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Human interleukin-1 receptor antagonist

High yield expression in *E. coli* and examination of cysteine residues

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The human IL-1 receptor antagonist (IL-1ra) was produced in a high yield *E. coli* expression system, and was purified in a rapid two-step purification. This recombinant IL-1ra molecule possessed full binding activity to the IL-1 receptor (type I) and totally inhibited IL-1-induced PGE_2 production by human dermal fibroblasts. Radioalkylation and analysis of V8-derived IL-1ra peptides indicate that the four cysteines present in the IL-1ra are not disulphide-linked.

IL-1 receptor antagonist; *E. coli* expression; Cysteine mapping

1. INTRODUCTION

The IL-1 receptor antagonist (IL-1ra) was originally described as a molecule which inhibits the activity of IL-1 [1]. cDNA [2] and genomic DNA clones have been isolated and characterized [3,4]. Using a polymorphism within the second intron [5] the chromosomal localization has been established by linkage analysis [6] and independently by in situ hybridization [4], showing that the genes for IL-1ra, IL-1α and IL-1β are located close to each other on chromosome two. Control of IL-1 expression level and/or therapeutic inhibition of the binding to the receptor may be important in the treatment of autoimmune and other IL-1-mediated inflammatory diseases, and IL-1ra may play an important role in development of such therapy [7].

In this study we describe a high yield expression of IL-1ra in *E. coli*, which produces a functionally fully active protein which is easy to purify. In addition our results indicate that the four cysteines are not disulphide-linked.

2. MATERIALS AND METHODS

2.1. *Vector construction*

The coding region for the IL-1ra (residues 3–152, numbering according to [2] was amplified from U937 cDNA by PCR with the introduction of an additional glycine residue and a *Bam*HI restriction site at the 5′ end and an *Eco*RI site at the 3′ end (5′ oligonucleotide CGG GAT CCG GGA GAA AAT CCA GCA AGA TG; 3′ oligonucleotide CGG AAT TCC CCT ACT CGT CCT CCT GGA). Using these primers the mature recombinant protein differs from the native protein in that its N-terminal sequence is GSGRK rather than RPSGRK as found in the native protein. The PCR product was introduced into the fusion protein expression vector pGEX-2T [8] and transformed in the *E. coli* strain NM554. The fusion partner glutathione S-transferase (GST) can be cleaved off after expression, using thrombin, to obtain an authentic recombinant IL-1ra protein.

An overnight bacterial culture was diluted 1:10 in fresh LB-medium and grown for 1 h at 37°C at which point IPTG was added (0.1 mM) and grown for a further 6 h. The cells were pelleted and resuspended in 1/50 of the starting volume in lysis buffer (PBS containing lysozyme, 0.2 mg/ml; DNase, 10 mg/ml; Triton X-100, 0.1% (v/v); PMSF, 0.1 mM; iodoacetamide, 5 mg/ml; benzamidine, 50 mM; EDTA, 5 mM; PBS is NaCl 150 mM; Na_2HPO_4 16 mM; NaH_2PO_4 54 mM; pH 7.2) for 1 h and thereafter sonicated 3 × 20 s using an MSE sonicator. After centrifugation (10,000 × *g*; 20 min) the supernatant was incubated with glutathione-agarose beads [8] (Sigma G-4510) at 4°C overnight. After washing the beads with PBS, the fusion protein was directly cleaved on the beads with human thrombin (1 Unit per 1 mg protein) (Sigma T-3010) at 37°C for 4 h. The beads were spun down and the recombinant IL-1ra was purified by passing through a Sephacryl HR S100 (Pharmacia) column of 950 mm × 25 mm diam. at a flow rate of 1.2 ml/min using 10 mM potassium phosphate (pH 7.3) with 0.05% (w/v) sodium azide as loading and elution buffer. Fractions were collected and the presence of the recombinant IL-1ra was checked by the OD_{280} and confirmed by SDS-PAGE. The purified material was also subjected to N-terminal amino acid sequence analysis (Applied Biosystems 470A) and electrospray mass spectrometry (VG BIO Q quadrupole mass spectrometer). These techniques confirmed that the recombinant material has been purified to > 98% and has the expected mol.wt. of 16,930 and expected N-terminal sequence. The extinction coefficient at 280 nm (1 cm, 1 mg/ml) was calculated by the method of Perkins [9] as 0.96. This value was used to calculate protein concentration.

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2.2. *Receptor binding studies with recombinant IL-1ra*

EL4.6.1.d10 cells were resuspended at 10^7 cells/ml in binding medium (RPMI 1640, 5% FCS, 20 mM HEPES pH 7.2), and 50 μl of cell suspension was added to each well of a 96-well filtration plate (Millipore Multiscreen). To each well 50 μl of ^{125}I-human-IL-1α (NEN Dupont) at a final concentrations of 50 pM was added. Unlabelled human IL-1β and human IL-1ra were serially diluted in binding medium, and 50 μl aliquots of unlabelled ligand were added to each well at the final concentration indicated on Fig. 2a. Receptor binding was allowed to proceed to equilibrium at 4°C. The plates were washed four times in binding medium by vacuum filtration, and the filters subsequently punched out and counted for bound radioactivity.

2.3. *Inhibition of PGE_2 production by IL-1ra*

The induction of PGE_2 production by IL-1 was assayed as described previously [10]. Briefly, human dermal fibroblasts were seeded at 10^4 cells per well in a 96-well plate. The cells were maintained for 3 days in culture to reach confluence, then washed with fresh medium and stimulated for 24 h with 50 pM human IL-1β in the presence of increasing concentrations of IL-1ra. At the end of the 24-h period, the culture supernatant was removed and immediately assayed for PGE_2 content using a scintillation proximity assay (Amersham).

2.4. *Cysteine residues in recombinant IL-1ra*

IL-1ra (30 μg) was dissolved into 1 ml of 0.1 M Tris-HCl, pH 8 and incubated at 37°C for 16 h with 3 μg endoproteinase Glu-C (V8 protease) (Boehringer-Sequencer grade) which cleaves after glutamic acid. The digested peptides plus a sample of starting material were run on a Brownlee Aquapore RP300 column (C8) in 0.1% TFA and peptides were eluted with a 2–50% gradient of acetonitrile over 50 min, then a 50–90% acetonitrile gradient over the next 15 min. The N termini of the main peptides were sequenced on an Applied Biosystems 470A protein sequencer with online PTH analysis, to determine whether pairs of disulphide linked peptides were present.

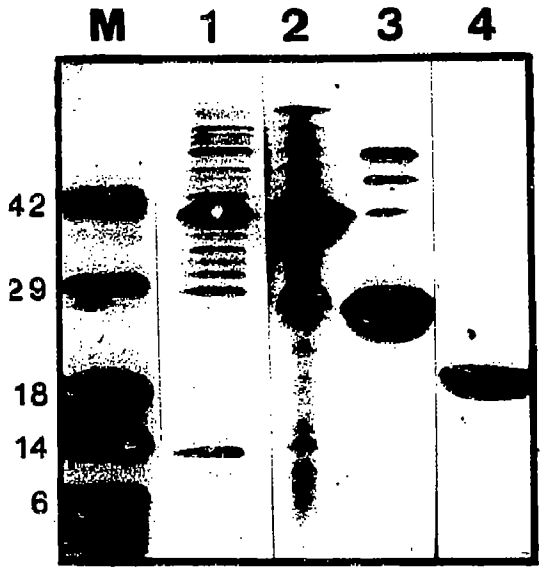

Fig. 1. SDS-PAGE analysis of the recombinant IL-1ra. Lane 1, total *E. coli* lysate. The major band represents the GST-IL-1ra fusion protein; lane 2, fusion protein attached to glutathione agarose beads after incubation with the total *E. coli* lysate; lane 3, material remaining bound to the glutathione agarose beads after thrombin cleavage. Only the GST is still bound to the beads; lane 4, supernatant after thrombin cleavage, containing the IL-1ra.

3. RESULTS AND DISCUSSION

The human IL-1ra was expressed as a GST-fusion protein in an *E. coli* expression system. After a simple two-step purification a yield of approximately 15 mg recombinant IL-1ra per litre of culture was obtained (Fig. 1). Due to the introduced thrombin cleavage site the IL-1ra starts with a glycine and the first two amino acids of the reported protein sequence [2] are absent. N-terminal amino acid sequencing and mass spectrometery, which indicated a precise mol.wt of 16,930 Da, confirmed the correct expression of the IL-1ra molecule.

In order to establish the binding activity of the recombinant protein, the IL-ra molecule was tested in an IL-1 receptor binding competition assay. The binding activity of the recombinant IL-1ra was only marginally less than that of IL-1β, indicating a similar affinity for binding to the type I IL-1 receptor (Fig. 2a). To investigate

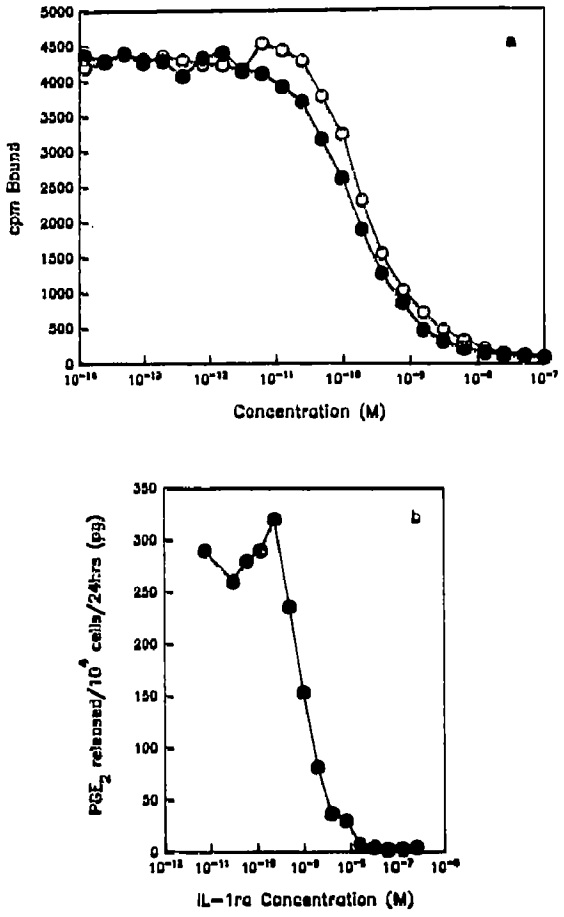

Fig. 2. The recombinant IL-1ra was tested in a competition assay for receptor binding activity. ^{125}I-labelled IL-1α was incubated with EL4 cells in the presence of increasing concentrations of unlabelled IL-1ra (open circles) or IL-1β (closed circles). (b) Inhibition of IL-1 induced PGE_2 production by IL-1ra. Human dermal fibroblasts were stimulated for 24 h with IL-1β, and the culture supernatant assayed for PGE_2 production. Increasing concentrations of IL-1ra were tested for their ability to inhibit this IL-1 induced PGE_2 production.

whether this molecule indeed inhibits IL-1 mediated activation and has no agonist activity, a PGE_2 production assay was performed. The recombinant IL-1ra totally inhibited PGE_2 production by human dermal fibroblasts induced by 50 pM IL-1β (Fig. 2b). Half maximal inhibition was obtained with a 25-fold molar excess of the IL-1 receptor antagonist. This is in line with the results obtained by other investigators, where a 10 to 100-fold excess was required (see refs. in [7]).

To establish whether or not the four cysteines present in IL-1ra are disulphide-linked, the recombinant protein was digested (without prior reduction) with the protease V8 and the N-termini of the major peptides obtained were sequenced. Human IL-1ra contains four cysteines at amino acid positions 66, 69, 116 and 122 (numbering of the amino acids is according to the IL-1ra cDNA derived sequence [2]). Two peptides were obtained which contained two cysteines each. One covered the amino acids from position 45 to 75 and the second from 113 to 126. As these two peptides were not eluted in the same fraction from reverse phase chromatography, they are not crosslinked by disulphide bridges. To determine whether neighbouring cysteines were disulphide linked, IL-1ra (135 μg) in 4 M guanidine, 0.2 M Tris-HCl, pH 8.2 was radioalkylated with [^{3}H]iodoacetic acid (50 μCi) (Amersham), with or without prior reduction by dithiothreitol. Uptake of radioactivity was identical with or without prior reduction, indicating that none of the 4-cysteines is involved in disulphide linkage, and that all 4 are titratable with iodoacetic acid in denaturing conditions. NMR-derived secondary IL-1ra structure [11] supports the view, that disulphide linkages are not formed.

IL-1ra shows, respectively, 18% and 26% amino acid identity with IL-1α and IL-1β [3]. IL-1α has only one cysteine and therefore no disulphide bridges. In the IL-1β molecule two cysteine residues are present, but crystal structure data clearly show that they are not disulphide linked [12]. Since this recombinant IL-1ra protein is as functionally active as the native protein, this strongly indicates that the native form of IL-1ra also lacks disulphide bridges.

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