Interleukin-7 (IL-7) is a pleiotropic cytokine originally detected by its ability to stimulate the growth of precursor B-cells in vitro (1). It was subsequently shown that immature and mature T cells also proliferate in response to IL-7 (2, 3). In addition, IL-7 stimulates the growth of B and T acute lymphoblastic leukemia cells (4) in vitro. Other studies demonstrate that IL-7 stimulates the proliferation of cells derived from patients with chronic lymphocytic leukemia and the Sezary syndrome of cutaneous T cell lymphoma (5). The observation that IL-7 induced an increase in DNA synthesis in acute myelogenous leukemia cells suggests that the pleiotropic effect of this cytokine is not restricted to cells from the lymphoid lineage (6).

Molecular and biochemical characterization of the IL-7 cytokine and the IL-7 receptor (IL-7R) have been described (7, 8). Human IL-7 was expressed in COS cells from a cDNA gene encoding a 177-amino acid (17,518 Da) glycoprotein preceded by a 25-amino acid signal sequence (7). Goodwin et al. (8) isolated cDNA clones that encode the murine and human forms of the IL-7R. Binding of radiolabelled IL-7 to recombinant IL-7R identified high and low affinity binding classes ($K_D = 1 \times 10^{-10}$ and $4 \times 10^{-7}$ M, respectively). Flow cytometric analyses have verified IL-7R expression on cells of both lymphoid and myelomonocytic origins (9) with T-cell and early B-cell lymphomas having between 2,000 and 4,000 IL-7 receptors/cell.

Mature IL-7 contains six cysteine residues. Although it has been reported that biologic activity is lost upon reduction with β-mercaptopetanol (10), the disulfide bond assignment of IL-7 has not been previously reported. Extensive work has been carried out on the biologic effects of IL-7; however, little is known of the cytokine's structure-function relationships. To begin an investigation of the tertiary structure of IL-7, we report on the construction of a synthetic gene encoding human IL-7 (hIL-7). We have biophysically and genetically mapped the disulfide bonds in hIL-7 using a combination of MALDI mass spectroscopy and site-directed cysteine to serine mutational analyses. MALDI mass spectroscopy performed on a tryptic digestion of hIL-7 under nonreducing conditions reveals a dimer of 68,500 M, respectively. In single disulfide bond-forming mutants of hIL-7, the ability to stimulate cell proliferation was abolished in the presence of 2 mM dithiothreitol. The results presented strongly suggest that only a single disulfide bond is required for hIL-7 to form a tertiary structure capable of stimulating precursor B-cell proliferation.

Disulfide Bond Assignment in Human Interleukin-7 by Matrix-assisted Laser Desorption/Ionization Mass Spectroscopy and Site-directed Cysteine to Serine Mutational Analysis*

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Interleukin-7 (IL-7) is a proteinaceous biological response modifier that has a bioactive tertiary structure dependent on disulfide bond formation. Disulfide bond assignments in human (h)IL-7 are based upon the results of matrix-assisted laser desorption/ionization (MALDI) mass spectroscopy and Cys to Ser mutational analyses. A gene encoding the hIL-7 was synthesized incorporating Escherichia coli codon usage bias and was used to express biologically active protein as determined by stimulation of precursor B-cell proliferation. MALDI mass spectroscopic analysis of trypsin-digested hIL-7 was performed and compared with the anticipated results of a simulated tryptic digestion. Many of the anticipated hIL-7 trypsin fragments were detected including one with a molecular mass equivalent to the sum of two polypeptides linked through a disulfide bond formed from Cys residues (Cys3 and Cys142). Subsequently, Cys to Ser substitution mutational analyses were performed. A hIL-7 variant with all six Cys substituted with Ser was found to be biologically inactive ($EC_{50} > 1 \times 10^{-7}$ M). In contrast, a family of single disulfide bond-forming variants of hIL-7 were constructed by reintroducing Cys pairs (Cys2-Cys142, Cys35-Cys130, and Cys48-Cys93), and each could stimulate cell proliferation with an $EC_{50}$ of $4 \times 10^{-8}$, $2 \times 10^{-8}$ and $2 \times 10^{-9}$ M, respectively. In single disulfide bond-forming mutants of hIL-7, the ability to stimulate cell proliferation was abolished in the presence of 2 mM dithiothreitol. The results presented strongly suggest that only a single disulfide bond is required for hIL-7 to form a tertiary structure capable of stimulating precursor B-cell proliferation.

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‡ The abbreviations used are: IL-7, interleukin-7; hIL-7, human IL-7; rhIL-7, recombinant hIL-7; IL-7R, IL-7 receptor; PCR, polymerase chain reaction; DTT, dithiothreitol; MALDI, matrix-assisted laser desorption/ionization; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yI)-2,5-diphenyl tetrazolium bromide salt.
DNA Synthesizer). The synthetic gene encoding rhIL-7 was designed using E. coli codon usage biases and constructed by polymerase reaction (PCR) amplification of four oligonucleotides spanning the 456-base pair length of the gene. The four IL-7 oligonucleotide DNA sequences were sequence 1, 5'-GTGCTTCTTACCTGCTGATG-3'; sequence 2, 5'-ACTACGCTGAGAGCTTTGTCG-3'; sequence 3, 5'-TGAGTCGCGCGTTGCTTCTG-3'; and sequence 4, 5'-TAGTCTGTTTTTGTTTATTTGTG-3'. The four IL-7 oligonucleotide primers used for PCR amplification were: NeoI primer, 5'-ACTACGCTGAGAGCTTTGTCG-3'; and HindIII primer, 5'-GTGCTTCTTACCTGCTGATG-3'.

Expression and Purification of hIL-7—Interleukin-7 and IL-7 mutant proteins were expressed under the control of a T7 RNA polymerase promoter in derivatives of pET11d. Bacterial expression cultures were induced by the addition of isopropyl-β-D-thiogalactoside to a final concentration of 1.0 mM (U. S. Biochemical Corp.). Following a 2-h incubation at 37 °C, bacteria were harvested by centrifugation (5000 g, 20 min, 4 °C). The insoluble inclusion body pellet was washed three times and resuspended in 10 ml of IL-7 free medium at a density of 1 × 10^8 cells/well. A 50-μl aliquot of protein was added to each well to give a range of concentrations from 10⁻¹² to 10⁻⁸ M in a final volume of 100 ml. After a 48-h incubation, a 30-μl aliquot of MTT (5 mg/ml) was added to each well and incubated at 37 °C for 2 h. The formazan crystals were then solubilized by adding 100 μl of 20% SDS in 50 mM 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide, pH 4.7, and incubating at 37 °C overnight (17). The following day the absorbance of the formazan product was measured on an enzyme-linked immunosorbent assay plate reader at 570 nm. Commercially available rhIL-7 preparations (Sterling Winthrop Inc., Collegeville, PA, and Upstate Biotechnology Incorporated, Lake Placid, NY) were used to establish the 2E8 Biosassay. Medium alone served as a negative control, and each sample was assayed in quadruplicate.

MALDI Mass Spectroscopy—Protein for MALDI mass spectroscopy was further purified using C4 reverse phase HPLC. Protein was eluted with an acetonitrile, 1% TEA gradient, fractions containing IL-7 were lyophilized and then resuspended in distilled water. Aliquots were digested with trypsin in 25 mM ammonium bicarbonate buffer, pH 7.8, for 18 h. Mass analysis was performed with a Vison 2000 reflection TOF mass spectrometer using a nitrogen laser 337-nm, 3-ns pulse width (Laser Science, Inc., Newton, MA). Data were collected and analyzed with a Pentium-based system with a 500 MHz sampling rate. Protein (10 pmol) was analyzed in a matrix of either dihydroxy benzoic acid for undigested samples or sinapinic acid for trypsin fragments.

RESULTS

Design and Construction of a Synthetic rhIL-7 Gene Sequence—The native DNA sequence encoding IL-7 (7) was modified in two ways to generate the synthetic sequence used in this work. First, all codons in the native hIL-7 sequence were changed to those favoring high level expression using E. coli codon bias (18). These changes do not alter the native IL-7 amino acid sequence, and this strategy has been shown to increase recombinant IL-2 production in E. coli (19). Secondly, using genetic wobble in the third position, individual codons were altered to introduce four unique restriction sites into the synthetic IL-7 gene: NdeI, AgeI, NaeI, and XhoI at positions 139, 217, 305, and 339, respectively. These sites facilitated cloning of the synthetic hIL-7 gene and the cassette mutagenesis leading to the construction of Cys to Ser substitution mutants (Table I). The sequence of the synthetic hIL-7 gene used in this study has been deposited in the GenBank™ data base (accession number AF019762).

Genetic Construction and Expression of hIL-7—The synthetic rhIL-7 gene was constructed from two pairs of overlapping oligonucleotides that spanned the entire length of the gene. The 5’ to 3’ construction of the gene was accomplished by PCR amplification of these oligonucleotide pairs. Oligonucleotides 1 and 2 (Oligo Set A) were PCR amplified to produce the 5’ half of the rhIL-7 gene, and the 3’ half of the gene was similarly amplified using Oligo Set B as the PCR template. The templates were designed such that both PCR products had a
unique AgeI restriction site that would allow for in-frame annealing during subsequent cloning, to produce the entire synthetic hIL-7 gene. The 5’ half of the synthetic hIL-7 gene was subcloned into pET11d using NcoI and HindIII restriction sites. Subsequently, the 3’ half was subcloned into the intermediate plasmid construct using AgeI and HindIII restriction sites to produce the IL-7 expression plasmid, pES5. In this construct, the structural gene encoding hIL-7 is under the control of a T7 promoter. Because the rhIL-7 gene in pES5 was constructed using PCR amplification, the intact gene was sequenced to ensure correct nucleotide incorporation.

E. coli HMS174(DE3)pLysS(pES5) were grown to an OD

A

of 0.6, and hIL-7 gene expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside. 2 h after induction, bacteria were harvested by centrifugation, and cell lysates were analyzed by SDS-PAGE under denaturing and reducing conditions. Following isopropyl-β-D-thiogalactopyranoside induction a protein band with a molecular mass of 17 kDa is present (Fig. 1A, lane 3). The apparent molecular mass is in excellent agreement with the predicted mass of 17,518 Da, calculated from the deduced amino acid sequence of hIL-7. Fig. 1B shows an immunoblot of the SDS-PAGE probed with anti-hIL-7 antibody and demonstrates that the 17-kDa protein is immunoreactive. The hIL-7 expressed in E. coli HMS174(DE3)pLysS(pES5) formed inclusion bodies that were purified by centrifugation (Fig. 1A, lane 4). Inclusion bodies were solubilized in 5 M guanidine hydrochloride, and the hIL-7 was further purified by HPLC molecular sizing (Fig. 1A, lane 5). Also, note that Fig. 1B (lanes 3 and 4) displays an anti-IL-7 immunoreactive protein with an apparent molecular mass of 34 kDa that corresponds to the size of an IL-7 dimer.

Refolding hIL-7 into a Biologically Active Conformation—To establish optimal refolding conditions for hIL-7, inclusion bodies were solubilized in 5 M guanidine hydrochloride, and the denatured protein was allowed to refold under various conditions. Denatured protein was refolded by dialysis against citrate-phosphate buffers at pH 4, 5, and 7, and glycine-NaOH buffer (0.05 M glycine, 0.03 M NaOH) at pH 10, where all buffers contained 6 mM DTT. Disulfide bonds were then allowed to form by removing DTT by dialysis. Once refolded, hIL-7 was assayed for biologic activity by measuring the stimulation of IL-7-dependent 2E8 cell proliferation in vitro. Maximal stimulation of proliferation of 2E8 cells occurred in the presence of hIL-7 refolded at pH 10 and 7 (data not shown). Although the addition of 50 mM NaCl did not appear to enhance the refolding, the addition of 0.4 M L-arginine to the buffers increased the yield of biologically active monomeric hIL-7 protein. Following purification and refolding the ED50 for hIL-7 stimulation of 2E8 cell proliferation was 2 × 10−10 M.

Biological Activity Based upon a Tertiary Structure Dependent on Disulfide Bond Formation—We next examined the sensitivity of hIL-7 to reduction in the presence of various concentrations of DTT. As shown in Fig. 2, 2E8 cells incubated with hIL-7 (1 × 10−8 M) resulted in a 2-fold increase in the A570 nm compared with control. The 2-fold increase in the A570 nm was lost when 2 mM DTT was added to the reaction. The loss of biologic activity in the presence of a thiol reducing compound suggests that the active tertiary structure of IL-7 is dependent on the formation of disulfide bonds.

MALDI Mass Spectroscopy of hIL-7 and Analysis of Trypsin-digested hIL-7—Mass spectroscopy of hIL-7 (17,518 Da) detected molecules with molecular masses of 17,519.1 Da and 8761.1 Da (Figs. 2A, 2B). The 8761.1-Da fragment represents a doubly ionized hIL-7 molecule. MALDI mass spectroscopy of trypsin-digested hIL-7 detected many protein fragments with masses corresponding to the size of predicted products (Table II). In addition, several of the polypeptides that contain a methionine appear to have been either oxidized as indicated by the addition of 15.9 Da to the predicted mass or coordinated with a Na+ that adds 22.9 Da. IL-7 trypsin fragments 7 and 12 were detected in both the oxidized and nonoxidized states, whereas fragment 15 was found with and without a bound Na+ (data not shown). The detection of molecules with a molecular mass of 1557.8 Da corresponds well to the size of hIL-7 tryptic...
fragments 1 and 23 linked through a disulfide bond (Fig. 2B). The association of these fragments physically maps the disulfide bond in hIL-7 to cysteine pair Cys<sup>3</sup>-Cys<sup>142</sup>. MALDI mass spectroscopy of hIL-7 tryptic digestion under reducing conditions did not detect the 1557.8-Da fragment (Fig. 2C).

Characterization of hIL-7 Cys to Ser Mutants—Individual site-directed Cys to Ser alterations were introduced into the hIL-7 DNA sequence using PCR mutagenesis. The mutated sequences were then subcloned into the hIL-7 structural gene by cassette replacement, and each mutant gene was then DNA sequenced to ensure that only the desired Cys to Ser mutation was introduced.
HIL-7 and Cys to Ser mutant proteins were then expressed and purified, and their ability to stimulate cell proliferation in the absence or the presence of DTT was measured (Fig. 3). Although each of the single Cys to Ser substitutions in hIL-7 were found to be biologically active (data not shown), the simultaneous replacement of all six Cys residues with Ser resulted in a mutant protein with an ED50 $< 1 \times 10^{-7}$ M. Based upon the MALDI mass spectroscopy analysis, Cys residues 3 and 142 were genetically reintroduced to form hIL-7 (C3S,C35S,C48S,C93S,C130S,C142S). This variant form of hIL-7 was found to have an ED50 $4 \times 10^{-9}$ M. Based upon computational molecular modeling and other site-directed mutagenesis experiments, cysteine pairs Cys48-Cys93 and Cys35-Cys130 were independently reintroduced into hIL-7 (C3S,C35S,C48S,C93S,C130S,C142S) producing hIL-7 (C3S,C35S,C130S,C142S) and hIL-7 (C3S,C35S,C48S,C93S,C130S,C142S). The latter two IL-7 variants were found to have an ED50 of $2 \times 10^{-9}$ and $2 \times 10^{-9}$ M, respectively. The reintroduction of Cys pairs Cys3-Cys142, Cys48-Cys93, and Cys35-Cys130 into hIL-7 (C3S,C35S,C48S,C93S,C130S,C142S) resulted in three variant forms of hIL-7. Each of these variants had the ability to form a single correctly paired disulfide bond. In each case the formation of a single correctly paired disulfide bond was sufficient to produce a biologically active conformation of IL-7 that was sensitive to 2 mM DTT (Fig. 3B). A summary of biological activity and IC50 for displacement of 125I-labeled IL-7 from the IL-7 receptor for native and Cys to Ser hIL-7 variants is presented in Table III.

**TABLE II**

| Fragment | Position | Average molecular mass | Amino acid sequence |
|----------|----------|------------------------|---------------------|
| 1        | 1–8      | 910.04                 | MDCDIEGK            |
| 2        | 9–11     | 318.33                 | DGK                 |
| 3        | 12–29    | 2099.48                | QYESVLMIDSQFLDSMK   |
| 4        | 30–44    | 1775.96                | EIGSNCLNMPFF    |
| 5        | 45–45    | 174.20                 | R                  |
| 6        | 46–52    | 799.91                 | HICDANK            |
| 7        | 53–59    | 899.08                 | EGMFLFR            |
| 8        | 60–62    | 316.36                 | AAR                |
| 9        | 63–63    | 146.19                 | K                  |
| 10       | 64–65    | 287.36                 | LR                 |
| 11       | 66–69    | 534.66                 | QFLK               |
| 12       | 70–82    | 1490.70                | MNSTGFDLHLLK       |
| 13       | 83–98    | 1662.93                | VSEGTILLNCTDGVK    |
| 14       | 99–100   | 231.26                 | GR                 |
| 15       | 101–102  | 1210.40                | KPAALGEOQFTK       |
| 16       | 113–118  | 718.76                 | SLEENK             |
| 17       | 119–121  | 346.43                 | SLK                |
| 18       | 122–124  | 403.44                 | EQR                |
| 19       | 125–125  | 146.19                 | K                  |
| 20       | 126–133  | 965.18                 | LNDLCFLK           |
| 21       | 134–134  | 174.20                 | R                  |
| 22       | 135–140  | 742.92                 | LLQEIK             |
| 23       | 141–145  | 650.76                 | TCKINK             |
| 24       | 146–151  | 661.87                 | ILMGTK             |
| 25       | 152–153  | 284.27                 | EH                 |

**DISCUSSION**

The disulfide bond assignments in hIL-7 were assigned based upon the combination of MALDI mass spectroscopy and site-directed Cys to Ser mutational analyses. Mature hIL-7 contains six cysteine residues and has been reported to lose all biological activity when incubated in the presence of a thiol. MALDI mass spectroscopic analyses were performed on trypsin-digested hIL-7 only and detected a pair of peptide fragments, 1 and 23, linked by a disulfide bond that physically maps the Cys pair Cys3-Cys142. Mass analysis of a trypsin-digested hIL-7 in the presence of 2 mM DTT fails to detect the paired fragments confirming the existence of the Cys3-Cys142 disulfide bond. Subsequently, site-directed Cys to Ser substitution mutational analysis of hIL-7 was performed to complement the MALDI mass spectroscopy work. A family of Cys to Ser substitution IL-7 mutants were constructed that included a biologically inactive non-disulfide
bond-forming mutant, hIL-7 (C3S,C35S,C48S,C93S,C130S, C142S). Ser was chosen to replace Cys residues in IL-7 because of its similarity in size and structure and the inability to form a disulfide bond. In the absence of disulfide bond formation, this variant of hIL-7 displayed an EC\textsubscript{50} \textgreater 1 \times 10^{-5} M for stimulation of 2E8 cell proliferation. Treatment of the non-disulfide bond-forming hIL-7 mutant with 2 mM DTT reduced biological activity to control levels, suggesting that disulfide bond formation in the 2E8 cell IL-7R is essential for stimulating cell proliferation. In fact, the IL-7R has been classified with a group of cytokine receptors based partially upon a predicted disulfide bonding pattern (20).

The site-directed mutational analysis of hIL-7 proceeded by reintroducing pairs of Cys residues into the non-disulfide bond-forming biologically inactive hIL-7 mutant. Reintroduction of Cys pair Cys\textsuperscript{3}-Cys\textsuperscript{142} formed a variant of hIL-7 that displayed a biologically active tertiary conformation. In comparison, MALDI mass spectroscopic analysis of trypsin-digested hIL-7 revealed a polypeptide with a molecular mass corresponding to the union of two fragments covalently linked through a disulfide bond between Cys pair Cys\textsuperscript{3}-Cys\textsuperscript{142}. Subsequently, the individual reintroduction of Cys pairs Cys\textsuperscript{35}-Cys\textsuperscript{130} and Cys\textsuperscript{48}-Cys\textsuperscript{93} into the non-disulfide bond-forming biologically inactive hIL-7 variant produced two single disulfide bond-forming mutants that were biologically active (EC\textsubscript{50} 4 \times 10^{-9}, 2 \times 10^{-9} M and 2 \times 10^{-9} M, respectively) in the in vitro precursor B-cell proliferation assay. All three single disulfide bond-forming hIL-7 mutants failed to stimulate precursor B-cell proliferation in the presence of 2 mM DTT. The Cys to Ser mutational analysis in this work has allowed us to assign three pairs of Cys residues involved in forming disulfide bonds in hIL-7. Mapping the disulfide bonds in hIL-7 increases the resolution involved in predicting the tertiary structure of this cytokine. An inverted protein structure prediction based on the amino acid sequence of hIL-7 has been combined with the data in this report and used to propose a hypothetical three-dimensional molecular model of the tertiary structure. It is anticipated that this model of IL-7 will be of some help in predicting those amino acid residues that play a role in receptor binding and activation.

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