Protein Engineering vol.5 no 5 pp.427–431, 1992

Permuteins of interleukin 1β—a simplified approach for the construction of permutated proteins having new termini

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A technique for the rapid and simple generation of permutated versions of the interleukin-1β (IL-1β) gene is described. In this method, the human IL-1β cDNA is twice amplified by the polymerase chain reaction (PCR) and the resulting DNA fragments are ligated in tandem. Between the two genes, the DNA sequence encodes a short four amino acid loop to link the native N- and C-terminal ends of the IL-1β protein. By using PCR amplification from this starting template, a new version of the IL-1β cDNA was obtained that encodes a permutated form of the IL-1β protein where the new N- and C-terminal amino acids correspond to residues 65 and 64 of the native IL-1β sequence, respectively. The name 'permutein' is proposed to describe proteins generated by this technology. The molecular profile (IL-1 receptor binding, biologic activity and solution properties) of the IL-1 permutein produced by this technology, permutein 65/64, is shown to be identical to that of native IL-1β. The approach should be useful to define further the structural features of this protein that are important for its function.

Key words: gene assembly/interleukin/*in vitro* mutagenesis/PCR/permutein

Introduction

A variety of approaches have been utilized to explore the interrelationship between protein structure and function. These include site directed mutagenesis and amino acid modifications based on the chemical reactivity of amino acid side chains (Imoto and Yamada, 1989; Sayers and Eckstein, 1989; Yanofsky and Zurawski, 1990). Occasionally the modified molecules undergo structural changes removed from the site of mutation making it difficult to localize which regions of the molecule are responsible for specific protein functions. In a novel approach to the study of the protein structure–function interrelationships, Goldenberg and Creighton (1983) chemically linked together the N- and C-termini of bovine pancreatic trypsin inhibitor followed by limited proteolysis to yield a circularly permutated version of the protein. Using a more general procedure, Luger *et al.* (1989, 1990) genetically engineered and expressed a circularly permutated version of the yeast phosphoribosyl anthranilate isomerase enzyme in *Escherichia coli*. These methods, however, required the use of specialized proteolytic enzymes or the introduction of unique restriction sites at particular locations within the structural gene. We describe in this manuscript a simplified approach for synthesizing circularly permutated proteins that obviates the need for restriction sites, and further, allows placement of N- and C-termini at any desired location within the gene of interest.

It has been estimated that 30% of proteins contain a tertiary structure in which the N- and C-terminal ends lie spatially very close to each other (Thornton and Sibanda, 1983). This makes permutagenesis potentially a very attractive method to use in the study of protein structure–function interrelationships. In the present study, we have designed a novel version of the human IL-1β protein in which the N- and C-termini are ligated together using an amino acid linker peptide. IL-1β offered a particularly attractive example for this type of approach since it has been demonstrated to play a central role in a variety of disease states including rheumatoid arthritis (Dinarello, 1989). Thus, an understanding of its structure–function interrelationships would be extremely useful in helping to design drugs aimed at antagonizing IL-1 action.

Using the PCR technology described below we have generated an IL-1β permutein (permutein 65/64) whose N- and C-terminal amino acids correspond to residues 65 and 64 of the native IL-1 sequence, respectively. Circular dichroic spectra of this protein are similar to that of native IL-1β. Furthermore, the receptor binding and biologic activities of the two proteins are indistinguishable. The construction of other IL-1β permuteins should prove to be useful in helping to define surface regions of the molecule that are important in receptor binding.

Materials and methods

Materials

Human recombinant IL-1β (rIL-1β) expressed in *E.coli* was purified to homogeneity according to methods previously described (Huang *et al.*, 1987). Human [^{125}I]IL-1β (specific activities 80–120 μCi/μg), was obtained from New England Nuclear. Reagents for electrophoresis were purchased from Bio-Rad. HEPES and all other reagent grade chemicals were purchased from Sigma. Restriction endonucleases, DNA polymerase (Klenow fragment) and T4 DNA ligase were obtained from New England BioLabs. DNA Taq polymerase and reagents for PCR amplification were purchased from Perkin-Elmer Cetus Co.

Cell culture

Raji and YT cells were maintained in RPMI 1640 medium containing 10% fetal calf serum. The cells were passaged weekly and the medium was changed two additional times weekly. For binding assays the cells were washed three times with RPMI 1640 and resuspended in binding buffer (RPMI 1640 containing 1% bovine serum albumin, 20 mM HEPES pH 7.2). Cell viability was assessed by trypan blue exclusion and by use of the redox dye MTT as described by Mossman (Mossman, 1983). Cell viability measured by these two assays ranged from 90 to 95%. Cell number was determined using a hemacytometer.

Construction of IL-1 permuteins

Forty-eight existing protein structures were searched (Finzel *et al.*, 1989b) for a loop that could serve to interconnect the native termini of the IL-1β molecule. Loops containing three, four, five and six amino acid insertions were examined. Insertions of four residues showed the least potential for structural alteration while minimizing the size of the linker. The sequence of the four residue linker, TAQT, represents a consensus sequence found in the 48 existing structures.

Primers used for the construction of the tandemly duplicated IL-1β template were designed as follows (sequences of primers are shown underlined in Figure 2). Primer A incorporates an ATG start codon and the N-terminal six codons of the first IL-1β gene. Primer B includes the last 6-2/3 codons of the IL-1β gene, the sequence encoding the suggested four amino acids for the spacer loop, and the first codon of the second gene. An interrupted palindromic restriction site (*Sfi*I) was incorporated into the nucleotide sequence. Primer C includes most of the sequence encoding the four amino acid linker region, the same *Sfi*I interrupted palindromic restriction site as found in primer B, and the first 8-2/3 codons of the second IL-1β gene. Primer D contains two TAA stop codons and the last three codons of the second IL-1β gene. The PCR conditions used for the construction of the IL-1β permutein were: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min for 25 cycles beginning with 0.1 ng template. The resulting two DNA fragments representing IL-1β genes 1 and 2 were purified by gel electrophoresis on a 1% agarose gel using GeneClean kits (Bio 101, San Diego, CA). The isolated fragments were digested with the restriction enzyme *Sfi*I (New England Biolabs) followed by phenol–chloroform extraction and ethanol precipitation. The two IL-1β gene fragments (50 ng each) were ligated together at room temperature and this material then served as the initial template for the construction of all subsequent permuteins. Permutein 65/64 was amplified from this template using oligonucleotide primers E and F (shown in Figures 1 and 2).

IL-1 permutein expression

Construction of the expression vectors was carried out using standard techniques (Ausubel, 1989). The PCR amplified permutein 65/64 sequence was digested with *Nde*I and *Nco*I and purified from agarose gels using GeneClean. The gene fragment was ligated into the bacterial expression vector pA266 (H.George, unpublished results) containing a pTAC promoter and subsequently transformed into the *E.coli* host strain W3110(F'iq). Overnight cultures of permutein plasmid-containing clones were grown at 37°C in L broth with ampicillin at 100 μg/ml. Cells were grown to late-log phase of growth and permutein protein synthesis induced by the addition of 100 mM isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Following induction, cells were incubated at 37°C with agitation for 4 h, the culture harvested by centrifugation and cell pellets quick frozen in a dry-ice–methanol bath and stored at −80°C. Protein production was analyzed in Coomassie Blue-stained PAGE gels (Laemmli and Favre, 1973) and quantitated using a scanning densitometer (LKB). *Escherichia coli* containing either the rIL-1 or permutein plasmids induced and grown by these methods were found to produce >10% of total cell protein and all of the recombinant protein was found in the soluble fraction.

Purification and characterization of IL-1 permuteins

Recombinant native Il-1β and the IL-1 permutein were purified using identical protocols. Cell pastes were suspended in 10 vol

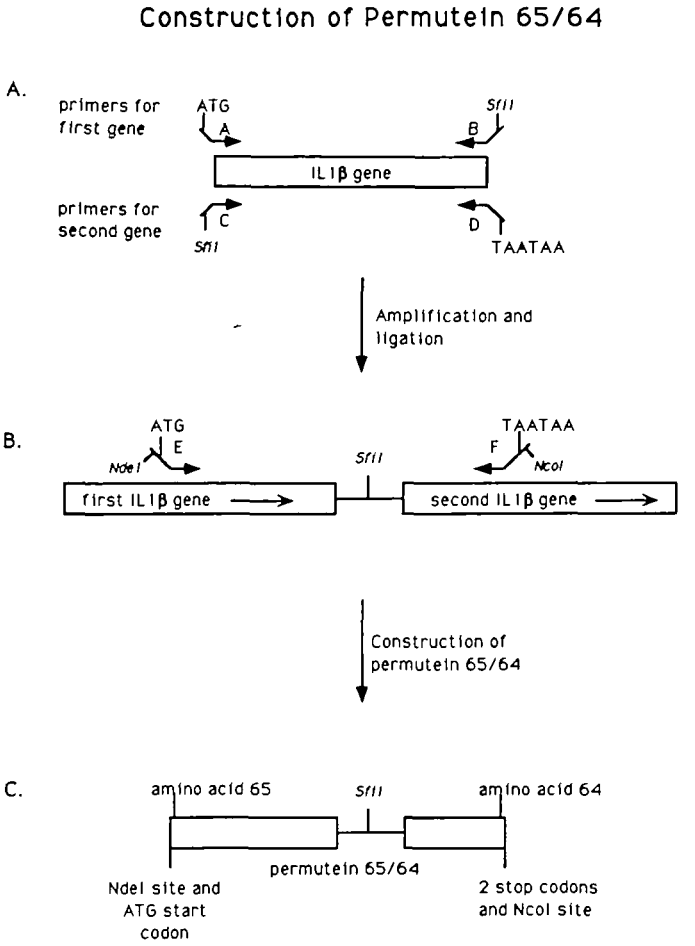

Fig. 1. Construction of permutein 65/64. Construction: the open rectangle represents the human IL-1β gene (British Biotech). Arrows labeled A–D represent the oligonucleotide primers used to amplify the first and second IL-1β genes for the tandem construct as shown. Arrows labeled E and F represent the primers used to amplify permutein 65/64 from the tandem template.

of 20 mM Tris–HCl, pH 7.0, 1 mM DTT, 1 mM EDTA. The cells were disrupted by high pressure homogenization and the suspension clarified by centrifugation. The soluble cellular components were applied to a Q-FF column (70 × 1000 cm, Pharmacia) equilibrated in 20 mM Tris–HCl pH 7.0. Flow-through fractions containing the recombinant IL-1s were diluted with an equal volume of 20 mM Tris–HCl, pH 8.5, the pH adjusted to 8.5, and applied to a DEAE-FF column (90 × 1000 cm) pre-equilibrated in 20 mM Tris–HCl, pH 8.5. The rIL-1s were eluted with 20 mM Tris–HCl, pH 7.0 and rIL-1-containing fractions were dialyzed against 10 mM sodium phosphate buffer, pH 6.3. This pool was then applied to an S-FF column (44 × 1000 cm) equilibrated in 10 mM sodium phosphate buffer, pH 6.3 and the rIL-1s were eluted with 10 mM sodium phosphate buffer pH 7.8. Endotoxin removal and buffer exchange into phosphate buffered saline (PBS, 10 mM sodium phosphate, 150 mM NaCl, pH 6.5) were performed by ion exchange chromatography. The identity and purity of the rIL-1s were confirmed by SDS–PAGE, IEF–PAGE, N- and C-terminal sequencing, and Western analysis. The endotoxin content of the samples was determined using the *Limulus* amebocyte lysate assay (Whittaker M.A. Bioproducts, Walkersville, MD). All preparations contained <10 endotoxin units per mg of protein.

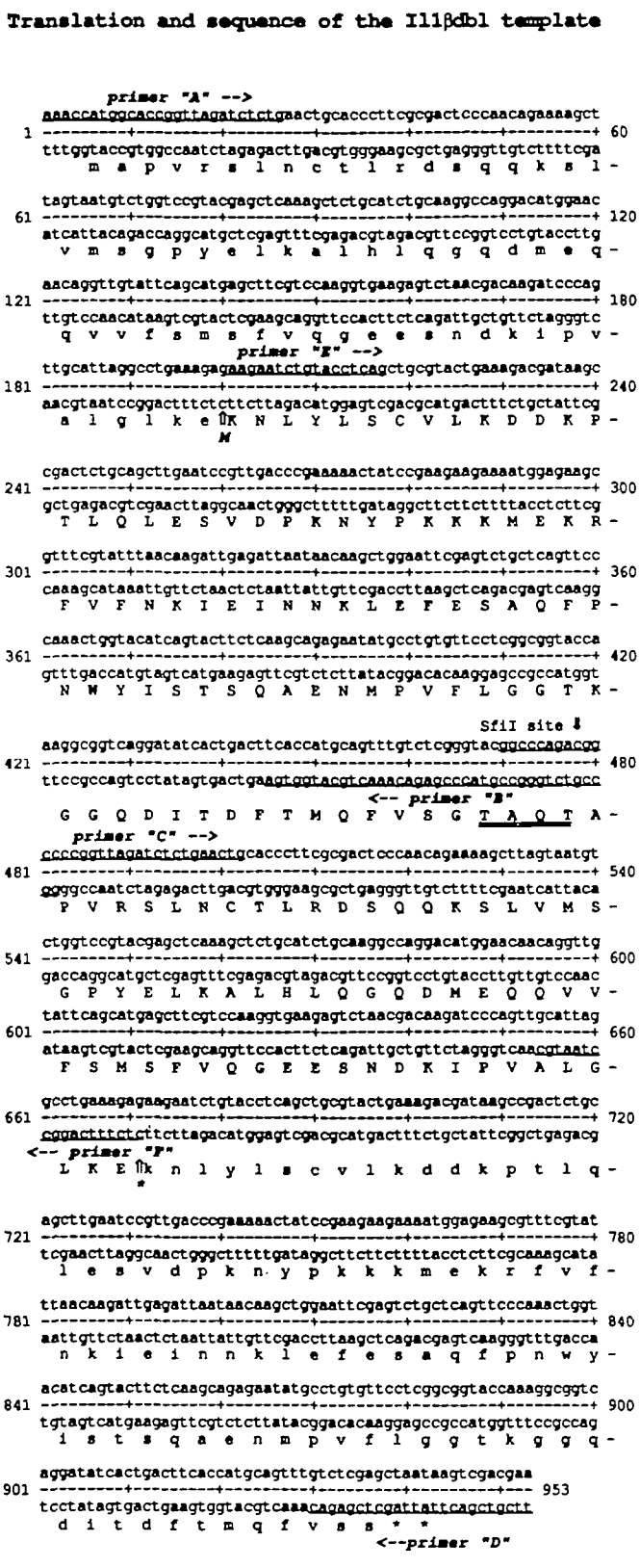

Fig. 2. Translation and sequence of construct schematized in Figure 1B: underlined nucleotide sequences represent primers used in PCR reactions. The single letter amino acid code is shown under nucleotide sequence; upper case letters represent amino acids contained within permutein 65/64 schematized in Figure 1C. Initiation methionine and termination positions are marked by ⇑ and represented by *M* and * respectively. Double underlined amino acids represent linker region. Position of the *Sfi*I site is marked by ⇕ .

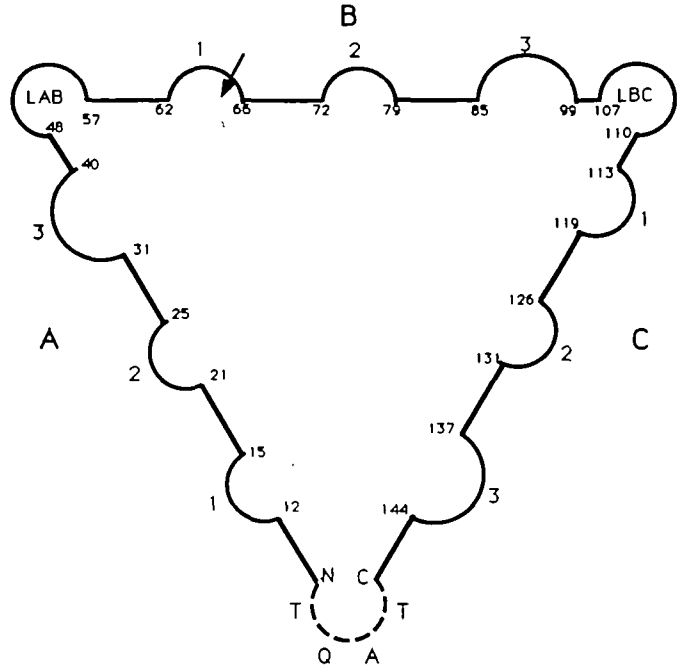

Fig. 3. Schematic depiction of IL-1β showing the location of surface loops. Large letters A, B and C represent the three domains of pseudosymmetry (Priestle *et al.*, 1988; Finzel *et al.*, 1989a). The larger numbers 1, 2 and 3 represent loops found between β pleated regions that comprise each of the three domains; smaller numbers refer to amino acid sequence. LAB and LAC represent the linker loops that connect domains A with B, and B with C, respectively. N and C are the N- and C-termini in native IL-1β, respectively. The dashed line represents the linker peptide, together with the sequence in single letter amino acid code (TQAT), used to ligate the N- and C-termini. Position of the new N- and C-termini created in permutein 65/64 is marked with ↓.

Biologic assays

Prostaglandin E2 (PGE_2) was assayed using a radioimmunoassay kit from New England Nuclear and the assay was performed as described by the manufacturer.

Radioreceptor binding assay

Raji and YT cells (1×10^7 cells per ml) were incubated with ^{125}I-labeled IL-1 in the presence and absence of unlabeled IL-1β or IL-1β permutein at the appropriate temperature for the designated times. The incubation was terminated by removing aliquots from the cell suspension and separating cells from buffer by centrifugation through silicon/paraffin oil mixture as described previously (Horuk *et al.*, 1987). Non-specific binding was determined in the presence of 1 μM unlabeled native rIL-1β. The biologic activity of the radiolabeled IL-1β in the murine thymocyte proliferation assay (Lachman *et al.*, 1985) was the same as that of unlabeled IL-1β (data not shown).

Circular dichroism

The circular dichroic (CD) spectra were obtained on a JASCO-600 spectropolarimeter. All samples were prepared in PBS buffer and concentrations adjusted to 350 μg/ml. A circular cell with light path length of 0.05 cm was used to record the spectra. A final spectrum of each sample was obtained by averaging 16 scans and the base line was corrected by subtracting the spectrum of the buffer. The secondary structure of human IL-1 and the permutein 65/64 was estimated using the method of Hennessey and Johnson (1981). The data in the 198–250 nm wave length region were fitted as a sum of the CD spectra of

α helix, β sheet, turn and random coil derived from the spectra of reference proteins with known crystal structures.

Data analysis

Equilibrium binding data were analyzed by LIGAND (Munson and Rodbard, 1980) as modified for the IBM PC by McPherson (1983).

Results and discussion

To facilitate subsequent construction of permutated versions of the human IL-1β coding region, a tandemly duplicated IL-1β template was first constructed. Synthesis of a tandem IL-1β template permitted positioning of the N- and C-termini at any location within the gene, eliminated the need for any special internal restriction sites, and further allowed cloning of the permutated cDNA product as a single fragment directly into the expression vector of choice. In order to construct the universal IL-1β tandem template, two pairs of oligonucleotide primers were designed to amplify the IL-1β coding region in two separate reactions. Primers A and B were used to amplify the first IL-1β gene, and primers C and D to amplify the second gene by the method of PCR.

The nature of the type of restriction site used in the construction of the tandem template was such that the unspecified overhanging sequence NNN within the *Sfi*I restriction site could be made non-palindromic and non-self-complementary. The two PCR amplified IL-1β genes could therefore be ligated together in a single tail-to-head monomeric gene1−gene2 orientation which thereby eliminated the need for cloning the template (Figure 1, panel B). Unligated templates were not amplifiable in the PCR reaction. This uncloned IL-1β tandem construct then constituted the starting material for permutein generation and an example of the construction of permutein 65/64 is given in Figure 1 (panel B). The sequence of permutein 65/64 as derived from the tandem IL-1β template is shown in Figure 2.

The crystal structure of IL-1β has recently been obtained at high resolution (Finzel *et al.*, 1989a; Priestle *et al.*, 1989). The structure consists of a core of interwoven β-strands with a series of 11 surface loops. The proximity of the N- and C-termini (14 Å) of the protein allowed the construction of an amino acid loop which could connect the N- and C-termini into an additional, twelfth, surface loop (Figure 3). This connection allowed the construction of novel IL-1β permuteins by varying the location of the N- and C-termini throughout the structure. The IL-1β permutein 65/64 described here retains many of the structural features of the naturally occurring molecule as indicated by circular dichroism (Figure 4). A secondary structure analysis using the method of Hennessey and Johnson (1981) estimates that the permutein 65/64 has about 18% α helix, 76% β sheet and 6% turn, very close to naturally occurring IL-1β with 8% α helix, 76% β sheet, 13% turn and 3% random coil.

The permutein 65/64 was tested for its ability to compete with radiolabeled IL-1β for binding to IL-1 receptors in cell types expressing either type I (YT) or type II (Raji) receptors. As shown in Figure 5, the permutein was equipotent in IL-1 binding studies with native IL-1β. In addition, the permutein had equivalent biological activity in a type I receptor related PGE_2 bioassay compared with that of native human IL-1β (data not shown). These data suggest that the 65/64 surface loop may not play a role in the expression of receptor binding and/or biological activity of IL-1β. Indeed, Labriola-Tompkins *et al.* (1991) have recently reported on the identification of the discontinuous binding site in human IL-1β for the type I receptor. The location of the

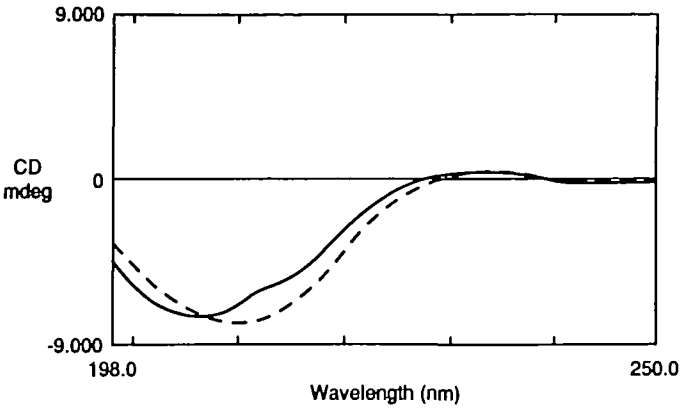

Fig. 4. CD spectra of human IL-1β (solid line) and permutein 65/64 (dashed line).

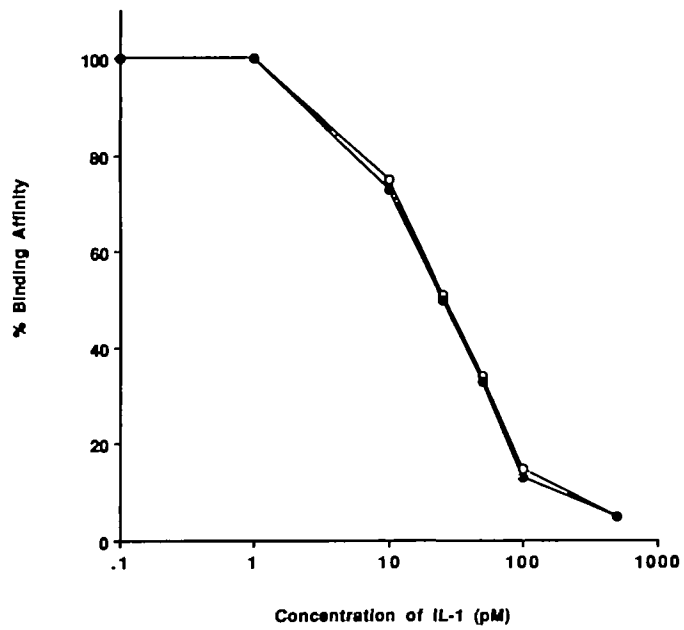

Fig. 5. Inhibition of binding of [^{125}I]IL-1 YT cells (type I IL-1 receptor) at 4°C by unlabeled IL-1β and permutein 65/64. Cells were incubated for 16 h with radiolabeled IL-1β and the indicated concentrations of IL-1β (●) and permutein 65/64 (○).

65/64 surface loop may be too distant from the site of receptor−ligand interaction to have any significant effect on binding to the type I receptor.

Although the specific example of a permutation of the Il-1β gene has been described here, the method for producing permuteins is applicable to any protein in which N- and C-termini are located spatially near each other. Furthermore, the method for constructing a tandemly duplicated template is also more generally applicable. Any gene for which a tandem duplication is desired can be easily constructed by one of two methods: the use of an appropriate restriction site, or through the method of splicing by overlap extension (Ho *et al.*, 1989; Horton *et al.*, 1989). The method of overlap extension uses overlapping DNA fragments representing the first and second genes generated by PCR. To synthesize the template, the DNA fragments (in the absence of oligonucleotide primers) are used together in a subsequent PCR reaction. Through their overlapping region, the fragments are able to serve as both primer and template for each other to generate a contiguous, tandemly duplicated gene

template. This procedure thereby eliminates the need for reliance on any particular restriction site and represents a simpler and more generally applicable method over those described previously (Goldenberg and Creighton, 1983; Luger *et al.*, 1989).

The fact that the recombinant, native IL-1β and permutein 65/64 exhibit virtually identical expression levels, solubilities during production in *E.coli*, require similar purification schemes, and exhibit similar folding characteristics as measured by CD spectra proves that we have succeeded in constructing and producing a novel, functional permutated form of the human IL-1β cytokine. Using these methods, it should be possible to scan the entire IL-1β molecule rapidly, specifically disrupting each of the 11 surface loops in turn, to look for altered binding affinities to either the type I or type II receptor, or for altered biological or physical properties of the hormone. This approach offers an exciting simplified and rapid approach for testing the structure–function relationships of a variety of other protein molecules.

Acknowledgements

We wish to thank Richard Yates for performing fermentations of bacteria producing the rIL-1s, Karen Krakowski for synthesis of oligonucleotides used in these experiments, Ramnath Seetharam for sequencing the purified, recombinant proteins, and Dianna Blessington, Andrea Daulerio and Joanne Ullman for technical assistance.

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Received on February 17, 1992; revised and accepted on March 30, 1992