Mutagenic Analysis of a Receptor Contact Site on Interleukin-2: Preparation of an IL-2 Analog with Increased Potency†

William G. Berndt,‡ David Z. Chang,‡ Kendall A. Smith,§ and Thomas L. Ciardelli*,‡,∥

Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, New Hampshire 03755, Department of Medicine, Cornell Medical College, New York, New York 10021, and The Veterans Administrations Hospital, White River Junction, Vermont 05001

Received February 3, 1994®

ABSTRACT: Interleukin-2 (IL-2) is a 133 amino acid α-helical protein secreted by activated T-cells. Combinatorial cassette mutagenesis was used to investigate the functional role of a contiguous five amino acid region of IL-2 suspected to interact with the intermediate-affinity IL-2 receptor. A limited random library of IL-2 mutants was constructed in which residues 17–21 (Leu-Leu-Leu-Asp-Leu) were simultaneously mutated. The proteins were produced in an *Escherichia coli* expression system and screened in a biological assay for their ability to mediate the proliferation of a murine IL-2-dependent cell line. From the over 2600 clones examined, only 42 exhibited significant activity, confirming the functional importance of this region. Selected clones were purified and further characterized by biological and receptor binding assays. Viewed in the context of the recently revised 2.5-Å crystal structure for IL-2, these results suggest the following conclusions: both Asp20 and Leu21, as shown by their sensitivity to mutation, are the functionally more important residues in this region, but for different reasons. Asp20 is solvent-accessible and likely plays a direct receptor contact role as previous studies have indicated. Leu21, in contrast, is completely buried in the hydrophobic core of the protein. Substitutions at this position, even a conservative Leu → Val substitution, were found to perturb the precise hydrophobic packing arrangements that are critical for activity, resulting in a significant loss of function. In addition, one of the analogs identified in the screen was found to be 2–3 times more potent than the wild-type protein.

Interleukin-2 (IL-2)[1] is a 15–18-kDa variably glycosylated protein secreted by activated T-cells. Originally recognized as the T-cell growth factor, IL-2 has since been shown to play an important role in modulating the activation, proliferation, and differentiation of other cells in the immune system, including NK-cells and B-cells (Smith, 1988). Because of its important immunoregulatory role, and the therapeutic promise it holds for the treatment of certain cancers and infectious diseases (Kaplan et al., 1992; Lotze et al., 1985, 1986; Smith et al., 1990), IL-2 has been the focus of several structure–function studies (Berndt & Ciardelli, 1992).

The biological effects of IL-2 on responsive cells are mediated through the interaction of the ligand with cell-surface IL-2 receptors (IL-2R). Three distinct IL-2R subunits have been characterized and cloned: the p55 α chain (Leonard et al., 1984; Nikaido et al., 1984), the p75 β chain (Hatakeyama et al., 1989), and the p64 γ chain (Takeshita et al., 1992a,b). Both p75 and p64 (but not p55) are members of the recently recognized hematopoietic receptor superfamily, that includes receptors for IL-2–IL-7, GM-CSF, erythropoietin, growth hormone, and prolactin (Bazan, 1989, 1990; Cosman, 1993; Cosman et al., 1990; Minami et al., 1993). These three transmembrane proteins cooperate noncovalently in the presence of IL-2 to form the physiologically important high-affinity receptor complex (Taniguchi & Minami, 1993). The high-affinity complex binds IL-2 with a dissociation constant (K_d) of $\approx 10^{-11}$ M. In addition to the high-affinity site, two other IL-2 binding sites have been characterized. The p55 subunit can bind IL-2 by itself to form the low-affinity IL-2R ($K_d = 10^{-8}$ M). Likewise, the p75 and p64 subunits associate in the presence of IL-2 to form the intermediate-affinity receptor complex which binds IL-2 with a $K_d = 10^{-9}$ M. It is the p75/p64 heterodimer that most likely forms the signaling complex (Takeshita et al., 1992a,b; Johnson et al., 1994), and the p64 γ subunit has recently been shown to be shared by both the IL-4 and IL-7 receptors (Kondo et al., 1993; Noguchi et al., 1993; Russell et al., 1993).

Numerous studies employing mutagenesis to probe IL-2 receptor binding have been reported, yet details of the IL-2/IL-2R interaction remain limited (Berndt & Ciardelli, 1992). In one study, a single mutation near the N-terminus of IL-2 (Lys for Asp20 or **D20K**) was reported to specifically perturb the interaction between IL-2 and the intermediate-affinity receptor (Collins et al., 1988). To further investigate the functional role of the region around Asp20, we have employed combinatorial cassette mutagenesis (Dunn et al., 1988; Reidhaar-Olson & Sauer, 1988) as a strategy to evaluate the structural significance of this region. Five contiguous amino acids (Leu17, Leu18, Leu19, Asp20, and Leu21) were simultaneously mutated to create a large library of IL-2 analogs. Residues 17–21 were selected based upon their probable conformation as derived from a reported 3-Å crystal structure of IL-2 (Brandhuber et al., 1987) that provided information on the peptide backbone conformation only. On the basis of that model, the 17–21 segment was located at the

† This work was supported by grants from the National Institutes of Health (AI23398), the Veterans Administration (VA002403234-05), and the American Cancer Society (FRA-385). Support provided by the Norris Cotton Cancer Center is gratefully acknowledged.

* To whom correspondence should be addressed at the Department of Pharmacology and Toxicology, Dartmouth Medical School.

‡ Dartmouth Medical School.

§ Cornell Medical College.

∥ The Veterans Administration Hospital.

® Abstract published in *Advance ACS Abstracts,* May 15, 1994.

[1] Abbreviations: CD, circular dichroism; DMSO, dimethyl sulfoxide; Gdn·HCl, guanidine hydrochloride; GM-CSF, granulocyte/macrophage colony stimulating factor; IL, interleukin; IPTG, isopropyl thiogalactoside; PBS, phosphate-buffered saline.

0006-2960/94/0433-6571$04.50/0

junction of an N-terminal α-helix and a loop leading to the next helical segment, and it seemed likely that most of the side chains were solvent-accessible and available for receptor interaction.

The protein library was expressed in bacteria, and crude bacterial lysates were screened in a biological assay for their ability to stimulate the proliferation of an IL-2-dependent murine cell line. By identifying active mutant clones and determining their sequences, a spectrum of functionally allowable substitutions at each of the mutated positions was determined. This information provided insight into the functional significance of the residues in this region. Furthermore, this approach served to generate a wide combination of electrostatic and stereochemical sequence combinations in a functionally sensitive region of the protein which lead to the production of the first IL-2 analog with improved activity.

Unfortunately, the 3-Å structure of IL-2 upon which the design for the mutagenesis studies was based has been shown to be seriously in error (Bazan, 1992). A revised structure (McKay, 1992) significantly realigned the peptide backbone through the electron density map, repositioning the N-terminus of the molecule. As a result, the 17–21 region of IL-2 was placed within the first of four core α-helices. In the context of the new structure, both Leu17 and Leu21 have their side chains buried in the hydrophobic interior of the protein. Although our original strategy was somewhat misdirected by the incorrect model, the new structure resolves the structural interpretation of the results determined in this study.

MATERIALS AND METHODS

Library Construction. The mutant IL-2 library was prepared by cassette mutagenesis on a synthetic IL-2 gene under the control of an IPTG-inducible *trc* promoter (Williams et al., 1988). A 93-mer oligonucleotide template corresponding to the coding strand of the mutant cassette was synthesized. Different mixtures of the 4 nucleotides were coupled at the 15 nucleotide positions constituting the codons for amino acids 17–21 (Table 1) to create the desired amino acid diversity in the library. 5′ and 3′ PCR primers were synthesized and used to enzymatically convert and amplify the single-stranded template into a double-stranded insert (Thiesen, 1990). The PCR product was digested with *Xho*I and *Mlu*I for directional cloning into the vector. To reduce the recovery of wild-type colonies, the synthetic IL-2 gene was modified. The wild-type DNA sequence between the *Xho*I and *Mlu*I sites in the gene was replaced with an unrelated 1.5-kb DNA insert to aid in isolation of doubly-digested vector by agarose gel electrophoresis. The cassettes containing the randomized sequences were cloned into the purified, doubly-digested vector and transformed by electroporation into the JM101 strain of *Escherichia coli* (Dower et al., 1988).

Biological Screen and Protein Preparation. Individual bacterial colonies harboring the mutant IL-2 plasmids were inoculated into 96-well microtiter plates containing 150 μL of M9 media plus ampicillin. Cultures were grown at 37 °C with shaking for 4–5 h, an aliquot (25 μL) was removed for storage in 10% DMSO at −70 °C, and IPTG was added (final concentration, 1 mM) to induce protein expression. After 2 h, duplicate 50-μL, aliquots of each culture were transferred to two new microtiter plates containing 100 μL of 7 M Gdn·HCl/50 mM Tris, pH 8.0. After shaking for 30 min, a 25-μL aliquot of each lysate was transferred to separate tubes containing 5 mL of PBS, pH 7.0, and mixed well; 25 μL of this solution was then added to individual wells of a 96-well microtiter plates containing 75 μL of BIDMEM media supplemented with 50 mg/mL gentamycin, 200 mg/mL L-glutamine, and 50 units/mL penicillin. One hundred microliters of CTLL-2 cells (4×10^4 cells/mL) was added to each well, and the mutant proteins were tested for their ability to maintain the proliferation of this IL-2-dependent cell line as described (Gillis et al., 1978). Four wild-type IL-2 standards and two Gdn·HCl blanks were included in every assay (due to the inherent variability in protein expression and folding between clones, this biological screen was not employed as a quantitative measure of biological activity). Clones producing biologically active protein were sequenced. A similar number of inactive clones were also sequenced. Selected analogs were expressed, refolded, and purified on a larger scale using standard methods for IL-2-related proteins (Landgraf et al., 1991) to obtain quantitative biological and receptor binding data. All of these analogs were monitored for purity and monomer content by reverse-phase and size-exclusion HPLC as described (Landgraf et al., 1991).

The IL-2 analog having a deletion of residues 17–31 (**Del1**) was identified from a separate mutagenesis experiment. It was expressed, refolded, and purified as described (Landgraf et al., 1991).

Purified samples of two IL-2 analogs (**7** and **Del1**) were subjected to 25 cycles of automated N-terminal sequence analysis on an Applied Biosystems Model 476A protein sequencer. The determined amino acid sequence correctly matched that predicted from the DNA sequence in both cases.

Competitive Radioreceptor Binding Assays. Competitive displacement of ^{125}I-IL-2 by mutant proteins was performed as described (Landgraf et al., 1992) on normal human T-cells induced to express high-affinity IL-2 receptors by stimulation for 72 h with OKT3 monoclonal antibody (Ortho Pharmaceuticals). Competitive binding at the intermediate-affinity (p75/p64) and low-affinity (p55) IL-2 receptors was accomplished using the cloned cell lines YT-2C2 (Teshigawara et al., 1987) and MT-1 (Miyoshi et al., 1980), respectively. For each protein, 3–5 separate assays were carried out with triplicate determinations, and curves were fit to the mean of the resulting data points employing a nonlinear least-squares procedure (Landgraf et al., 1992).

Bioactivity on Human Cells. Biological data obtained for the purified proteins were determined from human T-cells isolated (Ficoll–Hypaque purification) from peripheral blood following incubation for 3 days in the presence of OKT3 monoclonal antibody (Ortho Pharmaceuticals) and 2 days after antibody washout as described (Landgraf et al., 1989).

Circular Dichroism. Circular dichroism spectra for IL-2 and **L21V** were determined using an Instruments SA Jobin Yvon circular dichrograph calibrated with (+)-10-camphorsulfonic acid and epiandosterone. Near-UV CD spectra were measured from 250 to 320 nm in a 1.0-cm cell at 25 °C in 25 mM phosphate buffer, pH 6.5. Each protein sample was filtered (0.45-μm syringe filter) and the protein concentration determined by UV absorption at 280 nm in 6 M Gdn·HCl (Johnson, 1990). Spectra presented represent an average of 3–5 scans after subtraction of a buffer blank.

RESULTS

The objective of this study was to evaluate the use of limited simultaneous random mutagenesis as a probe for structurally important positions in a five-residue contiguous sequence of IL-2 centered around Asp20, a residue known to be critical for binding to the intermediate-affinity IL-2R (Collins et al., 1988). The intent was to limit the mutagenesis to proximal residues whose side chains were likely to interact directly with

Table 1: Distribution of Selected Residues for Active and Inactive 17–21 Analogs

IL-2[a]	Leu17	Leu18	Leu19	Asp20	Leu21
Codons[b]	NTS	NWS	NNS	NNS	NYS
residues observed[c]	(3)Leu-**27**,*18*	(3)Leu-**18**,*12*	(3)Leu-**11**,*9*	(3)Leu-**0**,*5*	(3)Leu-**28**,*21*
	(1)Phe-**4**,*2*	(1)Phe-**3**,*0*	(1)Phe-**3**,*0*	(1)Phe-**0**,*0*	(1)Phe-**2**,*3*
	(1)Ile-**2**,*2*	(1)Ile-**5**,*2*	(1)Ile-**2**,*1*	(1)Ile-**0**,*1*	(1)Ile-**0**,*3*
	(1)Met-**8**,*15*	(1)Met-**4**,*7*	(1)Met-**6**,*2*	(1)Met-**0**,*2*	(1)Met-**9**,*1*
	(2)Val-**0**,*5*	(2)Val-**4**,*5*	(2)Val-**6**,*3*	(2)Val-**0**,*3*	(2)Val-**3**,*1*
		(1)Tyr-**2**,*0*	(1)Tyr-**1**,*4*	(1)Tyr-**0**,*0*	
		(1)His-**0**,*0*	(1)His-**0**,*0*	(1)His-**0**,*2*	
		(2)Gln-**3**,*9*	(2)Gln-**0**,*8*	(2)Gln-**4**,*7*	
		(1)Asn-**0**,*0*	(1)Asn-**2**,*3*	(1)Asn-**1**,*0*	
		(1)Lys-**1**,*3*	(1)Lys-**0**,*3*	(1)Lys-**0**,*5*	
		(1)Asp-**0**,*0*	(1)Asp-**0**,*0*	(1)Asp-**14**,*0*	
		(1)Glu-**1**,*2*	(1)Glu-**0**,*1*	(1)Glu-**16**,*1*	(0)Glu-**0**,*1*
			(3)Arg-**2**,*0*	(3)Arg-**0**,*3*	
		(0)Ser-**1**,*0*	(3)Ser-**5**,*3*	(3)Ser-**4**,*4*	(2)Ser-**0**,*2*
			(2)Thr-**2**,*1*	(2)Thr-**2**,*3*	(2)Thr-**0**,*2*
			(2)Pro-**0**,*1*	(2)Pro-**0**,*1*	(2)Pro-**0**,*3*
			(2)Gly-**0**,*0*	(2)Gly-**1**,*0*	(0)Gly-**0**,*1*
			(1)Cys-**0**,*1*	(1)Cys-**0**,*1*	
			(1)Trp-**1**,*0*	(1)Trp-**0**,*1*	

[a] The IL-2 wild-type sequence over positions 17–21. [b] The mixture of nucleotides employed at each position: N = A, G, C, T,; S = G, C; W = A, T; Y = C, T. [c] Residues observed in 42 active and 40 inactive clones. (*X*) indicates the codon redundancy of the mixture; a value of (0) indicates that the residue appeared even though excluded from the nucleotide mixture. -**X**,*Y* indicates that the particular residue appeared **X** times in active proteins and *Y* times in inactive proteins.

the intermediate-affinity IL-2R. Residues 17–21 were chosen based upon their probable location suggested in the model of 3-Å crystal structure that was available at the beginning of this study (Brandhuber et al., 1987). Leu17, Leu18, and Leu19 were located at the C-terminus of helix A, an α-helix peripheral to the 4-fold helical core of the protein. Asp20 and Leu21 were placed adjacent to helix A at the beginning of a 13-residue loop leading to helix B in that structure. Given these structural limitations and the results of previous mutagenesis studies in other species (Zurawski & Zurawski 1989), a limited mutational analysis was performed at Leu17, Leu18, and Leu21 (Table 1) in order to increase the probability of recovering active proteins. Leu19 and Asp20 were allowed to sample all 19 other amino acids; thus, a limited random library of 216 000 different sequence combinations was possible (translational stops in this segment were avoided by codon selection and the use of *SupE* host, JM101).

A representative sample of 2610 clones was screened in duplicate for bioactivity on CTLL-2 cells. Although this cell line is of murine origin, it was chosen for the screen because the CTLL-2 IL-2 bioassay is well established, rapid, and highly reproducible (Gillis et al., 1978). Unlike the standard CTLL-2 bioassay designed to quantitate IL-2 activity, the biologic screen employed in this study measured activity at a single protein concentration. The conditions of the screen were tailored to the wild-type protein such that when the wild-type colonies were included as the positive control in each assay, the observable level of activity in these control wells would be approximately 50% of maximal activity based on a full dose response curve of the wild-type protein. For over 29 separate assays, the level of activity in the control wells included in each plate averaged 49 ± 18% of maximal activity. Each assay tested 90 mutant clones and included 4 wild-type positive controls and 2 Gdn·HCl blanks as negative controls. In each assay, the average maximum [^{3}H]thymidine incorporation obtained for the four standards determined the 100% activity level to which the mutant clones in that assay were compared. The negative control wells containing no IL-2-related protein displayed less than 2% of maximal activity. Wells providing greater than 5% of maximal activity were labeled positive in the screen. Only 42 clones, or 1.6% of the total, tested positive in the biological screen, confirming that the 17–21 region of IL-2 is a functionally sensitive region of the protein. Of the 42 individual active clones, 40 unique amino acid sequences were identified. The results of the initial biological screen are shown in Table 2.

The majority of the active clones encoded only two or three substitutions, although some clones encoding four and even five substitutions displayed activity. One sequence encoding only a single substitution was identified 3 times (Table 2, sequences 3, 4, and 5). That redundancy resulted from identical nucleotide sequences, suggesting that these were all derived from a single transformant. The fact that clones 4 and 5, although screened in the same assay, were found to differ in their initial activities reflects the inherent variability in expression and folding and exemplifies the caution that must be taken when ascribing quantitative significance to data derived from crude bacterial preparations. Another notable feature revealed from sequencing the active clones was the identification of mutations located outside the region targeted for study. Most of these nucleotide substitutions were silent at the amino acid level; however, for clones 17 and 22, substitutions were observed at residues not intended to be examined.

In addition to clones producing active protein, a total of 50 inactive clones were sequenced. Ten of these clones encoded either mutations outside of the target region or base deletions and insertions within the targeted region. This result suggests the possibility that up to 20% of the mutant library screened was found to be inactive not because of detrimental amino acid substitutions in the 17–21 region, but because of substitutions encoded outside of this region or errors in translating a full-length protein. Although not all of these unwanted mutations would lead to inactive protein, even after reducing the total number of 2610 clones tested by 20%, the frequency of active clones in the library remains at only 2%.

Due to the inherent limitations of the biological screening method, our goal was to sample enough of the library to categorize allowable functionality rather than to exhaustively evalute the entire library for all of the active sequence combinations. The 42 active sequences that were recovered provided some insights into the structural constraints of this region. Different amino acids were recovered at each of the five mutated residues, suggesting that none of the wild-type

Table 2: Active Sequences[a]

changes	sequence no.	activity	17	18	19	20	21	extra mutations
zero	wild type	+++	Leu	Leu	Leu	Asp	Leu	
one	1	+++	Met					
	2	+++		Phe				
	3	+++		Val				
	4	+++		Val				
	5	+		Val				
two	6	++			Phe	Ser		
	7	+++		Met	Ser			
	8	++		Gln		Glu		
	9	+++			Val	Glu		
	10	+++		Glu	Ser			
	11	+++			Met	Glu		
	12	+++				Glu	Met	
	13	+++		Gln	Ser			
	14	++	Ile		Met			
	15	+				Gly	Met	
	16	++			Val	Glu		
	17	+			Val		Met	Ile24Thr: C-A
	18	+			Val	Ser		
	19	+++		Tyr	Lys			
three	20	+++		Tyr	Met	Glu		
	21	++		Ser	Trp	Glu		
	22	+			Ile	Thr	Met	Gly27Met: G-A
	23	+++			Arg	Gln	Val	
	24	+++		Met	Arg	Glu		
	25	+++	Met	Ile	Asn			
	26	+	Met	Ile		Gln		
	27	+++	Met		Ser	Glu		
	30	+++	Met			Gln	Met	
	31	++		Ile		Glu	Met	
	32	+	Met	Phe	Asn			
	33	+		Val	Ile	Ser		
	34	+		Phe		Thr	Phe	
	35	+	Phe		Phe	Glu		
four	36	++	Ile	Ile	Thr	Glu		
	37	++	Phe		Met	Glu	Val	
	38	+	Met		Phe	Ser	Met	
	39	+	Phe	Ile	Thr	Glu		
	40	+		Met	Phe	Gln	Met	
five	41	+	Phe	Gln	Ala	Asn	Phe	
	42	+	Met	Met	Met	Glu	Met	

[a] Forty-two active clones were identified in the biological screen arranged by the total number of residue changes found. Activity: + (5–20% of the IL-2 standard); ++ (20–50%); +++ (50–100%). Extra mutations leading to residue changes outside of the targeted region are indicated (three silent mutations were also found).

residues are absolutely critical for conferring function (Table 1). In fact, in a related study, an IL-2 analog was identified that had the segment spanning residues 17–31 deleted (**Del1**), yet this protein retained full agonist activity (Figure 1). Position 20, originally identified as being important for binding, was confirmed to be the least tolerant to substitution. Acidic residues were recovered at this position for 30 out of 42 active clones with almost equal preference either for the wild-type aspartic acid or for glutamic acid (the expected frequency of these residues was ≈7%). The remaining active clones all contained hydrophilic residues at this position with the exception of a single Gly residue (**15**). A similar result has been reported for the murine protein (Zurawski & Zurawski, 1992). Although Leu18 and Leu19 are almost universally conserved among several species (Bazan, 1992), nonconservative substitutions were found to be permissible at both positions. Examination of the revised three-dimensional structure reveals that both residues are largely solvent-exposed and available for receptor contact; however, hydrophobicity at these positions is not strictly required. In contrast, at positions 17 and 21, a strong bias for the wild-type Leu residue was observed. Position 17 was found to accept all of the hydrophobes offered except valine; however, 27 out of 42 sequences recovered Leu. A similar result was observed for

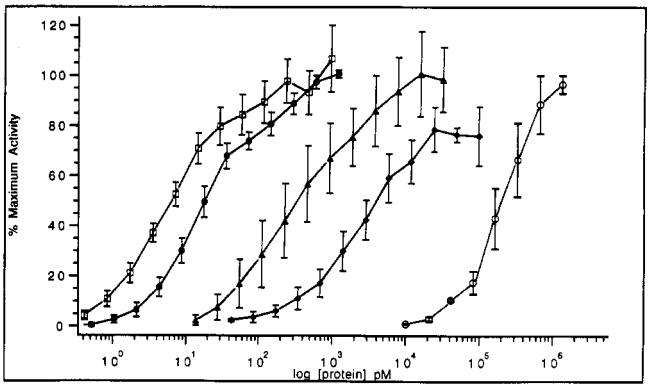

FIGURE 1: Bioactivity of selected IL-2 analogs on human peripheral blood lymphocytes. Activity was determined on day 5 OKT3-activated T-cells as described (Landgraf et al., 1989). Each point represents the mean (±SEM) of three to five independent assays employing triplicate determinations: (●) **IL-2**; (□) **7**; (▲) **L21V**; (♦) **29**; (○) **Del1**. For **IL-2** and analog **7**, seven independent assays were performed using different protein preparations and lymphocytes from different donors.

position 21 except that unlike position 17, Ser, Thr, and Pro were offered but not recovered in the active sample.

Another goal of this investigation was to identify any unusual or unexpected sequence combinations that might represent novel or improved functional domains of IL-2. A number of proteins from the active set were expressed in larger amounts and purified for quantitative biological characterization on human T-cells based upon their sequence or activity in the preliminary screen. Among this set, five mutants were recovered encoding a Ser at position 19. Four of these clones were found to elicit equal or greater activity than IL-2 in the biological screen although these sequences represented substitutions of a hydrophilic for a conserved hydrophobic Leu residue. Most of these proteins exhibited activity comparable to wild-type IL-2 in a quantitative bioassay employing normal activated T-lymphocytes. One analog (**9**), however, was found to be 2–3-fold more potent than IL-2 in this assay. This observation was reproducible with different protein preparations in multiple assays using lymphocytes from different donors ($p < 0.01$).

Of the Ser19 analogs that were found to have diminished potency, protein **29** was the most surprising. In addition to the Ser19 substitution, this clone encoded Glu20 and Val21 substitutions as well. On the basis of the frequency of recovery of glutamic acid at position 20, and a review of the active clones encoding position 21 substitutions, it seemed likely that the decreased potency observed for this clone was primarily a consequence of the Val21 mutation. This represented a conservative substitution for Leu21. To test this directly, an IL-2 analog was prepared with only the single Val for Leu21 replacement (**L21V**). The results from the human bioassay confirmed that even a conservative **L21V** substitution at position 21 decreased the potency of the protein 20-fold (Table 3).

To assess the origin of the observed biological effects, the receptor affinities of these three mutant proteins, **7**, **29**, and **L21V**, were tested by competitive binding assays to the low-, intermediate-, and high-affinity receptors (Figure 2). The 17–21 region of IL-2 was initially identified as a potential intermediate-affinity receptor binding site, so it was expected that the effect of mutations would be reflected at both this site and the high-affinity site, and have a minimal effect on binding to the low-affinity receptor. To an extent, this was found to be the case. On MT-1 cells that express the low-affinity (p55) receptor, however, analogs **29** and **L21V** were

Table 3: Bioactivity on Human Lymphocytes[a]

protein	sequence	EC_{50}[b] (pM)
IL-2	L-L-L-D-L	16.0 ± 2.0
7	L-M-S-D-L	7.6 ± 0.7[c]
15[d]	L-L-L-G-M	(1.4 ± 0.6) × 10^3
29	L-L-S-E-V	(2.6 ± 3.3) × 10^3
L21V	L-L-L-D-V	(4.0 ± 1.0) × 10^2
Del1	deleted	(2.4 ● 0.2) × 10^5

[a] Selected proteins were chosen for quantitative biological characterization. The analogs were expressed in 1-L cultures, purified, refolded, quantitated, and tested on human T-cells as described under Materials and Methods. [b] The concentration of protein (picomolar) generating 50% of maximal response as determined in the human T-cell bioassay. All proteins were full agonists. The results (±SEM) are from three to seven separate assays each determined in triplicate. [c] Satistically different from the proximate value ($p < 0.01$). [d] The results show the average and the range of two experiments carried out in triplicate.

found to suffer a 3–4-fold decrease in affinity. On the 2C2 cell line expressing the intermediate-affinity p75/p64 complex, the effect of the Val21 substitution was also evident, particularly in the context of the additional mutations in clone **29** where a decrease of more than 100-fold in the K_d was observed. At the high-affinity site expressed on human T-cells, the relative affinities of the three proteins appeared to parallel the biological data. Analog **7** displayed a slightly higher affinity although the difference was not statistically significant. Likewise, the decreased potency of **29** and **L21V** corresponded to decreased affinity at the high-affinity complex.

DISCUSSION

A combinatorial cassette mutagenesis approach (Dunn et al., 1988; Reidhaar-Olson & Sauer, 1988) was chosen to study the functional significance of an N-terminal region of IL-2 suspected to interact specifically with the intermediate-affinity IL-2R (Collins et al., 1988). In relation to the original 3-Å X-ray structure (Brandhuber et al., 1987), the 17–21 segment comprised the junction between the first α-helix and an extended loop leading to the second helical region. This three-dimensional model was subsequently shown to be seriously in error (Bazan, 1992; McKay, 1992). The proposed first helix was completely absent in the revised structure; the 17–21 region actually resides entirely within the first core α-helix (residues 6–30) on the opposite face of the molecule.

We restricted the randomness of the library at certain residues based on structural assumptions implied by the original model and the wish to favor active sequences. The limitations imposed by the necessity of using a biologic screen (rather than biologic selection) involving isolating, expanding, inducing, and diluting the proteins produced by each individual clone greatly reduced the scope of the study. Our goal was, therefore, only to study a large enough sample to define structural trends in this region. In addition, we employed nucleotide mixtures at each position that would only marginally favor wild-type residues so that sequences with multiple amino acid substitutions would predominate. This would afford an opportunity to examine sequence combinations that diverge greatly from the wild type in a region that is highly conserved among species (Bazan, 1992). Less than 2% of the 2600 clones examined were positive in the biologic screen. Despite the limitations of the method that include variability in expression levels and susceptibility to folding irregularities, the low frequency of active sequences confirms that this region of the molecule is functionally important. The sensitivity of this region appears primarily due to selectivity at positions 17, 20, and 21.

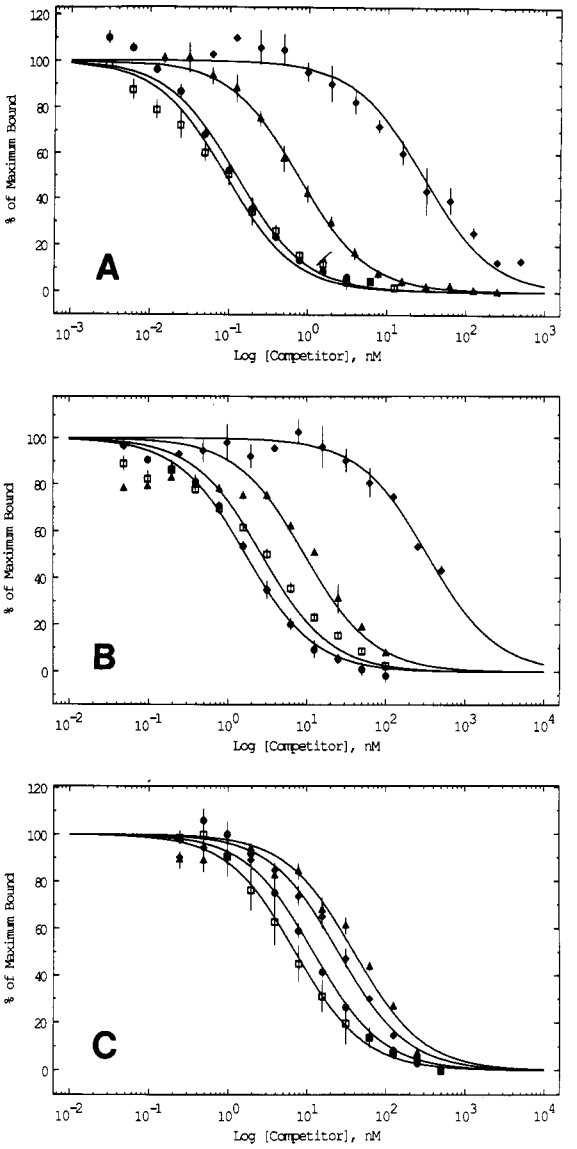

FIGURE 2: Competitive radioreceptor binding assays on (A) high-affinity receptors on activated T-cells, (B) intermediate-affinity receptors on YT-2C2 cells, and (C) low-affinity receptors on MT-1 cells were carried out as described (Landgraf et al., 1992). Each point represents the mean (±SEM) or three to five separate assays carried out in triplicate: (●) **IL-2**; (□) **7**; (▲) **L21V**; (♦) **29**. K_d values determined from nonlinear least-squares curve-fitting (Landgraf et al., 1992) were (A) 11.1 ± 0.4 pM, 8.2 ± 0.2 pM, 67.2 ± 1.4 pM, and 2.6 ± 0.5 nM, respectively; (B) 1.1 ● 0.2 nM, 1.8 ± 0.4 nM, 6.2 ± 0.1 nM, and 220 ± 5.2 nM, respectively and; (C) 11.5 ± 0.2 nM, 6.7 ● 0.1 nM, 36.8 ± 0.8 nM, and 23.9 ± 0.5 nM, respectively.

The stringency of the requirement for wild-type Leu residues at positions 17 and 21 can be understood in the context of the three-dimensional structure; the side chains of these residues participate in core packing. The reduction in bioactivity observed in the **L21V** analog illustrates the importance of core structure for this protein. Of particular significance was the observation that this analog suffered a 4-fold reduction in affinity to the low-affinity receptor, the interaction site of which has been putatively assigned distal to the first helix in the 35–45 region (Suave et al., 1991). However, unlike mutations in this segment that reportedly abrogate low-affinity receptor binding but have little influence on bioactivity (Suave et al., 1991), the **L21V** analog suffers losses in both parameters. Thus, this single alteration in core packing changes the tertiary conformation sufficiently to influence all aspects of IL-2 receptor recognition. The alteration in tertiary structure

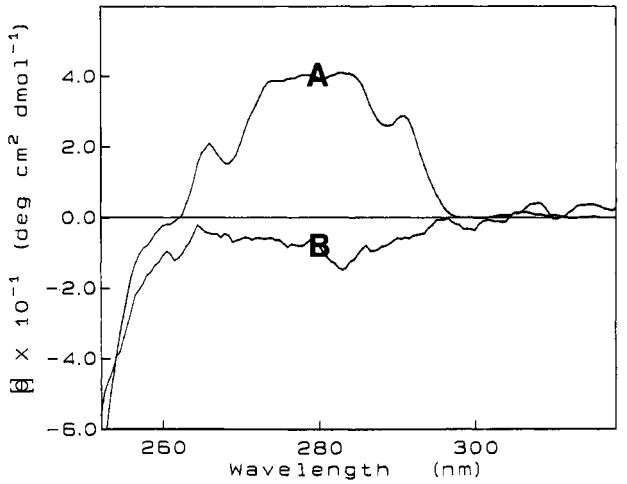

FIGURE 3: Near-ultraviolet CD spectra of (A) wild-type **IL-2** and (B) **L21V** in 25 mM phosphate buffer, pH 6.5 at 25 °C. Spectra represent the average of three to five scans with buffer blank subtracted.

induced by this mutation was easily observable in the near-UV CD (Figure 3), a region of the spectrum diagnostic of the asymmetric environment of the buried Trp121 chromophore. The influence of such a conservative substitution on both structural and biological parameters is significant in light of a recent report suggesting that it is the pattern of hydrophobicity in 4-fold α-helical proteins rather than the exact nature of the side chains that determines whether a protein will fold into a compact and stable structure (Kamtekar et al., 1993). Although folding may be resilient to the nature of the hydrophobic side chain, stability (Sandberg & Terwilliger, 1989), molecular recognition, and subsequent function are much more sensitive to minor perturbations of the protein core. The fact that none of the active sequences were found to possess Val at position 17 may suggest that this position is also sensitive to core packing, but this was not tested directly.

The importance of position 20 for IL-2 function was confirmed in this study. The selection of an acidic residue such as Asp20 within the first core helix is apparently one that has been conserved for nearly all members of the helical cytokine family of proteins (Shanafelt et al., 1989; Lopez et al., 1992b). Of the 42 active IL-2 analogs in this study, 30 possessed an acidic residue at this position, with the wild-type aspartic acid or its conservative analog glutamic acid being recovered with about equal frequency. Only one of the inactive sequences had an acidic resdue at position 20 although several inactive sequences had hydrophilic amino acids at this position (Table 1). Although tolerant to some other hydrophilic amino acids, Asp and Glu were clearly preferred. This indicates that the requirement for an acidic side chain at residue 20 was the primary reason for the low frequency of clones producing active protein in the library.

In addition to finding Asn, Gln, Ser, and Thr at this position in active proteins, the Gly was also recovered. This protein (**15**) was purified and tested in the human bioassay and found to be a full agonist with an EC_{50} = 1.5 nM, about 100-fold less potent that IL-2 itself (Table 3). This result suggests that Asp20 contributes significantly to the receptor binding affinity, but is not critical for activating the receptor and eliciting a full biological response. Examination of an analog in which this position had been deleted (**Del1**) also supports this conclusion. In this protein, more than half of the first core helix has been removed, including the entire segment examined in this study; nevertheless, this analog retains sufficient structural information to be a full agonist, albeit with greatly reduced potency. This suggests that many of the proteins in the library are "inactive" only within the limits of the screen employed and will display activity if high enough concentrations are used. Therefore, although position 20 is important in conferring high affinity, it as well as the surrounding residues is not essential for receptor triggering.

Identification of a single contact residue does not necessarily indicate that any of the neighboring residues serve a similar function. Leu18 and Leu19 were much more tolerant to substitution than Asp20, possibly indicating that they do not contribute directly to receptor contact. One analog, however, containing mutations at these positions (Met18, Ser19; **7**) was found to be more active than the wild-type protein (Figure 1). This observation was due to an increase in receptor affinity, demonstrating that it is possible for one or both of these positions to influence receptor binding in either a direct or an allosteric fashion. Although the increase in bioactivity for this analog was only 2–3-fold, it is the first report of a purified IL-2 analog with greater potency that the wild-type protein, a result apparently achieved for only one other lymphokine (Lopez et al., 1992a). This finding also provides support for more powerful approaches to affinity enhancement such as phage display methods (Lowman et al., 1991) although the complex nature of the IL-2 receptor makes selection of higher affinity binding proteins particularly challenging.

REFERENCES

Bazan, J. F. (1989) *Biochem. Biophys. Res. Commun. 164*, 788–796.

Bazan, J. F. (1990) *Immunol. Today 11*, 350–354.

Bazan, J. F. (1992) *Science 257*, 410–412.

Berndt, W. G., & Ciardelli, T. L. (1992) in *Interleukin-2* (Waxman, J., & Balkwill, F., Eds.) pp 12–28, Blackwell Scientific Publications, Oxford.

Brandhuber, B. J., Boone, T., Kenney, W. C., & McKay, D. B. (1987) *Science 238*, 1707–1709.

Collins, L., Tsien, W. H., Seals, C., Hakimi, J., Weber, D., Bailon, P., Hoskings, J., Greene, W. C., Toome, V., & Ju, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7709–7713.

Cosman, D. (1993) *Cytokine 5*, 95–106.

Cosman, D., Lyman, S. D., Idzerda, R. L., Beckmann, M. P., Park, L. S., Goodwin, R. G., & March, C. J. (1990) *Trends Biochem. Sci. (Pers. Ed.) 15*, 265–270.

Dower, W. J., Miller, J. F., & Ragsdale, C. W. (1988) *Nucleic Acids Res. 16*, 6127–6145.

Dunn, I. S., Cowan, R., & Jennings, P. A. (1988) *Protein Eng. 2*, 283–291.

Gillis, S., Ferm, M. M., Ou, W., & Smith, K. A. (1978) *J. Immunol. 121*, 2077–2032.

Hatakeyama, M., Tsudo, M., Minamoto, S., Kono, T., Doi, T., Miyata, T., Miyasaka, M., & Taniguchi, T. (1989) *Science 244*, 551–556.

Johnson, K., Choi, Y., Wu, Z., Ciardelli, T., Granzow, R., Whalen, C., Sana, T., Pardee, G., Smith, K., & Creasy, A. (1993) *Eur. Cytokine Network* (in press).

Johnson, W. D. (1990) *Proteins: Struct., Funct., Genet. 7*, 205–214.

Kamtekar, S., Schiffer, J. M., Xiong, H., Babik, J. M., & Hecht, H. M. (1993) *Science 262*, 1680–1684.

Kaplan, G., Cohn, Z. A., & Smith, K. A. (1992) *Bio/Technology 10*, 157–162.

Kondo, M., Takeshita, T., Ishii, N., Nakamura, M., Watanabe, S., Arai, K., & Sugamura, K. (1993) *Science 262*, 1874–1877.

Landgraf, B. E., Cohen, F. E., Smith, K. A., Gadski, R., & Ciardelli, T. L. (1989) *J. Biol. Chem. 264*, 816–822.

Landgraf, B. E., Williams, D. P., Murphy, J. R., Smith, K. A., & Ciardelli, T. L. (1991) *Proteins: Struct., Funct., Genet. 9*, 207–216.

Landgraf, B. E., Goldstein, B., Williams, D. P., Murphy, J. R., Sana, T. R., Smith, K. A., & Ciardelli, T. L. (1992) *J. Biol. Chem. 267*, 18511–18519.

Leonard, W. J., Depper, J. M., Crabtree, G. R., et al. (1984) *Nature 312*, 626–631.

Lopez, A. F., Shannon, M. F., Barry, S., Phillips, J. A., Cambareri, B., Dottore, M., Simmons, P., & Vadas, M. A. (1992a) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11842–11846.

Lopez, A. F., Shannon, M. F., Hercus, T., Nicola, N. A., Cambareri, B., Dottore, M., Layton, M. J., Eglinton, L., & Vadas, M. A. (1992b) *EMBO J. 11*, 909–916.

Lotze, M. T., Matory, Y. L., Ettinghausen, S. E., Rayner, A. A., Sharrow, S. O., Seipp, C. A. Y., Custer, M. C., & Rosenberg, S. A. (1985) *J. Immunol. 135*, 2865–2875.

Lotze, M. T., Chang, A. E., Seipp, C. A., Simpson, C., Vetto, J. T., & Rosenberg, S. A. (1986) *JAMA, J. Am. Med. Assoc. 256*, 3117–3124.

Lowman, H. B., Bass, S. H., Simpson, N., & Wells, J. A. (1991) *Biochemistry 30*, 10832–10838.

McKay, D. B. (1992) *Science 257*, 412–413.

Minami, Y., Kono, T., Miyazaki, T., & Taniguchi, T. (1993) *Annu. Rev. Immunol. 11*, 245–267.

Miyoshi, I., Kubonishi, I., Mitsuo, S., Hiraki, S., Tsubota, T., Kimura, I., Miyamoto, K., & Sato, J. (1980) *Gann 71*, 155–156.

Nikaido, T., Shimizu, A., Ishida, N., Sabe, H., Teshigawara, K., Maeda, M., Uchiyama, T., Yodoi, J., & Honjo, T. (1984) *Nature 311*, 631–635.

Noguchi, M., Nakamura, Y., Russell, S., Ziegler, S. F., Tsang, M., Cao, X., & Leonard, W. J. (1993) *Science 262*, 1877–1880.

Reidhaar-Olson, J. F., & Sauer, R. T. (1988) *Science 241*, 53–57.

Russell, S. M., Keegan, A. D., Harada, N., Nakamura, Y., Noguchi, M., Leland, P., Friedmann, M. C., Miyajima, A., Puri, R. K., Paul, W. E., & Leonard, W. J. (1993) *Science 262*, 1880–1883.

Sandberg, W. S., & Terwilliger, T. C. (1989) *Science 245*, 54–58.

Shanafelt, A. B., Miyajima, A., Kitamura, T., & Kastelein, R. A. (1991) *EMBO J. 10*, 4105–4112.

Smith, K. A. (1988) *Science 240*, 1169–1176.

Smith, K. A. (1990) in *Regulatory Cytokines: Action and therapeutic strategies* (Jennings, J., Ed.) pp 9–29, Mediscript, London.

Suave, K., Nachman, M., Spence, C., Bailon, P., Campbell, E., Tsien, W.-H., Kondas, J. A., Hakimi, J., & Ju, G. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4636–4640.

Takeshita, T., Asao, H., Ohtani, K., Ishii, N., Kumaki, S., Tanaka, N., Munakata, H., Nakamura, M., & Sugamura, K. (1992a) *Science 257*, 379–382.

Takeshita, T., Ohtani, K., Asao, H., Kumaki, S., Nakamura, M., & Sugarmura, K. (1992b) *J. Immunol. 148*, 2154–2158.

Taniguchi, T., & Minami, Y. (1993) *Cell 73*, 5–8.

Teshigawara, K., Wang, H.-M., Kato, K., & Smith, K. A. (1987) *J. Exp. Med. 165*, 223–238.

Thiesen, H.-J. (1990) *DNA Protein Eng. Tech. 2*, 93–96.

Williams, D. P., Regier, D., Akiyoshi, D., Genbauffer, F., & Murphy, J. R. (1988) *Nucleic Acids Res. 16*, 10453–10467.

Zurawski, S. M., & Zurawski, G. (1989) *EMBO J. 8*, 2583–2590.

Zurawski, S. M., & Zurawski, G. (1992) *EMBO J. 11*, 3905–3910.