1β (VQGEESNDK), which con-

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**TABLE II**

| Protein          | Binding | Bioactivity |
|------------------|---------|-------------|
|                  | IC50    | ED50        |
| IL-1β            | 2.18    | 2.04        |
| IL-1ra           | 0.47    | >10,000     |
| IL-1ra K145D     | 0.53    | 342         |
| IL-1ra K145D/H54P/QGEESN | 0.63 | 38          |

*Not applicable.*
tains the six β-bulge amino acids, has immunostimulatory but no inflammatory effects normally associated with IL-1 (33–35). Additionally, this same group has shown that the insertion of VQGEESNDK into recombinant human ferritin H chain and recombinant flagellin from Salmonella muenchen increased the immunogenicity of these antigens in mice (36).

We compared the activities of purified IL-1β, IL-1ra K145D, and IL-1ra K145D/H54P/QGEESN to further characterize the changes that lead to agonist activity. Since we have postulated that the IL-1R AcP is involved in IL-1 signal transduction, we tested the ability of mAb 4C5 (anti-muIL-1R AcP) to inhibit the bioactivity of the purified IL-1ra mutants. As expected, the bioactivity of IL-1β was fully inhibited by mAb 4C5 (Fig. 3A). This mAb was also able to inhibit completely the activity of both IL-1ra K145D and IL-1ra K145D/H54P/QGEESN (Fig. 3, B and C). The observation that 4C5 inhibits the activity of the IL-1ra analogs confirms that the agonist activity of IL-1ra K145D and IL-1ra K145D/H54P/QGEESN involves an association with the IL-1R AcP. These data would suggest that IL-1ra fails to elicit biological responses because it is unable to interact productively with the IL-1R AcP. Consistent with this hypothesis, we have shown previously, using protein cross-linking, that both IL-1α and IL-1β associate with a complex of the Type I IL-1R and IL-1R AcP on the cell surface, whereas IL-1α associates only with the Type I IL-1R (37). Cross-linking studies to demonstrate direct interactions between the IL-1ra analogs and the IL-1R AcP are in progress.

Since the bioactivity of IL-1ra K145D is completely inhibited by mAb 4C5, it appears that this single amino acid substitution is sufficient to allow IL-1ra to establish a productive interaction with the IL-1R AcP. It is not clear whether this association is through the direct binding of Asp-145 to the IL-1R AcP or if the K145D substitution affects local secondary structure, allowing other amino acids to interact. The latter possibility is unlikely, since the crystal structure of the IL-1β D145K analog is identical to wild-type IL-1β (23). The mechanisms by which the insertion of VQGEESN and the H54P substitution increase agonist activity of IL-1ra are also not known. Interestingly, the ~8–9-fold increase in bioactivity of IL-1ra K145D/H54P/QGEESN compared to IL-1ra K145D appears to be the cumulative effect of all three mutations, suggesting that each mutation can contribute in an additive manner to enhance agonist activity.

The cumulative effects of these three mutations are also interesting since their positions on the IL-1ra protein appear to be spatially separated (Fig. 4). Ile-51 and His-54 are located on the open face of the β-barrel of IL-1ra, whereas Lys-145 is located away from the open barrel end (20). In IL-1β, the same relative positions of the β-bulge and Asp-145 are observed (15, 16, 18, 30) with the two regions separated by the known Type I IL-1R binding site (Fig. 4). As discussed above, it is difficult to assess whether the β-bulge or residue 145 is important for direct intermolecular interaction (e.g. with the IL-1R AcP) or to maintain the local conformation.

A direct measurement of binding of the IL-1ra analogs to the IL-1R AcP is not possible because the accessory protein does not bind IL-1 ligands except in the presence of the Type I IL-1R (37). However, the observation that ~25-fold more mAb 4C5 is needed to inhibit IL-1β bioactivity on D10 cells compared to the amount of mAb 4C5 necessary to block the IL-1ra analogs (Fig. 3) suggests that the interaction of the analogs with the IL-1R AcP may be of lower avidity than that of IL-1β. The relatively weak interaction of the IL-1ra analogs with the accessory protein would then account for their lower agonist activity compared to IL-1β.

We attempted to assess the ability of the VQGEESN insertion to enhance agonist activity of IL-1ra in the absence of the K145D substitution. Whereas the insertion of the β-bulge into IL-1ra K145D augmented agonist activity, the insertion of the β-bulge into IL-1ra appeared to reduce receptor binding as well as bioactivity. We also attempted to remove the six β-bulge amino acids from IL-1β. This deletion led to very poor protein production and poor receptor binding, suggesting that this region plays a role in the structural stability of IL-1β.

In addition to the β-bulge insertion, an alternative strategy was the substitution of amino acids in IL-1ra that were predicted to be in close proximity to Lys-145 with the corresponding aligned residues in IL-1β. None of the single amino acid substitutions that we created yielded a significant increase in the biological activity of IL-1ra K145D (Table I). The Tyr at position 147 appears to be the most important of the amino acids we tested since substitution of this residue abolished the biological activity of IL-1ra K145D. Substitution at this position with Thr also abolished binding, indicating that the structure of this analog was drastically altered. Residue 116 also appears to be important since substitution at this site with Phe abolished the biological activity of IL-1ra K145D. It is unclear what role(s) Tyr-147 and Cys-116 play in agonist activity since neither residue is conserved in IL-1β or IL-1α.

The observation that His-54 → Pro substitution is able to augment agonist activity of both IL-1ra K145D and IL-1ra

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4 G. J. u and E. Labriola-Tompkins, unpublished results.
IL-1ra Agonist Activity

K145D/QGEESN (Table I) indicates that the residue at position 54 plays an important role independent of the β-bulge insertion. Although it is offset by one residue in the structural alignment, the mutant Pro-54 in IL-1ra may mimic effects of Pro-57 in IL-1β. We have not determined whether the His-54 → Pro substitution enhances activity in the absence of the K145D substitution. The analogous substitution in IL-1β (Ile-56 → Pro) led to a complete loss of binding and bioactivity. These data are not surprising, since Ile-56 has been identified as a critical residue in the binding site in IL-1β (23). These data suggest that His-54 in IL-1ra may not play the same role as Ile-56 in IL-1β.

The effect of insertion of the β-bulge into IL-1ra K145D confirms the importance of this region for IL-1 biological activity. The inhibition of both IL-1ra K145D and IL-1ra K145D/H54P/QGEESN by mAb 4C5 (anti-muIL-1R AcP) supports the hypothesis that IL-1R AcP is involved in IL-1 agonist activity. Our results also begin to elucidate the mode of action of IL-1ra. The antagonist function of IL-1ra is most likely the result of two characteristics: 1) the ability to compete with IL-1α and IL-1β for binding to the Type I IL-1R, and 2) the inability to form a productive complex with the Type I IL-1R and IL-1R AcP. Since IL-1ra K145D/H54P/QGEESN only regains ~5% of the agonist activity of IL-1β, insertion or substitution of other amino acid residues may lead to additional increases in biological activity.

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