Site-Specific Mutagenesis of the Human Interleukin-2 Gene: Structure-Function Analysis of the Cysteine Residues

Abstract. *The gene encoding human interleukin-2 (IL-2) has been cloned from human spleen cells, peripheral blood lymphocytes, and the Jurkat cell line. Nucleotide sequence analysis of the gene revealed that the encoded IL-2 protein has three cysteines located at amino acid residues 58, 105, and 125 of the mature protein. Site-specific mutagenesis procedures were used to modify the IL-2 gene by changing each of the cysteine codons individually to serine codons. Substitution of serine for cysteine residues at either position 58 or 105 of the IL-2 protein substantially reduced biological activity, indicating that the cysteines at these positions are necessary for maintenance of the biologically active conformation and may therefore be linked by a disulfide bridge. The modified IL-2 protein containing a substitution at position 125 retained full biological activity, suggesting that the cysteine at this position is not involved in a disulfide bond and that a free sulfhydryl group at that position is not necessary for receptor binding.*

Human interleukin-2 (IL-2) is a protein of approximately 15,000 daltons produced by human lymphocytes on stimulation with mitogens or antigens. In addition to its ability to support the long-term growth of T cells in culture (*1*), it augments the generation of cytotoxic T lymphocytes (*2*), enhances the activity of natural killer cells (*3*), stimulates lymphokine-activated killer cell activity (*4*), and reconstitutes in vitro the immune response in acquired immunodeficient states (*5*).

Although the IL-2 protein was purified to homogeneity from the Jurkat cell line (*6*) and from induced human peripheral blood lymphocytes, it has not been available in sufficient quantity for structural characterization of the protein. The availability of the cloned IL-2 gene (*7, 8*) permits us to study the disulfide bond structure of the IL-2 protein by manipulations at the genetic level.

We modified the three cysteine codons present in the IL-2 gene by changing each one separately to a serine codon. This type of mutation causes a minimal alteration to the structure of the protein because the substitution of an oxygen atom (serine) for a sulfur atom (cysteine) prevents disulfide bond formation at the site of the mutation. The mutagenesis was accomplished by extending synthetic oligonucleotide primers carrying the desired base changes and by the action of DNA polymerase I Klenow fragment on a single-stranded M13 phage DNA template containing the IL-2 gene.

The oligonucleotide primer CTTCTAGAGACTGCAGATGTTTC (DM27) (C, cytosine; T, thymine; A, adenine; G, guanine) creates two single-base mutations (Fig. 1A), one of which converts the UGU (U, uracil) cysteine codon to a UCU serine codon; the other mutation generates a new Pst I restriction site without changing the encoded amino acid (silent mutation). This new restriction enzyme site provides a convenient method for distinguishing this mutation (Ser^{58}) from the parent and from the other mutants (Ser^{105} and Ser^{125}). The scheme for the mutagenesis of Cys^{58} to Ser^{58} is shown in Fig. 1B (*9–15*). After hybridization of the primer to the single-stranded DNA template and extension of the primer with a DNA polymerase I Klenow fragment, the hybrid phage DNA molecules were transformed into competent cells of *Escherichia coli* K12 strain JM103 (*13*) and plated onto agar plates to obtain phage plaques. The phage plaques were transferred to nitrocellulose filters, and mutant plaques were identified by hybridization with ^{32}P-labeled primer DM27. The primer preferentially hybridized with the mutant DNA, which matched the primer sequence completely, and did not hybridize with parent DNA, which has two single-base mismatches (Fig. 1B). The replicative form DNA (RF DNA) of prospective mutants was then further characterized by restriction enzyme mapping to confirm the presence of a new Pst I site. Nucleotide sequence analysis of the phage DNA confirmed the presence of the two desired single-base mutations.

Similarly, two other primers, CATCAGCATACTCAGACATGAATG (DM28) and GATGATGCTCTGAGAAAAGGTAATC (DM29), were used to generate the Ser^{105} and Ser^{125} mutations, respectively. Each of these primers generates a

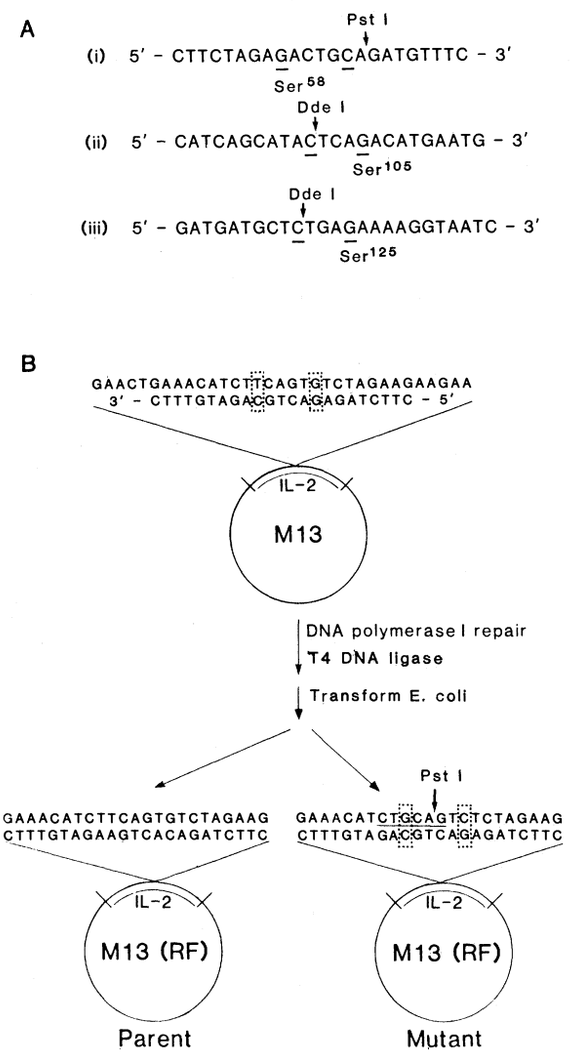

Fig. 1. (A) Oligonucleotide primers (i) DM27, (ii) DM28, and (iii) DM29 were used to substitute a serine for a cysteine at amino acid positions 58, 105, and 125, respectively. The two single-base changes (underlined), the corresponding position for the serine codon, and the newly generated restriction enzyme sites are shown. (B) Schematic describing the use of DM27 in the mutation of Cys^{58} to Ser^{58}.

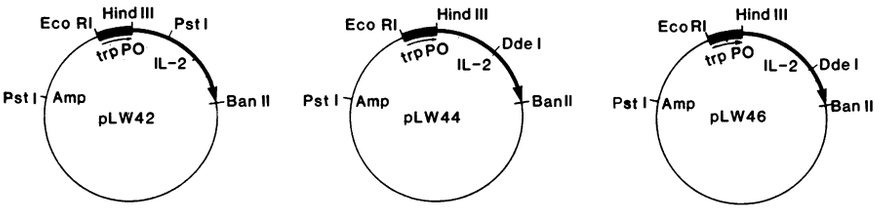

Fig. 2. Recloning of the mutagenized IL-2 gene for expression in *E. coli*. Replicative form DNA from all three mutagenized clones (Fig. 1) were each digested with restriction enzymes Hind III and Ban II and the insert fragments purified from a 1 percent agarose gel. Similarly, the plasmid pTrp3 (*8*) was digested with Hind III and Ban II. The large plasmid fragment containing the *E. coli trp* promoter was purified on an agarose gel and then ligated to each of the insert fragments. The ligated plasmids were transformed into competent *E. coli* K12 strain MM294 (*19*). The plasmid DNA's from these transformants were analyzed by restriction enzyme mapping to confirm the presence of the plasmids pLW42 (Ser^{58}), pLW44 (Ser^{105}), and pLW46 (Ser^{125}).

new Dde I restriction site instead of a Pst I site (Fig. 1A). After identification and confirmation of these mutations by sequence analysis, the three mutants were individually subcloned into an expression vector under the control of an *E. coli trp* promoter as shown in Fig. 2. Cell-free extracts of *E. coli* harboring these plasmids were assayed for IL-2 activity on the murine HT-2 cell line (*16*). The results obtained are shown in Table 1. Extracts from cells containing pLW21 (parent) had 9×10^5 units of IL-2 activity per milliliter, and of the three mutants, only extracts containing pLW46 (Ser^{125}) had comparable levels of IL-2 activity. Extracts containing pLW42 (Ser^{58}) had less than 0.5 percent of wild-type activity, and extracts of pLW44 (Ser^{105}) consistently showed a low level of IL-2 activity, generally about 1 to 2 percent of the activity found in extracts of pLW21 or pLW46.

Table 1. Biological activities of serine-substituted IL-2. Bacterial cultures of MM294 cells carrying different plasmids were grown in minimal medium in the absence of tryptophan to induce the *E. coli trp* promoter. Cultures were harvested when they reached an absorbance of 1.0 at 600 nm. They were then concentrated by centrifugation, suspended in a one-tenth volume of buffer containing 50 m*M* tris (*p*H 8.0), 50 m*M* EDTA, 15 percent sucrose and 1 percent SDS, and sonicated in a cup sonicator (Branson) for 2 minutes. Cell-free extracts of bacterial strains were assayed in duplicate on a murine IL-2-dependent cell line, HT-2, using a [^{3}H]thymidine incorporation assay (*16*).

Extracts	IL-2 activity expressed as	
	Unit*/ml	Percent†
pIL2-7 (negative control)	<69	<0.01
pLW21 (positive control)	9.00×10^5	100.0
pLW42	4.41×10^3	0.49
pLW44	1.68×10^4	1.87
pLW46	1.18×10^6	130.11

*A unit of activity is defined as the reciprocal of the dilution necessary to obtain half-maximal incorporation of [^{3}H]thymidine into HT-2 cells. †Expressed as percentage of activity relative to the wild-type protein in *E. coli* (pLW21).

The dramatic loss of IL-2 activity when cysteine is replaced with serine at positions 58 and 105 supports the hypothesis that these two cysteines are necessary for full biological activity and may be involved in a disulfide bridge that holds the protein in the biologically active conformation. If the putative disulfide bridge between Cys^{58} and Cys^{105} is absolutely required for biological activity, then a mutation at either site would be expected to abolish activity completely. The low level of residual activity found in extracts of the Ser^{58} (pLW42) and the Ser^{105} (pLW44) mutants of IL-2 may be due to the ability of a small proportion of the mutant protein to exist transiently in a biologically active conformation, thus allowing binding to the IL-2 receptor. Alternatively, it is possible that a disulfide bridge may be formed between Cys^{58} and Cys^{125}, or between Cys^{105} and Cys^{125}, allowing the proteins to fold into a conformation that can still bind to the IL-2 receptor but with a much lower affinity and therefore with apparently lower level of biological activity. The latter alternative is unlikely, because when the pLW46 extracts are mixed with 100-fold excess of extracts of pLW42 or pLW44, the activity is additive (data not shown). If the IL-2 proteins encoded by pLW42 and pLW44 have a lower affinity for the receptor than does the protein encoded by pLW46, one would expect the activities to be nonadditive when mixed with increasing amounts of the higher affinity pLW46-encoded Ser^{125} IL-2. Purification of both Ser^{58} and Ser^{105} mutant proteins would permit competitive receptor binding studies to be performed to compare the affinity of these two modified IL-2 proteins for the IL-2 receptor.

The level of biological activity found in extracts of pLW42 is one-fourth to one-third that of extracts of pLW44. If both Cys^{58} and Cys^{105} are involved only in structurally holding the IL-2 protein in a biologically active conformation, a mutation at either site that prevents the formation of disulfide bonds would be expected to inactivate the mutant proteins to a similar extent. The consistently lower activity of the Ser^{58} mutant leads one to speculate that the regions of the

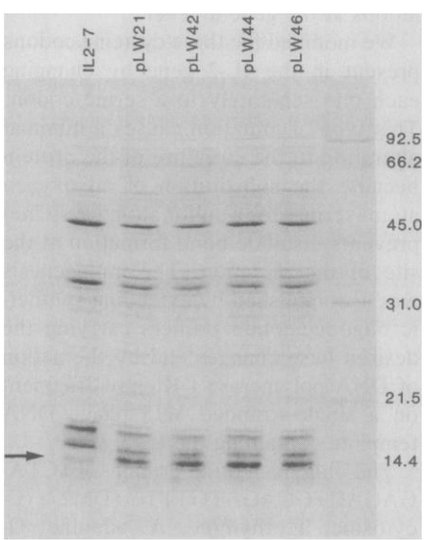

Fig. 3. Sodium dodecyl sulfate–polyacrylamide gel analysis of proteins in extracts of *E. coli* harboring plasmids expressing IL-2. All bacterial extracts were prepared as described in Table 1. Cell-free extracts were subjected to SDS-polyacrylamide gel electrophoresis as described by Laemmli (*20*). Proteins were visualized by Coomassie blue staining.

IL-2 protein surrounding the Cys^{58} residue may be involved in the interaction with the IL-2 receptor on cell surfaces, and therefore slight perturbations of the amino acid sequence (cysteine → serine) in that region may adversely affect the receptor binding capability of the protein. To demonstrate that Cys^{58} is directly involved in receptor binding, it is necessary to study the structure-function relationship of the surrounding amino acids, either by site-specific mutagenesis, in order to identify the effects of these changes on receptor binding, or by coupling of the protein to the receptor, in order to identify the amino acids involved in the direct interaction with the receptor.

Our results clearly demonstrate that Cys^{125} of the human IL-2 protein is not involved in a disulfide bridge and is not necessary for the interaction with the IL-2 receptor. However, both Cys^{58} and Cys^{105} are necessary for biological activity and therefore may be involved in a disulfide bridge that holds the IL-2 protein in a biologically active conformation. In addition, the phenotype of the Ser^{58} IL-2 suggests that Cys^{58} may also be involved in receptor binding. Purification of these three mutant IL-2 proteins should help to determine in what ways their protein conformations differ from that of the wild-type protein.

Using the same technique to replace a free cysteine residue in human fibroblast interferon while retaining biological activity helped us to overcome difficulties encountered during the purification of the recombinant interferon from *E. coli* (*11*). The purified recombinant wild-type interferon protein had a low specific activity and poor stability because of the preferential formation of incorrect disulfide bridges between the three cysteine residues of the protein (*11*). Substituting a serine for one of the cysteines stabilized the recombinant fibroblast interferon, so that it had a specific activity comparable to that of native interferon (*11*). In the case of recombinant human IL-2, we have found that the purified protein is unstable in the reduced form and readily oxidizes into oligomeric forms with loss of biological activity. After controlled oxidation, the purified IL-2 protein had a higher specific activity and greater stability, but the oxidized product is heterogeneous and all possible disulfide bridges are formed (*17*). Therefore, the ability to replace one of the cysteine residues with a serine residue while retaining biological activity is likely to help generate a recombinant IL-2 that is both molecularly more homogeneous and has a reproducibly higher specific activity. On the basis of our present results and our results on human fibroblast interferon (*11*), we suggest that this could be a general method for preparing biologically active "muteins" (*18*) from proteins containing nonessential cysteine residues.

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Identification and Location of Brain Protein 4.1

Abstract. *Protein 4.1 is a membrane skeletal protein that converts the low-affinity interaction between spectrin and actin into a high-affinity ternary complex of spectrin, protein 4.1, and actin that is essential to the structural stability of the erythrocyte. Pig brain was shown to contain an 87-kilodalton immunoreactive analog of protein 4.1 that has partial sequence homology with pig erythrocyte protein 4.1 and the same location as spectrin in the cortical cytoplasm of neuronal and glial cell types of the cerebellum.*

Protein 4.1, spectrin, and actin are major components of a fibrous meshwork of proteins located on the cytoplasmic surface of the erythrocyte membrane, which is now commonly referred to as the membrane skeleton. The membrane skeleton is a strong, flexible, elastic structure that appears to be responsible for the maintenance of the erythrocyte shape, reversible deformability, and membrane structural integrity (*1*). Erythrocyte protein 4.1 from human, pig, and mouse is a phosphoprotein of about 80,000 daltons (80 kD) (*2*) that binds to the terminal ends of the fibrous spectrin $(\alpha\beta)_2$ tetramer (α = 240 kD, β = 220 kD) and thereby stimulates the weak, end-on, bivalent binding of spectrin tetramer to F actin probably through formation of a ternary complex of spectrin,