DELETION MUTANTS OF HUMAN INTERLEUKIN 1β WITH SIGNIFICANTLY REDUCED AGONIST PROPERTIES: SEARCH FOR THE AGONIST/ ANTAGONIST SWITCH IN LIGANDS TO THE INTERLEUKIN 1 RECEPTORS

András Simoncsits,[1] Jesper Bristulf,[1] Marie Louise Tjörnhammar,[1] Miklos Cserzö,[2,3] Sandor Pongor,[2,4] Elena Rybakina,[5] Silvia Gatti,[6] Tamas Bartfai[1]

The existence of an endogenous high affinity interleukin 1 receptor antagonist (IL-1ra) suggests that this molecule lacks some structural motif(s) which are present in the closely homologous agonist interleukin 1β (IL-1β) and which serve as the 'agonist switch' causing signal transduction by the agonist-receptor complex. The primary sequence alignment of IL-1β and IL-1ra sequences from different species reveals a six amino acid long motif that is quasi conserved among IL-1β sequences, but is missing from the IL-1ra sequences. The three-dimensional structure of human IL-1β was used as a template for building structural models of deletion mutants (ΔSND 52–54 and ΔEESNDK 50–55) using molecular graphics. These models indicated that the middle three residues SND 52–54 from the EESNDK 50–55 loop may be deleted without causing major changes in the tertiary structure of the mutant as compared to that of IL-1β. Residues SND 52–54 from the above loop were deleted. When compared with IL-1β the IL-1β-ΔSND analog (ΔSND 52–54) binds with the same affinity to type 2 IL-1 receptor but with a more than 10-fold lower affinity to type 1 IL-1 receptor. Despite of this small decrease in affinity at the type 1 receptor the ΔSND 52–54 has a 1000-fold lower biological activity than IL-1β when tested in a thymocyte activating factor assay. In vivo the ΔSND 52–54 mutant has also significantly reduced efficacy in stimulating serum corticosterone and IL-6 levels. The above findings suggest that the SND loop plays an important role in the agonist type interactions between IL-1β and IL-1 receptors.

The cytokines interleukin 1α and β (IL-1α and IL-1β) are mediators of a multitude of processes involving the immune, endocrine and nervous system.[1] These two small proteins of approximately 150 amino acids have been extensively studied during the past years and the cDNA sequences for IL-1α and β are known from several species as well as the three-dimensional structure for human IL-1α and human IL-1β. Althought IL-1α and β only show about 25% amino acid sequence homology, they seem to adopt the same type of three-dimensional folding pattern[2] and appear to be recognized with high affinity by the same IL-1 receptors.[3,4] Discovery and cloning of an endogenously occurring IL-1 receptor antagonist (IL-1ra) which belongs to the same protein family as IL-1α and β,[5–7] and which antagonizes the action of these agonists by binding to the same receptor with high affinity has further accelerated studies on the IL-1 receptor–ligand interactions, especially since lowering of mortality in septic shock by the IL-1ra were demonstrated.[8] Due to the biological and pharmaco-

From the [1] Department of Neurochemistry and Neurotoxicology, Stockholm University, Stockholm, Sweden; [2] International Centre for Genetic Engineering and Biotechnology, Trieste, Italy; [3] Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences, Budapest, Hungary; [4] ABC Institute for Biochemistry and Biotechnology, Gödöllö, Hungary; [5] Institute of Experimental Medicine, Russian Academy, St Petersburg, Russia; [6] Mario Negri Institute for Pharmacological Research, Milan, Italy.

Correspondence to: Tamas Bartfai, Department of Neurochemistry and Neurotoxicology, Stockholm University, 106 91 Stockholm, Sweden.

In memory of the late Professor Bertil Åberg, a pioneer in biotechnology who died in May 1992.

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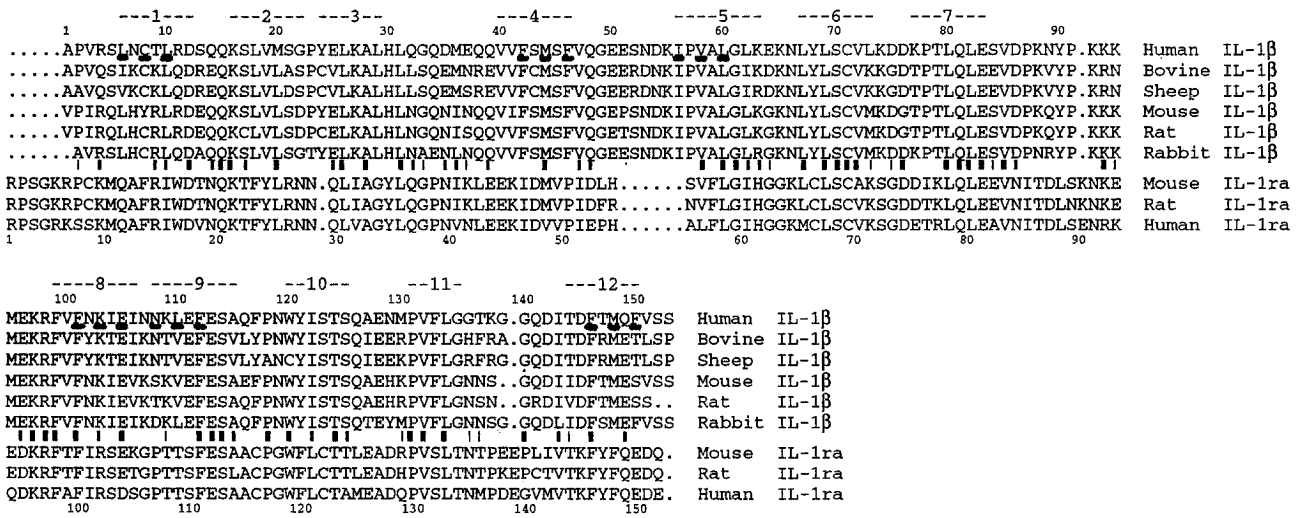

Figure 1. Primary sequence alignment between IL-1β and IL-1ra from various species.

Broken lines contain the numbering of the β-strands according to Murzin et al.[24] Underlined residues in human IL-1β shows residues inside the barrel formed by β-strands 1, 9, 5, 8, 3 and 12. Homologies between IL-1β and IL-1ra sequences are indicated by: (▌) where seven out of nine sequences have identical residues; (❙) seven out of nine sequences have similar residues; and (❘) where a residue in any IL-1ra sequence is also present in at least one of the IL-1β sequences. Numbering is for human IL-1β and IL-1ra, respectively.

logical importance of Il-1α and β a large number of mutants and analogs of both proteins have been examined concerning their effect on biological and receptor binding properties. Important amino acid residues for binding to IL-1 receptors and for agonist properties have been identified throughout the IL-1β molecule.[9–16] The IL-1α molecule has been subject to similar studies.[17–19]

We decided to base our structure activity relation studies on the information concerning the primary structure of the IL-1ra and set out to compare the primary sequences of IL-1β with IL-1ra in the hope of finding regions that might explain the functional (agonist/antagonist) differences between these two molecules.

RESULTS

Multiple alignment of IL-1ra and IL-1β sequences from different species (Fig. 1) shows that conserved residues are predominantly located in the β-strands which constitute the barrel core of the IL-1β molecule.[11,20,21] The most prominent differences between IL-1ra and IL-1β at the primary sequence level were the different N-terminals and a gap in IL-1ra sequence where there is a loop between the fourth and the fifth β-strand of the IL-1β molecule (Fig. 1). Sequence alignment between sequences of IL-1α and IL-1β shows that this region is also preserved, albeit with somewhat different residues in IL-1α (data not shown), and these residues are located between the fourth and the fifth β-strand of the IL-1α molecule.[2] Hence, the EESNDK (50–55 in human IL-1β) motif was the focus of our interest as we assumed that it may be involved in inducing those conformational changes in the receptor that distinguish the binding of an agonist from that of an antagonist. As this region is a surface loop in the IL-1β structure, it can be expected that some mutations can be accommodated within the structure, but complete deletion of this six amino acid loop could prevent the proper folding of the molecule. Three-dimensional model building based on the IL-1β structure and using energy minimization techniques reveals that the deletion of a part of the above motif, SND 52–54 can be accommodated without major changes to the adjacent and 'wide' type β-bulge[10] and β-strand regions (Fig. 2). On the other hand, deletion of the whole EESNDK 50–55 motif results in a distortion of the β-bulge which seems to be further propagated along the chain (Fig. 2). Indeed, in expression experiments in *Escherichia coli*, the ΔEESNDK 50–55 mutant yielded an insoluble protein that could not be released by the applied freeze–thaw technique while ΔSND 52–54 could be expressed as a soluble protein of good stability although the yields are lower than for the native IL-1β using the same expression vector and fermentation conditions. The ΔSND 52–54 as well as D54R were purified to homogeneity and their receptor binding properties were compared to that of human recombinant IL-1β (hrIL-1β) and

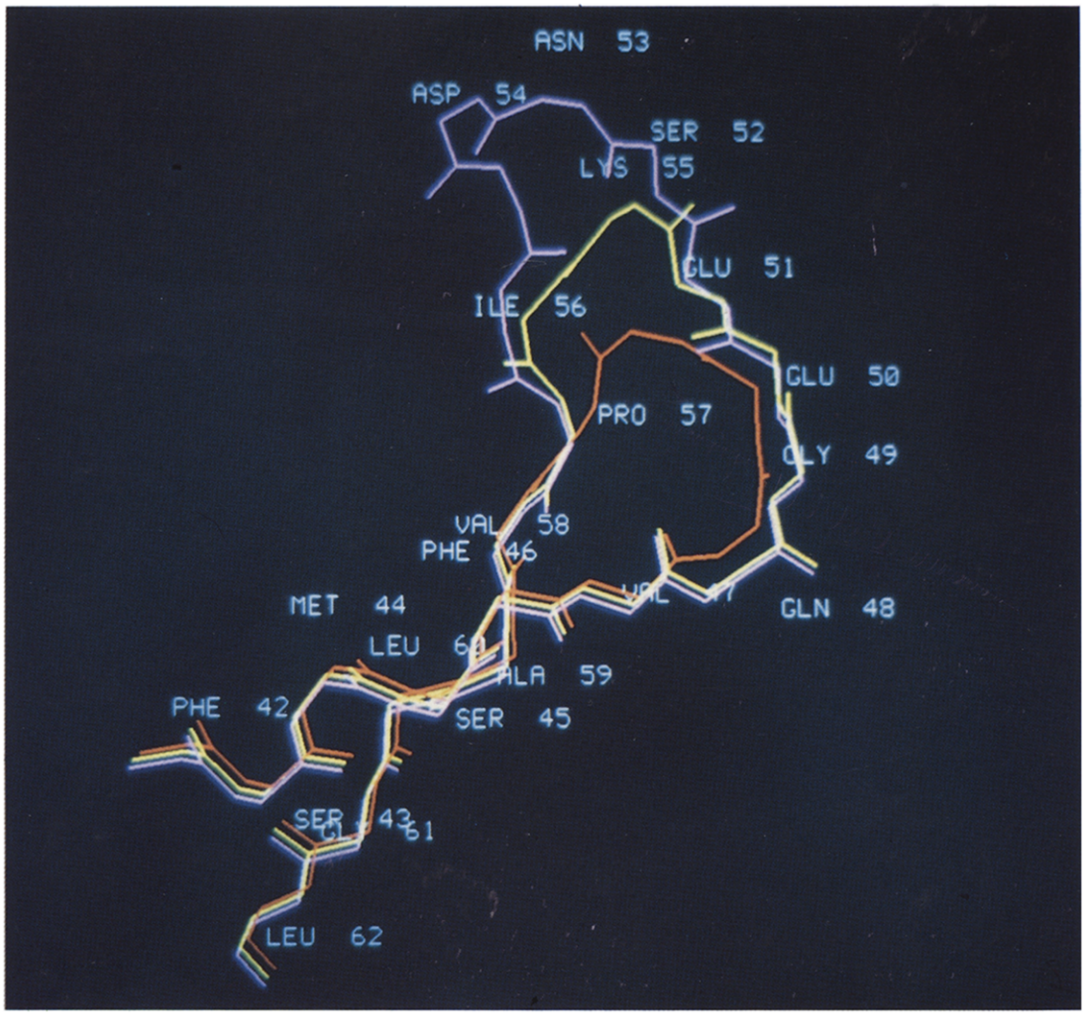

Figure 2. Three-dimensional models of the ΔSND 52–54 and ΔEESNDK 50–55 compared with IL-1β.

The region 42–65 of IL-1β is labelled purple and numbered, while the corresponding regions of ΔSND 52–54 and ΔEESNDK 50–55 are labelled yellow and red, respectively. IL-1β and ΔSND 52–54 show little structural differences while the β-bulge region of ΔEESNDK 50–55 is distorted.

human recombinant IL-1ra (hrIL-1ra) by competitive binding assays using EL-4 mouse thymoma cells, carrying predominantly type 1 IL-1 receptors,[22] and human Raji cells carrying predominantly type 2 IL-1 receptors.[4]

The ΔSND 52–54, like the endogenously occurring IL-1β and IL-1ra, has high affinity for both IL-1 receptor types. The affinity of ΔSND 52–54 and of hrIL-1β is very similar concerning binding to the type 2 IL-1 receptor where an IC_{50} value for ΔSND 52–54 of ≈5 nM and an IC_{50} value for hrIL-1β of ≈4 nM are found (Fig. 3A). At the type 1 IL-1 receptor, ΔSND 52–54 possesses however a 10-fold lower affinity than hrIL-1β thus the IC_{50} value for ΔSND 52–54 is ≈17 nM and the IC_{50} for hrIL-1β is ≈1.4 nM (Fig. 3B). The D54R mutant showed high affinity binding to both IL-1 receptor types (Fig. 3). In agreement with earlier work,[23] hrIL-1ra exhibits lower affinity towards the type 2 IL-1 receptor on Raji cells (IC_{50} ≈ 25 nM) than towards the type 1 IL-1 receptor on T cells, where it binds with an IC_{50} value of ≈0.5 nM (Fig. 2).

We then set out to examine the effects of the deletion of SND on the efficacy of IL-1β as an agonist in different IL-1 responses. Binding of hrIL-1β to mouse thymocytes stimulates ^{3}H-thymidine incorporation into these cells with a half maximum stimulation concentration of approximately 100 pg/ml (Fig. 4) while ΔSND 52–54 stimulates ^{3}H-thymidine incorporation at a 1000-fold higher concentration (Fig. 4). The hrIL-1ra shows no such agonist activity in the concentration range tested and thus behaves as a bona fide antagonist (Fig. 4). Preliminary experiments with *E. coli* extracts of the D54R analog indicate that it has an intermediate proliferating effect on mouse thymocytes compared with similar extracts of hrIL-1β and ΔSND 52–54 (data not shown).

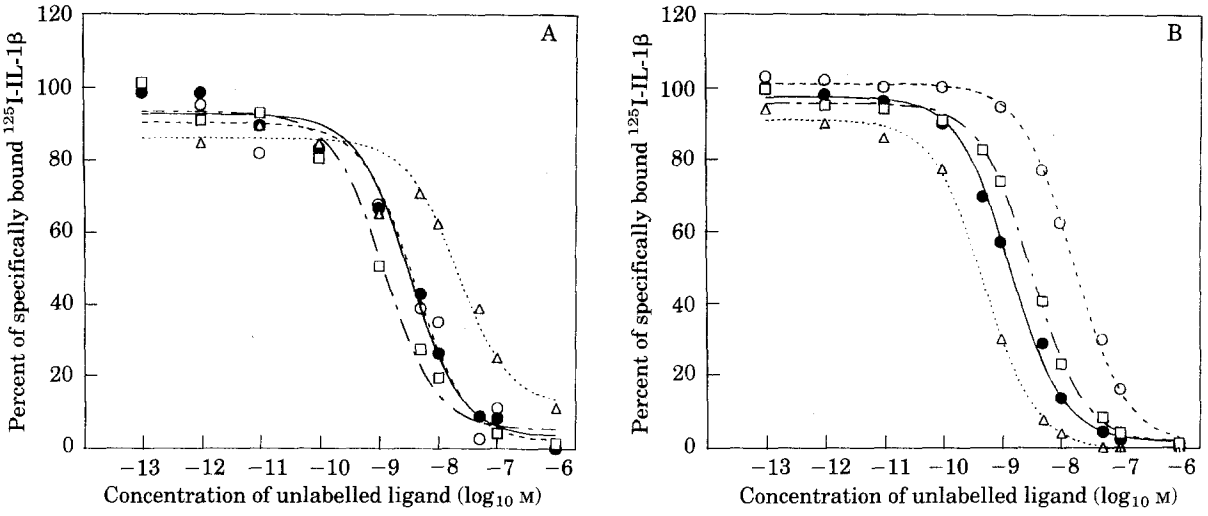

Figure 3. Competitive binding analysis of IL-1 analogs.

Raji cells (A) and EL-4 cells (B), where used as sources for type 2 and type 1 IL-1 receptors, respectively. Increasing amounts of hrIL-1β (●), ΔSND 52–54 (○), hrIL-1ra (△) and D54R (□) were used to inhibit the binding of approximately 3 nM ^{125}I-IL-1β to 5 × 10^5 cells in a total volume of 100 μl during 60 min in room temperature. Data are mean values of several independent experiments and expressed as percent specific binding of ^{125}I-IL-1β as compared to untreated controls.

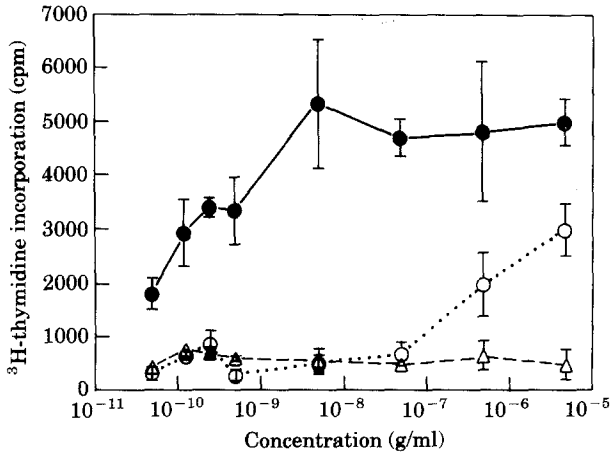

Figure 4. Biological activity of hrIL-1β, ΔSND 52–54 and hrIL-1ra.

The incorporation of ^{3}H-thymidine into mouse T cells was measured after incubation of various concentrations of hrIL-1β (●), ΔSND (○), and hrIL-1ra (△). Mean values of quadruplicates and standard deviations are shown for a typical experiment.

In vivo experiments at two dose levels of hrIL-1β and ΔSND 52–54 (100 ng and 1000 ng, i.p.) (Tables 1 and 2) with CD1 mice indicate that ΔSND 52–54 acts as a weak partial agonist in the examined IL-1-mediated responses. It is noteworthy that the ratio of efficacy of hrIL-1β and ΔSND 52–54 varies with the different responses. While 1000 ng ΔSND 52–54 causes 75% of the hrIL-1β effect with respect to glucose suppression it causes only 50% of the serum amyloid response and only 10% of the IL-6 response in mice (Table 2).

The data in Tables 1 and 2 show that the efficacy of ΔSND 52–54 as an IL-1β agonist is more significantly reduced than the binding affinity of this deletion mutant.

DISCUSSION

It is very unusual that a naturally occurring ligand acts as a bona fide antagonist and that the antagonism to a response is not exerted at another receptor or at a post-receptor level but directly at the same receptor which binds the agonists of the response. This therefore is an interesting challenge in receptor–ligand studies to define the structural prerequisites of agonist/antagonist type binding at the same receptor. This has not yet been achieved satisfactorily for small ligand interactions with their receptor (e.g. norepinephrine/propanolol-β receptor).

Since IL-1ra binds with high affinity but in an antagonistic fashion to the IL-1 receptors we hypothesized that the surface of the IL-1ra molecule should have structural features similar to IL-1β. More importantly, there must be structural features which at the same time distinguish these two molecules and render one an agonist and the other antagonist at the same receptor.

The work presented here is an attempt to evaluate the significance of the surface loop connecting the

TABLE 1. Effects of the i.p. injection of hrIL-1β and ΔSND 52–54 (100 ng/mouse) in CD1 mice.

	Saline	IL-1β	ΔSND 52–54	Percentage of IL-1β response
Glucose (mg/dl)	238 ± 15	187 ± 11*	237 ± 16	
Corticosterone (ng/ml)	156.3 ± 55	559.2 ± 95**	176 ± 60	5
IL-6 (U/ml)	<50	1439 ± 671	188.4 ± 97.5°	13

Effects of IL-1β and ΔSND 52–54 on different biological parameters in CD1 mice injected i.p. with (100 ng/mouse). Serum levels of glucose, corticosterone and IL-6 were measured 2 h after the injection. Data are mean ± SE (five mice per group). At this dose ΔSND 52–54 is only able to induce IL-6 in the serum; this effect is almost 10 times lower than the effect of the same dose of IL-1β ($P < 0.05$ vs IL-1β).
* $P < 0.05$ vs saline; ** $P < 0.01$ vs saline; ° $P < 0.05$ vs IL-1β (Dunnet's test).

TABLE 2. Effects of the i.p. injection of hrIL-1β and ΔSND 52–54 (1000 ng/mouse) in CD1 mice.

	Saline	IL-1β	ΔSND 52–54	Percentage of IL-1β response
Food-intake (g/day/mouse)	5.76	3.64	4.6	
Glucose (mg/dl)	172.2 ± 16.3	102.6 ± 2**	119 ± 8**	75
Corticosterone (ng/ml)	22.2 ± 7.6	403 ± 40**	252 ± 90*	60
SAA (mg/ml)	<0.02	191.3 ± 33	88.6 ± 5°°	46
IL-6 (U/ml)	<50	150 000	16 432 ± 6263°°	11

Serum levels of glucose, corticosterone and IL-6 were measured 2 h after the injection, whereas serum amyloid A levels were measured 8 h after the injection at the peak time. The food-intake was evaluated 1 day after the i.p. injection. Data are mean ± SE (five mice per group).
ΔSND 52–54 has an effect on the serum levels of glucose, corticosterone, IL-6 and SAA whereas only a small effect was observed on food-intake. At this dose ΔSND 52–54 induces significantly lower IL-6 and SAA serum levels if compared with IL-1β.
* $P < 0.05$ vs saline; ** $P < 0.01$ vs saline; °° $P < 0.01$ vs IL-1β (Dunnet's test).

fourth and the fifth β-strand of the IL-1β molecule in the agonist/antagonist distinction between IL-1β and IL-1ra since this loop seems to be absent from the IL-1ra molecule (Fig. 1). The three-dimensional structure of IL-1ra is not yet determined but several lines of evidence suggest that it may possess the same type of tertiary structure as IL-1α and IL-1β although at the primary structure level human IL-1ra shows only 18% homology with human IL-1α and 26% homology with human IL-1β.[7] IL-1α and IL-1β along with several other proteins such as fibroblast growth factors and the Kunitz protease inhibitor show a characteristic type of folding pattern (β-trefoil fold) where the requirement for medium or large hydrophobic residues at 18 sites in the inner core of the molecule is essential for the proper folding.[24] Many of these 18 inner residues of IL-1β have identical or similar counterparts in the primary sequences of IL-1ra (Fig. 1). Furthermore, the amino acid residues 92–105 in IL-1β that seem to be involved in receptor binding[25] show high degree of homology to corresponding residues in the IL-1ra sequences (Fig. 1). Point mutations such as D145K in the human IL-1β were shown to cause a marked reduction of bioactivity[26] and conversely the K145D mutation rendered the IL-1ra a partial agonist. Based on the above findings we assumed that also IL-1ra belongs to the β-trefoil family. Results with the point mutant D54R and with the point mutant, N53E[9] showed that rather large changes in charge in this loop do not substantially affect receptor binding as these mutants showed high affinity binding at both IL-1 receptor subtypes (for data on D54R cf Fig. 3). One could therefore expect that the deletion of amino acids SND 52–54 from this loop (EESNDK 50–55) causes the reduction of agonist activity not via a removal of negative charge (D54R) but rather by disturbing the tertiary structure of this part of the IL-1β molecule.

A putative binding epitope on IL-1β that seems to be specifically involved in the interaction of IL-1β with the type 1 IL-1 receptor has been described as a cluster of residues located at the end of the open barrel in proximity to both the N- and C-terminals of IL-1β.[9] These residues were found to be important for the interaction of IL-1β with the type 1 IL-1 receptor. The loop (EEDNDK 50–55) which we were concerned with is located close to this putative binding epitope. Point mutants of this binding epitope similarly to the deletion mutant ΔSND 52–54 did not differ substantially from IL-1β concerning their binding affinity to the type 2 IL-1 receptor. The model of ΔSND 52–54, based on the IL-1β structure (Fig. 2), indicates that apart from the deleted residues the major distortion is caused at the Ile 56 residue, which is part of the 'wide' β-bulge in IL-1β. The observed partial receptor subtype specificity of ΔSND 52–54 is

therefore congruent with the postulation by Labriola-Tompkins et al.[9] that Ile 56 is an essential residue of IL-1β for type 1 IL-1 receptor but not for type 2 IL-1 receptor interactions.

The major findings of this report are concerned with the fact that the efficacy of ΔSND 52–54 has shown a major decrement as compared to that of IL-1β while the affinities of the two ligands are comparable. This suggests that the contribution of the SND residues to the triggering of the biological response is larger than its contribution to the overall free energy of binding. This finding may serve as a basis for the design of low molecular weight IL-1 receptor agonists.

Furthermore, the differences in the efficacies of hrIL-1β and ΔSND 52–54 in eliciting different in vivo IL-1 receptor mediated responses (Tables 1 and 2) suggest that these ligands have different biological concentrations, or that more than one type of IL-1 receptor is involved in mediating the responses to hrIL-1β and ΔSND 52–54 and thus mutants with partial agonist properties such as ΔSND 52–54 may be useful also in addressing the significance of different IL-1 receptor subtypes. NMR and X-ray studies of the IL-1 receptor ligand complex will be needed for the final assessment of the interactions between the SND motif and the IL-1 receptors. Such studies have been initiated.

MATERIALS AND METHODS

Sequence Alignment and Model Building

The protein sequences of IL-1α, IL-1β and IL-1ra were taken from the Swiss-Prot database, or alternatively, nucleotide sequences from EMBL Data Bank were translated into protein sequences. Both databases are located at the European Molecular Biology Laboratory, Heidelberg. Multiple alignment of the sequences was performed with programs from the University of Wisconsin Genetics Computer Group[27] on a VAX computer. The three-dimensional structure of human IL-1β[28] was used as a template for building a structural model of ΔSND 52–54 and ΔEESNDK 50–55 molecules using the molecular graphics program package Insight (Biosym Technologies, Barnes Canyon Road, San Diego, CA 92121, USA) installed on a Silicon Graphics 4D workstation. The structure of IL-1β was first subjected to energy minimization using the CVFF force-field with distance dependent dielectric constant and 14 Å cut-off distance. After 100 steps of steepest descent minimization a conjugate gradient minimization was performed until the maximal energy derivative decreased under 0.01 J/Å. The initial models of the ΔSND 52–54 and ΔEESNDK 50–55 molecules were built on the basis of the minimized IL-1β structure and refined with the procedure described above.

Enzymes, Bacterial Strains and Vectors

Restriction enzymes and T4 ligase were from New England Biolabs, T4 polynucleotide kinase from Boehringer Mannheim and Klenow fragment of DNA polymerase I from Pharmacia. The M13 in vitro mutagenesis kit was obtained from Bio-Rad, including the *E. coli* strains CJ236 and MV1190.[29] *Escherichia coli* strains JM101[30] and JM109[31] as well as the M13K07 helper phage[32] and the M13mp18 vector[31] were from Pharmacia. pZ152 phagemid[33] was purchased from Anglian Biotec (Colchester, England) and α-[^{35}S]-dATP was obtained from Amersham. The oligonucleotides used for mutagenesis, vector construction and for sequencing were synthesized by the phosphoramidite method using PAC-amidites[34] on a Pharmacia Gene Assembler. The deprotected oligonucleotides were purified by polyacrylamide gel electrophoresis in the presence of 8 M urea. Recombinant vectors were purified on Qiagen-tips (Diagen, GmbH, Germany) as recommended by the supplier.

Construction of the Mutant IL-1β Genes

The human IL-1β gene was purchased from British Bio-technology (BBG25). It was subcloned as an *Eco* RI–*Hin*d III fragment into M13mp18 and was then subjected to oligonucleotide-directed in vitro mutagenesis[29] using an M13 in vitro mutagenesis kit (Bio-Rad). Deletion mutagenesis was performed with the following oligonucleotide primers: TCCTTTGTACAAGGA|ATACCTGTGGCCTT (ΔEESNDK 50–55 mutant), TGTACAAGGAGAAGAA|AAAATACCTGTGGCC (ΔSND 52–54 mutant) where the vertical bars separating the mutagenic oligonucleotides into two arms show the fusion points obtained after deletion. The D54R substitution mutant was obtained by using the AAGGAGAAGAAAGTAATCGCAAAATACCTGTGGC oligonucleotide, where the mismatched residues are underlined. The template-oligonucleotide annealing temperatures for the arms were calculated as described,[35] otherwise the suppliers' instructions were followed. Recombinants were randomly picked (two to four plaques per mutagenesis), sequenced[36] and replicative forms were prepared from the correct clones as described.[37]

Construction of the Expression Vector pRIZ'

The pPEX *E. coli* expression vector was constructed from pM23, a pBR322 based expression vector which utilizes the *lac* repressor controlled *rrnB P2* promoter of *E. coli*,[38] as previously described.[39] It was further modified to obtain the pRIZ' vector as follows. (1) The ribosomal binding site of the β-galactosidase gene was replaced with the AGGAGGAAATAACCATGG sequence, which contains the consensus Shine-Dalgarno sequence GGAGG and includes the ATG initiation codon as part of the CCATGG *Nco* I restriction site. (2) The M13 phage intergenic region was introduced by using the *Nde* I–*Pst* I fragment of pZ152[33] to obtain a phagemid. (3) The 1.7 kbp *Eco* RI fragment of pMC9[40] containing the *lac* I^q gene, the *lac* Z control region and a part of the *lac* Z gene was inserted at the unique *Nde* I site after rendering the cohesive ends blunt with Klenow polymerase and dNTP. The resulting pRIZ' vector was then cleaved with *Nco* I and *Eco* RI and the genes coding for human recombinant IL-1β (hrIL-1β) or its

analogs were cloned between these sites, using JM109 as *E. coli* host for transformation and for recombinant phagemid isolation. The recombinants were sequenced using the oligonucleotide primer TGTAGCGGGAAGGCGTATTAT which corresponds to a part of the promoter region (from −28 to −8) and allows checking the amino terminal half of the IL-1β genes.

Expression and Purification of IL-1β Analogs

JM101 *E. coli* cells transformed with the expression constructions were grown in LB medium containing 100 mg/l ampicillin at 37°C and the exponentially growing cultures ($Abs._{600}$ = 0.3–0.4) were induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.7 mM and further grown for 4–6 h. Pellets of the induced cells were extracted by using a repeated freeze–thaw procedure followed by hydrophobic interaction chromatography over phenyl Sepharose CL-4B column.[41] Further purification was done with FPLC chromatography on a Mono-S column (Pharmacia) with a linear gradient of NaCl (0–0.2 M) in 50 mM sodium acetate, pH 5.5. The elution was followed by SDS-polyacrylamide gel electrophoresis[42] and the homogeneous fractions were pooled and dialysed against 5 mM sodium phosphate, pH 7.5.

IL-1 Receptor Binding Studies and Biological Assays of the Mutants of IL-1β

IL-1β (recombinant human IL-1β, specific activity 1×10^7 U/mg) was a kind gift from Sclavo, Siena, Italy.

The EL-4 mouse thymoma cells were grown in RPMI-1640 medium, supplemented with 5% (v/v) fetal calf serum, 25 μM β-mercaptoethanol and 50 μg/ml gentamycin. The human B-lymphoma cell line Raji was maintained in RPMI-1640, containing 10% (v/v) fetal calf serum and 50 μg/ml gentamycin. Human recombinant IL-1β was radiolabelled using the [^{125}I]-Bolton–Hunter reagent (DuPont, NEN) in accordance with the supplier's instructions. Briefly, 10 μg hrIL-1β in 10 μl 100 mM sodium phosphate, pH 8.5 was reacted with the ^{125}I-Bolton–Hunter reagent for 1 h at 0°C followed by 3 h at 4°C. Separation of protein from unreacted reagent was done through gel filtration on a NAP-5 column (Pharmacia) and labelled hrIL-1β was stored as an approximate 50 nM solution in binding medium (RPMI-1640, 25 mM HEPES, pH 7.2, 1% (w/v) BSA, 0.1% (w/v) sodium azide) at −70°C. The specific activity was approximately 30 μCi/μg. Competition binding was performed as follows: cells (EL-4 or Raji) were harvested washed in Hank's balanced salt solution, resuspended in binding medium (10^7 cells/ml) and incubated at room temperature for 1 h with increasing concentrations of hrIL-1β, ΔSND 52–54, D54R or IL-1ra, and a constant concentration of labelled tracer in 96-well plates. Each well thus contained 25 μl of unlabelled ligand (final concentrations 10^{-12}–10^{-6} M), 25 μl [^{125}I]-hrIL-1β (≈3 nM, ≈2×10^5 cpm/well) and 50 μl of cell suspension (5×10^5 cells/well). The cells in each well were collected by centrifugation through 150 μl of a phthalate oil mixture (60% dibutylphthalate, 40% dinonylphthalate, Aldrich) at 12 000 rpm for 0.5 min, the supernatants were aspirated and the radioactivity of the pelleted cells was measured in a gamma-counter. Binding data were analysed by non-linear regression with the Kaleidagraph program (Abelbeck Software) on a Macintosh computer.

T cell Assay

Biological activity of the ΔSND 52–54 was assessed using the thymocyte-activating factor (TAF) assay.[43]

Animals and in vivo Treatments

Male, adult (25–30 g) CD-1 mice (Charles River, Calco, Italy) were used. Animals were housed five per cage in air-conditioned quarters (60% relative humidity, 22°C) with a 12 h light/dark cycle, and were given standard laboratory chow (Altromin, Rieper, Bolzano, Italy).

Food Intake and Body Weight

These were measured at day 0 and at day 1. IL-1β and ΔSND 52–54 were dissolved in 0.15 M sodium phosphate buffer, pH 5.7, and diluted in saline solution.

Animals were treated with IL-1β or ΔSND 52–54 at the doses indicated i.p. in a final volume of 0.2 ml. Control mice received saline solution. All treatments were performed between 9.00 and 11.00 am.

Blood was obtained from the retroorbital plexus under light ether anaesthesia and serum prepared for IL-6, glucose, corticosterone and SAA determination. Blood was collected at 2 h for IL-6, corticosterone and glucose determinations and at 8 h for SAA determination, since previous experiments indicated that, at these times, peak levels were observed.

Sera

Serum IL-6 levels were measured as hybridoma growth factor using 7TD1 cells (a kind gift from Dr van Snick, Bruxelles, Belgium) as previously described.[44] IL-6 is expressed as costimulatory units/ml using a standard curve with human recombinant IL-6 (Immunex Corp., Seattle, WA; specific activity 10^7 U/ml). One unit in the 7TD1 assay corresponded to 1 pg of hrIL-6.

Serum corticosterone was measured by a RIA using a polyclonal antibody to corticosterone from Sigma, following the manufacturer's instructions. (^{3}H) corticosterone was purchased from Amersham.

Serum glucose was measured by the glucose oxidase/peroxidase method with a commercially available kit (Boehringer, Mannheim).

Serum amyloid-A (SAA) was measured in sera by an ELISA[45] using a polyclonal rabbit anti mouse SAA (a kind gift from Dr J. Sipe, Boston).

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