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Mouse interleukin-2 structure-function studies: substitutions in the first α-helix can specifically inactivate p70 receptor binding and mutations in the fifth α-helix can specifically inactivate p55 receptor binding

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The function of two α-helical regions of mouse interleukin-2 were analyzed by saturation substitution analysis. The functional parts of the first α-helix (A) was defined as residues 31-39 by the observation that proline substitutions within this region inactivate the protein. Four residues within α-helix A, Leu31, Asp34, Leu35 and Leu38, were found to be crucial for biological activity. Structural modeling suggested that these four residues are clustered on one face of α-helix A. Residues 31 and 35 had to remain hydrophobic for the molecule to be functional. At residue 38 there was a preference for hydrophobic side chain residues, while at residue 34 some small side chain residues as well as acidic or amide side chain residues were functionally acceptable. Inactivating changes at residue 34 had no effect upon the ability of the protein to interact with the p55 receptor. Disruption of the fifth α-helix (E), which had little effect upon biological activity, resulted in an inability of the protein to interact with the p55 receptor. Mutagenesis of the α-helix E region demonstrated that α-helicity and the nature of the side chain residues in this region were unimportant for biological activity. The region immediately proximal to α-helix E was important only for the single intramolecular disulfide linkage.

Key words: receptor binding / saturation substitution mutagenesis/structure—function

Introduction

Interleukin-2 (IL-2), a protein hormone produced by activated T cells, is a mediator of T and B cell function (O'Garra *et al.*, 1988). Both the human (hIL-2) and mouse (mIL-2) proteins have been the subject of studies aimed at defining structure-function relationships within this hormone (Wang *et al.*, 1984; Zurawski *et al.*, 1986; Ju *et al.*, 1987; Zurawski and Zurawski, 1988; Collins *et al.*, 1988). Parallel studies have determined that the functional receptor for IL-2 contains two non-covalently linked binding polypeptides, the low affinity p55 chain and the intermediate affinity p70 chain (reviewed by Smith, 1988). These chains act together to bind IL-2 with a high affinity (Smith, 1988).

The three-dimensional structure of hIL-2 at 3.0 Å resolution revealed a backbone of six α-helices. This structure was insufficiently detailed to define side chain locations (Brandhuber *et al.*, 1987). A fine structure deletion analysis of mIL-2 identified three regions totalling 25% of the molecule as being most critical to the activity of the protein (Zurawski and Zurawski, 1988). This work addresses the precise molecular requirements of the most N-terminal of these critical regions (residues 31-40) and the nature of the interaction of this region with the p70 receptor subunit. In addition, we examine the role of the fifth α-helix, the proximal part of the most C-terminal critical region, in interaction with the p55 receptor.

Results and discussion

Substitution mutagenesis of residues 31-40

Residues 31-40 of mIL-2 comprise the most N-terminal region critical to activity as defined by our previous fine structure deletion analysis (Zurawski and Zurawski, 1988). Although this deletion analysis identified the importance of residues 31-40, it could not be used to identify the crucial residues within this region. We used a form of combinatorial cassette mutagenesis (Reidhaar-Olsen and Sauer, 1988) to saturate this critical region with substitutions. This method can be used as a probe for the informational content of protein sequences and can identify those residues within a region that have the highest informational content (Reidhaar-Olsen and Sauer, 1988). Mutagenesis was accomplished by recloning this mIL-2 region as a synthetic DNA fragment in which the codons specifying residues 31-40 were synthesized with various levels of incorrect nucleotides. The plasmids encoding the resultant mIL-2 proteins were characterized by DNA sequence analysis and the biological activity of each selected protein was estimated relative to normal mIL-2.

We anticipated that not all residues within the first critical region would be important. Unimportant residue positions would be those capable of accepting major changes in the residue side chain without inactivating biological activity. Conversely, important residue positions would be capable of accepting only limited changes, or no changes, in the amino acid side chains. Several single or multiple substitution proteins with changes at Leu31, Asp34 and Leu33 had $<1\%$ biological activity relative to normal mIL-2 (Figure 1). Also, some substitutions at Leu38 resulted in at least a 10-fold reduction in activity of the protein. These data identified Leu31, Asp34, Leu33 and Leu38 as important residues. In contrast, most substitution proteins that only involved changes at Leu32, Met33, Gln36, Glu37, Leu39 and Ser40 had normal or near normal activity (Figure 1).

The above analysis permitted identification of four important residues within the first critical region. However, the set of mutants was insufficient for characterizing the nature of the side chains that would be acceptable at each of these residues. To supplement this information, we undertook individual cassette mutagenesis studies at each of these important residue positions. This was accomplished by cloning synthetic DNA fragments in which the nucleotide sequence encoding each of these important residues was

	31 40		
	LLMDLQELLS		100
1*	M.....		95
2*	V.....		38
3*	I.....		24
4	.R.....		90
5	..L.....		112
6	..K.....		100
7	..I.....		140
8ϕ	..W.....		41
9	...G.....		53
10*	...N.....		36
11	...E.....	44N→S	76
12*	...T.....		121
13	....V.....		29
14	....M.....		84
15	....I.....		42
16	.....H....		71
17	.....G....		50
18	......G...		160
19*	.....V..		63
20*	.....I..		90
21*	.....T..	42M→I	77
22*	.....T..		39
23*	.....S..		33
24	.....K		67
25*	.....P		108
26	.....	46N→D	127
27	.Q.....T		129
28	.R.....V.		34
29	.F....A...		28
30	.H....M..		29
31	..L.....L		56
32	..K...V..		36
33	..I.....R.		24
34	..K.....L		144
35	..V.....L		84
36	...N...M..		23
37	...AM.....		20
38	....M....T		43
39	.....H..V.		65
40	......V..A		73
41	.....T	41R→Q	125
42	V.T...D...		16
43	..LE.A....		40
44	..T..K...A		65
45	..T.....QV		51
46	.....HQ..W		59
47*	G.....		3
48*	A.....	41R→Q*	2
49*	...V.....		3
50*	....T.....		10
51*	....S.....		4
52*	.....Y..		8
53*	.....H..		4
54*	.....R..		2
55*	.....N..		9

	31 40		
	LLMDLQELLS		100
56*	.....E..		7
57	.....Q..		8
58	..IV.....		5
59	....CR....		2
60	.R.N.....T		9
61	..TEV.....		3
62	.....KM.P		6
63	.V...HD.R.		8
64*	P.....		0.1
65*	W.....		0.2
66*	C.....		0
67*	E.....		0.1
68*	Q.....		0.1
69*	R.....		0
70*	K.....		0.3
71	.P.....		1
72	....Y.....		0
73*	...W.....		0
74*	...F.....		0
75*	...K.....		0
76*	...C.....		0.5
77*	...M.....		1
78*	...L.....		0.6
79	....P.....		0
80*	....G.....		1
81*	....W.....		0.5
82*	....K.....		0.4
83*	....R.....		0
84*	....E.....		0.1
85*	....N.....		0.1
86	....Q.....		0.6
87	R.I.....		0
88	P...Q.....		0
89	M....P....		0
90	Q.....T..		0.5
91	P.....V.		0
92	..A.N.....		0
93	...V...R..		0
94	....Q....R		0
95	....RH....		0
96	....VL....		1
97	.....PL		0
98	M..G....M.		0.2
99	.R.V.P....	46N→C	0
100	.R.G..G...		1
101	.Q.H.....T		0
102	.P.....K.K.		0
103	..DGP.....		0
104	V..QR....L		0
106	..IGRR....		0
107	...V.LK..T		0.4
108	....QHG.I.		0
109	D...IHD..W		0
110	.VIEQ...R.		0
111	..VK.H..ML	51R→S	1

Fig. 1. mIL-2 proteins with substitutions in residues 31–40. The left column shows the protein number and the right column shows the biological activity of each protein expressed as a percentage relative to normal mIL-2. The center column shows the sequence differences found in each protein compared to the normal sequence shown in the first row. Some proteins had substitutions occurring outside of the residue 31–40 region and these are listed separately. * indicates that the protein was from a cassette mutagenesis experiment where residues encoding only one codon were randomized. ϕ indicates that the protein was from a site-directed mutagenesis experiment. Proteins 1–46 are the set with normal or near normal activities, proteins 47–63 are the set with moderate activities, and proteins 64–111 are the set with low or undetectable activities.

randomized. These studies (also included in Figure 1) greatly expanded the data set for these important residues and aided in the analysis described below for both types of mutagenesis.

The combined data set for the substitution analysis of residues 31–40 is presented in Figure 1 and includes 56 single substitution proteins, 31 double substitution proteins and 23 proteins with more than two substitutions in this region. To analyze the data for proteins with multiple substitutions we assumed that each substitution acted independently and that changes of noncritical residues 41–51 did not affect the function of the protein. We divided the proteins into three sets. The first set included those proteins with normal or near normal activities (i.e. >10% compared to the parental mIL-2). This set defined those substitutions for each of the residues 31–40 that did not disrupt the function of mIL-2. Table I shows that acceptable substitutions were isolated for each of residues 31–40. Although no one particular side chain residue in this region is absolutely required for function of mIL-2, the pattern of acceptable, tolerable, and detrimental substitutions revealed which residues in this region were the most important and defined the nature of the side chains required at these locations. Residues Leu32, Met33, Gln36, Glu37, Leu39 and Ser40 were recognized as relatively unimportant on the basis that each of these residues accepted substitutions that resulted in a radical change of the side chain. For example, the hydrophobic non-polar side chain residues of Leu32, Met33 and Leu39 accepted replacement with the basic side chain residues of Arg or Lys, while the uncharged polar side chain residues of Gln36 and Ser40 or the acidic side chain residue of Glu37, accepted replacement with basic side chain residues or non-polar side chain residues. Of the 46 acceptable substitutions, 23 were inferred from single substitution data (Table I).

The second set of proteins had moderate activities (i.e. between 1 and 10% compared to the parental mIL-2). This set included those substitutions for each of residues 31–40 that were functionally tolerable but not optimal. Of the 15 tolerable substitutions, 10 were inferred from single substitution data (Table I).

The third set of proteins had low or undetectable activities (i.e. ≤1% compared to the parental mIL-2) and defined those substitutions for each of residues 31–40 that were detrimental to the function of mIL-2. Of the 29 detrimental substitutions, 23 were inferred from single substitution data (Table I).

The above analysis of the data revealed a high degree of internal consistency. For example, every multiple substitution protein that contained a change defined as detrimental had very low or undetectable activity. Acceptable changes occurred in various combinations in active proteins. Only one multiple substitution protein (protein 100, Figure 1) with ≤1% activity had no changes that were classified as detrimental. A possible explanation of the phenotype of protein 100 is presented below. Of the moderately active proteins with multiple changes (set 2, Figure 1), only proteins 60 and 61 should have been slightly more active if each substitution acted independently.

The remarkable internal consistency of these results supports the validity of the premise that each change in a protein with multiple substitutions in this region is acting independently. An analogous study of eight residues of the helix 5 region of λ repressor concluded that most functionally acceptable substitutions in multiple mutant backgrounds would behave similarly to single substitutions (Reidhaar-Olsen and Sauer, 1988). The data presented here extend this observation from functionally acceptable substitutions to inactivating substitutions.

Characterization of the spectrum of acceptable side chain residues at four functionally important residues in the α-helix A region

Two of the four functionally important residues, Leu31 and Leu35, accepted substitutions of only the hydrophobic non-polar side chain residues of Val, Ile and Met. Small non-polar side chain residues or the small uncharged polar side chain residues were tolerated substitutions for Leu31 and Leu35, while all other substitutions for these residues were detrimental to the protein. Leu38 had a substitution spectrum similar to that of Leu31 and Leu35, except that the acceptance of small uncharged polar chain residues and tolerance for all other residue side chains suggested that this residue is of lesser importance. The last crucial residue,

Table I. Summary of acceptable, tolerable and detrimental substitutions at residues 31–40 of mIL-2

b[a]			LEU				LEU			GLU
h[b]			LEU				MET	ILE		ASN
m[c]	LEU	LEU	MET	ASP	LEU	GLN		LEU	LEU	SER
	31	32	33	34	35	36	37	38	39	40
1[d]	VAL[g]	phe[h]	val	glu	VAL	glu	asp	VAL	val	thr
1	ILE	his	LEU	ASN	ILE	GLY	gln	ILE	gln	ala
1	MET	gln	ILE	GLY	MET	ala	GLY	met	arg	val
1		ARG	thr	ala		HIS	ala	SER		leu
1			TRP	THR		lys	val	THR		trp
1			LYS							LYS
1										PRO
2[e]	GLY	val		VAL	SER	arg	lys	ASN		
2	ala				THR			GLU		
2					cys			GLN		
2								TYR		
2								HIS		
2								ARG		
3[f]	PRO	PRO		PHE	PRO	pro			pro	
3	CYS			TYR	GLY	leu			met	
3	asp			TRP	ASN					
3	TRP			CYS	GLU					
3	GLU			LEU	GLN					
3	GLN			MET	TRP					
3	LYS			his	LYS					
3	ARG			LYS	ARG					

[a]Sequence differences for the analogous region of bovine IL-2.
[b]Sequence difference for the analogous region of human IL-2.
[c]The sequence of residues 31–40 of mIL-2.
[d]Acceptable substitutions (see Figure 1).
[e]Tolerable substitutions (see Figure 1).
[f]Detrimental substitutions (see Figure 1).
[g]Residues in capitals were inferred from single substitution proteins.
[h]Residues in lower case were inferred from multiple substitution data.

Asp34, ranked equally in importance with Leu31 and Leu35 on the basis of the preponderance of detrimental substitutions that were assigned to this residue. Acceptable substitutions at Asp34 were confined to amino acids with acidic side chains, small uncharged polar side chains (except Cys) or very small non-polar side chains. All other side chain residues, except that of barely tolerable Val (Figure 1), were detrimental. The substitution spectrum for Asp34 revealed that the primary requirement at this residue position was modest side chain bulk.

The functionally important side chains in residues 31–39 must be presented in an α-helical context

A common feature in the substitution spectrum for residues 31–40 was that proline substitutions for the important residues Leu31 and Leu35, and the unimportant residues Leu32, Gln36 and Leu39, were all detrimental to the activity of the protein. A structural consequence of proline substitution is that it imposes a constraint on the peptide bond that is unfavorable for α-helicity. The three-dimensional structure of hIL-2 at a resolution 3.0 Å showed that hIL-2 residues 11–19 form an α-helical segment, and this segment is followed by an extended loop of 12 residues (Brandhuber *et al.*, 1987). Assuming structural homology (64% sequence homology) between the functionally interchangeable mIL-2 and hIL-2 molecules, the analogous α-helical region in mIL-2 would extend from residues 25–33. The proline substitution data for this region indicated that it is likely that the functional part of this α-helical region in mIL-2 extends up to and including residue 39, but not to residue 40 where proline substitution was acceptable. Our previous fine structure deletion data indicated that the functional N-terminal boundary of this α-helix was Gln30 (Zurawski and Zurawski, 1988). Glycine residues, although sometimes found in α-helices, are not preferred for α-helical regions (Chou and Fasman, 1974). Glycine substitutions were acceptable or tolerable for four of the residues in this α-helical region and only for protein 100, which had Gly changes at residues 34 and 37 (Figure 1), did such substitution result in a protein of low activity.

Given the likelihood that the functionally important information in residues 31–39 is presented in an α-helical context, we examined the spatial relationships of the four important residues of this region. An α-helical model of residues 30–39 (Figure 2) shows that the three important hydrophobic residues, Leu31, Leu35 and Leu38, would be clustered on one face of the α-helix and are adjacent to the important Asp34 residue. The spatial proximity of the three important Leu residues may permit this surface to participate in a single area of hydrophobic interaction. It is possible that disruptive changes at Asp34, which is important only in the sense that the side chain at residue 34 must be small, act through steric hindrance of this hydrophobic interaction. There are three logical targets for this postulated hydrophobic

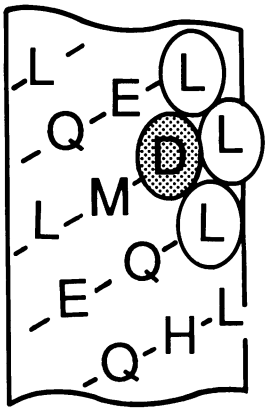

Fig. 2. Cylindrical plot of α-helix A. mIL-2 residues 26–39 are represented commencing at the bottom of the cylinder. The four crucial residues within this region are circled and the crucial Asp34 residue is shaded.

interaction—the p55 receptor, the p70 receptor or an intramolecular interaction with another part of IL-2 itself.

Effects of substitutions in residues 31–40 on binding to the p55 receptor

Several proteins with substitutions of residues 31–40 were examined for their ability to bind to the p55 receptor. For the purpose of these studies, the use of a L cell line stably transfected with a mouse p55 cDNA, and that expresses high numbers of p55 receptors, permitted the unambiguous examination of p55 subunit binding in the absence of p70 subunit or p70 + p55 receptor binding. We used a competitive binding assay with [^{125}I]hIL-2 as the labeled ligand and partially purified mutant mIL-2 proteins as the competing ligands to measure the ability of these mutant mIL-2 proteins to interact with the p55 receptor (Figure 3).

Our p55 receptor-binding studies showed that proteins with substitutions of residues 31–40 fell into three classes (Table II and Figure 3). The first class had moderate or near normal biological activities and had near normal abilities to bind to the p55 receptor. The changes in these proteins defined residues that are not critical for either p70 or p55 receptor interaction. The second class of proteins represented a subset of the substitutions at Asp34 and had very low or undetectable biological activity. This class bound normally to the p55 receptor. Since p70 receptor binding is required for biological activity (Smith, 1988), the normal p55 receptor binding of the biologically inactive subset of Asp34 substitution proteins indicated that they have a deficiency in p70 receptor binding or activation. The third class of proteins had very low or undetectable biological activity and had very low or undetectable binding to the p55 receptor. The mutations in this class of mIL-2 protein thus presumably disrupt interactions with both the p70 and p55 receptors.

In hIL-2, substitution of Asp20 (analogous to mIL-2 Asp34) with Lys also inactivated biological activity, inactivated p70 receptor binding, and had no effect upon p55 receptor binding (Collins *et al.*, 1988). As determined by circular dichroism, the hIL-2 20D→K protein appeared to be unaltered in α-helical content and the authors indicated this to be strong evidence that this residue interacts directly with the p70 receptor (Collins *et al.*, 1988). On the basis of a limited substitution analysis, these authors concluded that this Asp residue takes part in a direct ionic interaction

Table II. p55 receptor binding of mIL-2 mutant proteins

Protein	Mutation	Biological activity (%)		p55 binding (%)
9[a]	34D→G	20[b]	(50)[c]	100[d]
10	34D→N	10	(36)	100
12	34D→T	20	(120)	100
13	35L→V	3	(34)	20
86	35L→Q	2	(0.2)	5
65	31L→W	0.3	(0.2)	1
64	31L→P	0.3	(0.1)	0
71	32L→P	0.1	(1)	0
79	35L→P	0	(0)	≤0.05
83	25L→R	0	(0)	0
91	31L→P,39L→V	0	(0)	0
97	39L→P,40S→L	0	(0)	0
72	34D→Y	0	(0)	33
73	34D→W	0	(0)	100
74	34D→F	0	(0)	100
75	34D→K	0	(0.2)	100
114[e]	Δ123–124	16	(2)	0
115[e]	i124GP	49	(17)	≤0.05
60[e]	Δ73–77	0	(0)	0
63[e]	Δ77–79	0.1	(0.2)	0
131[e]	Δ137–138	0	(0)	0

[a]Protein numbers refer to Figure 1.
[b]Specific activity relative to mIL-2 of partially purified protein as determined by the HT2 proliferation assay.
[c]Specific activity relative to mIL-2 of SDS extracted crude lysates from Figure 1.
[d]Relative to mIL-2 prepared in an identical manner.
[e]Mutant protein numbers are taken from Zurawski and Zurawski (1988).

with a specific residue on the p70 receptor. However, our observation that the mIL-2 34D→G protein was active despite the absence of a side chain at this position shows that this side chain can play no such active role in interaction with the p70 receptor. Rather, our finding that the important criterion for this residue is that the side chain is small suggests that this residue has an indirect role. It is possible that this indirect role could be a consequence of the close spatial proximity of this Asp residue to the three important hydrophobic α-helix A residues that we have identified.

In hIL-2, Leu17 is analogous to one of these important hydrophobic residues (mIL-2 Leu31) and the hIL-2 17L→N protein was low in biological activity and was also low in p55 and p70 receptor binding (Collins *et al.*, 1988). Physical studies demonstrated that this protein was greatly reduced in α-helical content (Collins *et al.*, 1988). These data suggested that at least this residue of the hydrophobic patch on the first α-helix normally engages in an intramolecular interaction which helps to structurally stabilize the protein. Disruption of this intramolecular interaction must therefore disturb certain structural features that are required to present crucial residues to both receptor subunits. The inability of several proteins with changes in the first α-helix to interact with both receptor subunits may reflect a more global involvement of this region in a crucial intramolecular interaction. Further physical studies are required to address this issue.

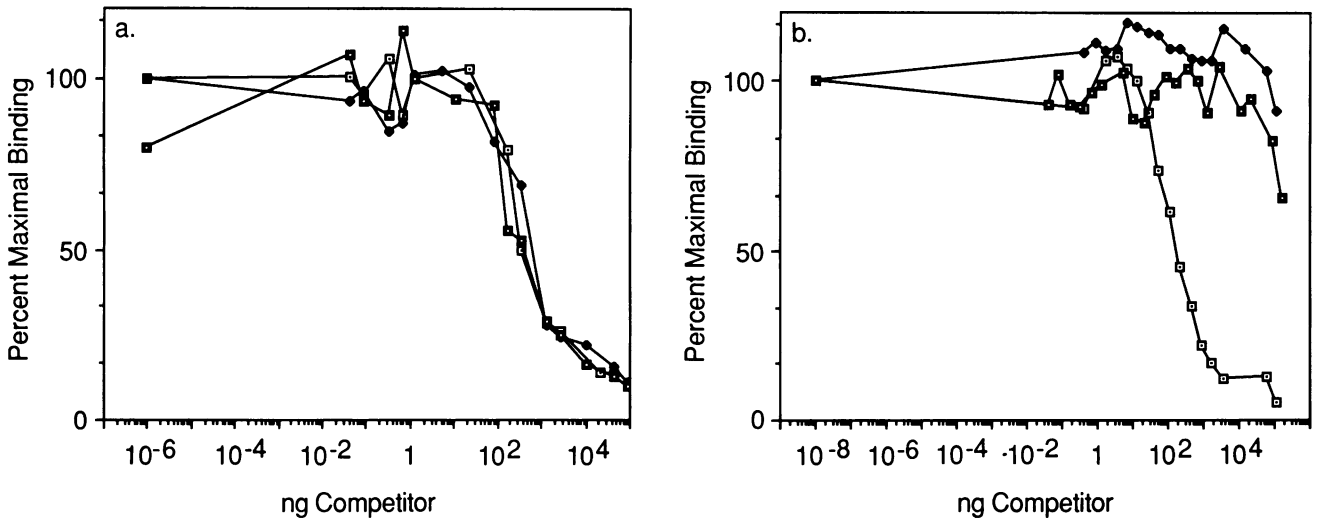

Fig. 3. Competitive inhibition of binding of [^{125}I]hIL-2 to transfected L cells bearing p55 receptors by mIL-2 and mIL-2 mutant proteins. The data are presented as a percentage of binding observed in the presence of defined amounts of competitor protein compared to binding observed in the absence of competitor protein. (**a**) Open squares are mIL-2, closed squares are protein 74 (Figure 1), closed diamonds are protein 9 (Figure 1). (**b**) Open squares are mIL-2, closed squares are protein 114 (Zurawski *et al.*, 1986), closed diamonds are protein 115 (Zurawski *et al.*, 1986).

The data presented above provide strong support for identity of functionality between hIL-2 and mIL-2 in the N-terminal region. Our assertion that mIL-2 has an N-terminal α-helix analogous to the hIL-2 α-helix A is supported by the proline substitution data for this region. Also, it is striking that the functional properties of substitution mutations at the hIL-2 Leu18 and Asp20 residues are identical to similar mutations at the analogous Leu31 and Asp34 residues of the proposed α-helix A of mIL-2. In fact, in this and previous works (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988), we have not identified any mutational changes that would suggest that there are fundamental differences in the structure–function relationships of these two homologous proteins.

A full understanding of the nature of the α-helix A intramolecular interaction would be facilitated by refinement of the three-dimensional structure of hIL-2 to a degree that would permit identification of IL-2 residues that interact with this region. At the present degree of resolution, the most likely location for such an intramolecular interaction is the middle section of the second α-helix (Brandhuber *et al.*, 1987). Phe58, Tyr59 and Leu60 are three mIL-2 residues in this region that offer potential for hydrophobic interaction. Small deletions involving these residues resulted in 100- to 500-fold reductions in biological activity (Zurawski and Zurawski, 1988).

In the three-dimensional structure of hIL-2, the middle section of the second helix also appears to be in close proximity to hydrophobic side chains located on the C-terminal α-helix F (Brandhuber *et al.*, 1987). Deletion of one of these residues in hIL-2 (Phe124) resulted in a protein which was biologically inactive yet had near normal binding to the p55 receptor (Collins *et al.*, 1988). We anticipate that mutagenic studies such as described here for α-helix A will be required for the remaining critical regions that we identified by fine structure deletion analysis. In combination with appropriate receptor binding and physical studies these data should permit elucidation of both the intramolecular and intermolecular interactions that govern the ability of IL-2 to function.

Deletion/addition changes in the fifth mIL-2 α-helical region specifically inactivate p55 receptor binding

Several proteins with addition or deletion mutations in regions of mIL-2 other than the first α-helix were examined for their abilities to bind the p55 receptor. These studies revealed a class of protein that had near-to-normal biological activity but very low or undetectable binding to the p55 receptor (Table II and Figure 3). Both representatives of this class were mIL-2 proteins with alterations in the region analogous to the fifth α-helix of hIL-2 (called α-helix E, Brandhuber *et al.*, 1987; Zurawski and Zurawski, 1988). The phenotypes of these proteins demonstrated that this region is unimportant for p70 receptor binding.

The mIL-2 α-helix E region does not need to be α-helical and contains no residues that are important for biological activity

The short α-helix E region immediately follows the second of the disulfide-linked Cys residues in hIL-2 and corresponds to mIL-2 residues 122–128. Most small deletions in this region of mIL-2 inactivated the protein; however, deletion of a single residue (Asp124) or insertion of two residues (Gly Pro at Asp124) in this region resulted in biologically active proteins (Zurawski and Zurawski, 1988). To investigate the role of this region in the structure–function relationships of mIL-2, we utilized a modified form of combinatorial cassette mutagenesis (Reidhaar-Olsen and Sauer, 1988) to ask what types of residues in the α-helix F region were acceptable for a biologically functional protein. In this technique, a number of adjacent residues are randomized by cassette mutagenesis and functional proteins are identified and characterized from a large pool of plasmids encoding such proteins.

We used synthetic DNA to mutagenize residues 121–123 and 125–127 such that we obtained a large pool of plasmids that encoded random sequences at these residue positions. Several hundred such plasmids were then examined for their ability to direct the synthesis of mIL-2 proteins of normal or near normal activity. Five per cent of the candidates met this criteria and 16 of these were further characterized by

Table III. Biologically active mIL-2 proteins containing randomized sequences in the α-helix E region

	121[a]	122	123	124	125	126	127	
	GLN[b]	PHE	ASP	ASP	GLU	SER	ALA	100
112[d]	THR[e]	LEU	SER	[f]	ALA	GLY	THR	144
113	LEU	LEU	[g]			LYS	THR	98
114	LEU	VAL	SER		THR	HIS		38
115	ARG	LEU	ILE		LYS	GLY	VAL	36
116	VAL	MET	GLY		ASP	VAL	GLU	138
117	ASP	SER	SER		THR	PRO	THR	38
118	ILE	MET	SER			ARG	GLN	98
119	LEU	ASP	PHE		SER	HIS	GLY	45
120	SER		GLU		LEU	ASN	VAL	75
121	LEU	MET	ASN		GLY	PRO	ILE	34
122	ALA	LEU	CYS		SER	THR	TYR	35
123	THR	PRO	THR		ILE	GLY	LEU	10
124	HIS	PRO	CYS		ASP	PRO	LEU	30
125	VAL	SER	SER			TYR	THR	60
126	ARG	LEU	ILE		PRO	ALA	VAL	62
127	SER	VAL			THR	HIS		36

[a]Row indicates the residue number.
[b]Row indicates the normal mIL-2 sequence.
[c]Column indicates the biological activity of the protein (in %).
[d]Column indicates the protein number.
[e]Row indicates the sequence changes present in the protein.
[f]Residue 124 was not randomized since deletion of this residue resulted in a protein of almost normal activity (Zurawski and Zurawski, 1988).
[g]Blank spaces signify no change to the protein sequence at that position.

DNA sequence analysis (Table III). A wide spectrum of substitutions was observed for all six randomized residues and from these data we concluded that the nature of the side chains of each of these residues is unimportant for biological activity. Five of these active mIL-2 proteins had proline substitutions within the region that should encode α-helix E (residues 122–128). These data, together with the previously characterized active mIL-2 protein with Gly Pro inserted in this region, suggested that this region does not have to be α-helical for biological activity.

Combinatorial cassette mutagenesis of the α-helix F region suggested that these residues are low in informational content. However, 95% of proteins with random sequences in this region had significantly reduced biological activity or were inactive. Some of these inactive proteins (15%) can be accounted for by the random inclusion of translation stop codons in this region. The reduced activity of the remaining proteins must reflect subtle and as yet unrecognized, structural requirements. These requirements could reflect factors such as acceptable charge distributions and appropriate distribution of hydrophobic and hydrophilic residues. It is likely that an exhaustive analysis of many more active proteins with sequences randomized in this region may reveal these subtle features. However, our goal was to use the technique to appraise rapidly the informational content of the randomized region and to identify residues with high informational content. Our results further support the validity of combinatorial cassette mutagenesis as a method to achieve this goal and extend the technique to the randomization of several residues simultaneously.

A single Cys residue is required at positions 118–120 for biological activity

Previous studies had identified the single disulfide linkage in IL-2 as an important structural feature (Wang *et al.*, 1984). Conservative substitutions at the second Cys residue of this disulfide linkage of hIL-2 resulted in at least a 10-fold reduction in activity (Ju *et al.*, 1987) and deletion of the analogous Cys120 of mIL-2 resulted in an inactive protein (Zurawski and Zurawski, 1988). Deletion analysis of mIL-2 revealed that residues 114–118 were expendable for activity, but was insufficiently detailed to address the role of the residues immediately flanking Cys120 (Zurawski and Zurawski, 1988).

We undertook a combinatorial cassette mutagenesis study in which residues 114–120 were randomized. Of the 960 proteins that we examined, 2.5% had normal or near normal activities. Sequence determinations of nine such proteins (Table IV) demonstrated that each of residues 114–120 accepted many substitutions. Also, all active proteins had a single Cys residue present at positions 118–120. These data suggested that the only residue in this region with a high informational content is Cys120, and that this Cys residue is also functionally accepted at positions 118 and 119. Since seven residues were randomized at once, we expected that 66% of the proteins would not have translational stop codons in the randomized region. If the only function criterion for residues 114–120 is the presence of a single Cys residue at positions 118–120, then ~6.5% of the proteins should be functional.

In the two combinatorial cassette mutagenesis studies described here, we observed a difference between expected and observed frequencies of functional proteins. For the analysis of residues 114–120, this disparity was much less than that observed for analysis of residues 121–123, 125–127. This agreement between expected and observed frequencies in the residue 114–120 analysis supports our conclusion that the only informational content in these residues is the presence of a Cys residue at positions 118–120. This data reveals the necessity for evaluating

Table IV. Biologically active mIL-2 proteins containing randomized sequences in the region proximal to α-helix E

	114[a]	115	116	117	118	119	120	
	SER[b]	ASP	ASN	THR	PHE	GLU	CYS	100[c]
128[d]	LEU[e]	LEU	GLU	GLU	CYS*			
						SER	ALA	33
129	ALA	ASN	GLU	ALA	CYS*	ARG	GLN	56
130	HIS	ALA	ALA	MET	CYS*	ASN	GLN	42
131	GLY	LEU	LYS	GLU	ASN	CYS*	ILE	15
132	THR	[f]	TRP	ARG	ASP	CYS*	LEU	23
133	ARG		VAL	PHE	LEU	CYS*	THR	17
134	TYR	LEU	THR		TRP		CYS*	24
135	PRO	THR	LEU	ASN	LYS	VAL	CYS*	23
136	GLY	SER	PRO	SER	HIS	ARG	CYS*	75

[a]Row indicates the residue number.
[b]Row indicates the normal mIL-2 sequence.
[c]Column indicates the biological activity of the protein (in %).
[d]Column indicates the protein number.
[e]Row indicates the sequence changes present in the protein.
[f]Blank spaces signify no change to the protein sequence at that position.
*Highlights the Cys residues.

observed versus expected frequencies of functional proteins in such experiments. The joint consideration of the observed sequences and the observed frequencies of functional proteins makes this type of genetic analysis a powerful tool for structure–function studies.

The two randomized substitution studies described here have clarified the role of residues 114–128 which include part of the critical region III that we identified previously by fine structure deletion analysis (Zurawski and Zurawski, 1988). The data suggested that the single most important requirement within this region for biological activity is the presence of a single Cys residue at positions 118–120. The chemical nature of the other residues in this region is relatively unimportant. However, deletion analysis demonstrated that these residues may provide the correct spatial placement of α-helix F relative to the rest of the protein. In addition, the p55 receptor binding data have identified α-helix E as being essential for p55 receptor interaction. We anticipate that analysis of the p55 receptor binding abilities of the randomized mIL-2 proteins described here should permit us to address both the side chain and α-helical requirements of this region for binding.

Conclusions

The work described here advances several aspects of our understanding of structure–function relationships for IL-2. In the p55/p70 receptor model, separate regions of IL-2 are recognized by the two receptor chains in the context of a functional high affinity complex (Smith, 1988). This model predicts that it should be possible to isolate mutant forms of IL-2 that recognize only the p55 or p70 receptor. The data presented here confirm this prediction and also provide confirmation for two suppositions inherent in this model. Firstly, binding of IL-2 to the p55 receptor alone will not trigger a biological response. Secondly, only binding of IL-2 to the p70 receptor is necessary for triggering a biological response. In view of the above results, it is of interest that high levels (>1000-fold excess) of mutant mIL-2 proteins that are biologically inactive but fully active for p55 receptor binding do not antagonize the activity of normal IL-2 in the standard T cell proliferation assay (S.Zurawski, unpublished). This surprisingly irrelevant role for the p55 receptor in such an assay is supported by our finding that it is possible to isolate mIL-2 mutant proteins that fail to recognize the p55 receptor, yet have near-to-normal biological activities. Since p55 contributes an ~100-fold increase in affinity for IL-2 to the functional high affinity receptor (Smith, 1988), an expectation might have been that $p55^{-}p70^{+}$ mutant IL-2 proteins would be substantially reduced in activity. The high activities of $p55^{-}p70^{+}$ mIL-2 proteins, which refute this expectation, may simply be a reflection that the biological and receptor binding assays measure different parameters. In the biological assay, the ability of the mutant protein to drive proliferation of target cells is measured over a 24 h period. In receptor binding assays, the interaction is measured over minutes and the phenomena of internalization, activation and signal transduction are not addressed. It is possible that the ability of IL-2 to interact with the p55 receptor is important in localized *in vivo* situations where, for example, it may help to entrap large amounts of IL-2 for activation of the p70 receptor.

The IL-2 molecule can be viewed as a structural platform that presents particular side chains to the high affinity receptor complex. It is the goal of structure–function studies to identify the specific nature of this structural platform and the functional residue side chains. Two classes of functional residue side chains may be expected. The first class would be involved in facilitating binding of IL-2 to the appropriate receptor subunits. The second class would be involved in activation of the biological response that results from binding. Mutations in this latter class of residue would be recognized by their ability to act as antagonists in biological assays.

The work described here represents the completion of a search for both classes of such functionally important residues in the N-terminal half of mIL-2. A combination of fine structure deletion analysis and saturating substitution analysis has now been applied to the first 56 residues (or 58%) of the 149 residue mIL-2 protein. In this region there is only a single important structural feature, i.e. α-helix A extending functionally from residue 31–39. Three Leu residues in α-helix A present a critical hydrophobic face, at least a part of which contributes to an intramolecular interaction which helps to structurally stabilize the protein.

A fourth residue in α-helix A (Asp34) defines the vicinity, but not the exact location, of one site of IL-2–p70 receptor interaction.

The work described here also defines the region of mIL-2 that includes and immediately surrounds α-helix E as important for biological activity only in the sense that it provides an anchor point for the single intramolecular disulfide linkage. The α-helix E region itself is, however, vital for IL-2–p55 receptor interaction. Although this work has permitted a mutational separation of IL-2 binding to the p55 and p70 receptors, mutational uncoupling of p70 receptor binding and activation has not been observed. We anticipate applying the methods described here to the remaining critical residues identified by fine structure deletion analysis (Zurawski and Zurawski, 1988). Such experiments should lead to a complete identification of residues involved in receptor binding and may possibly lead to the discovery of mutant IL-2 proteins that are antagonists.

Materials and methods

Recombinant DNA protocols

Materials and protocols for recombinant DNA work, including synthesis, cloning of synthetic DNA and sequencing have been described previously (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988). The mIL-2 expression plasmid exTRP IL-2 (Zurawski *et al.*, 1986) containing a synthetically derived coding region (Zurawski and Zurawski, 1988) was used for the construction of a null vector that had a deletion of the internal TGCA nucleotides of the *Pst*I site encoding residues 35–36. Mutagenesis of residues 31–40 involved the cloning of synthetic DNA fragments encoding the region between the *Xba*I site at residue 28 and the *Sph*I site at residue 53. Random substitution mutagenesis was accomplished by incorporating, during DNA synthesis, 1 and 5% incorrect nucleotides at the nucleotides encoding residues 31–40. Cloned synthetic DNAs from both types of syntheses were pooled prior to further analyses. Randomization of individual codons (residues 31,34,35,38) was accomplished by incorporating during DNA synthesis equimolar amounts of G,A,T,C at the first and second codon position and equimolar amounts of G,A,T,C or G,C at the third codon position. For the randomization of individual residues, the codon for Ser40 in the synthetic DNA was changed to TCT thereby altering the *Nru*I recognition site at residues 40–41. This enabled the null vector background to be reduced in cloning experiments by treating the ligations with *Nru*I prior to transformation. Transformants which expressed mIL-2 protein were identified by colony immunoblots (Zurawski and Zurawski, 1988).

Randomization of residues 114–120 involved the cloning of synthetic DNA fragments encoding the region between the *Mlu*I site at residue 105 and the *Sal*I site at residue 130 into a null vector that had the region between these sites substituted with the sequence AAATAGGCCTTTA. The *Stu*I site within this sequence permitted the null vector background to be reduced by treating the ligations with *Stu*I prior to transformation. Randomization of residues 121–123,125–127 involved the cloning of synthetic DNA fragments encoding the region between the *Bam*HI site at residue 113 and the *Sal*I site at residue 130 into the synthetic mIL-2 expression vector. Removal of vector background was accomplished by changing the codon for Ser114 to TCT which altered the *Bam*HI site and permitted treatment of the ligations with *Bam*HI. For all randomization clonings the transformants were pooled and plasmid prepared and retransformed into the *lon*$^-$ *Escherichia coli* strain AB1899 (Howard-Flanders *et al.*, 1964) which was used as the host for expression of all mIL-2 proteins. Nine hundred and sixty transformants were picked into individual wells of 96-well microtiter dishes and assayed using the rapid bioassay screen described previously (Zurawski and Zurawski, 1988) at a 1:4000 or 1:20 000 dilution.

The plasmids encoding all selected proteins were sequenced through the entire region that was replaced by synthetic DNA and the activities of the encoded proteins relative to the parental mIL-2 were determined as described previously (Zurawski and Zurawski, 1988).

Purification of mutant mIL-2 proteins

Cells harboring mutant mIL-2 expression plasmids were grown overnight at 37°C in 1 l of L broth with 270 μl ampicillin. The cells were harvested by centrifugation, washed in buffer (50 mM Tris–HCl, pH 8.0), resuspended in 5 ml buffer and frozen in liquid nitrogen. After thawing, the suspension was made 200 μg/ml lysozyme, 1 mM $MgCl_2$, 5 μg/ml DNAse, 100 μM phenylmethylsulfonyl fluoride and then refrozen and thawed. Insoluble material was recovered by centrifugation (14 000 r.p.m., 30 min, Sorvall SS34 rotor) and resuspended in 5 ml phosphate-buffered saline (PBS, Sigma) by sonication. Triton X-100 was added to 0.5% and the suspension was incubated at 25°C for 30 min. The insoluble material was recovered by centrifugation and resuspended in 10 ml 5 M guanidine–HCl in PBS by sonication. After incubation at 25°C for 30 min the soluble material was recovered by centrifugation and dialyzed against PBS. The recovered soluble mIL-2 protein was 50–90% pure and the yield varied from 1 to 10 mg. The concentration of the mIL-2 proteins was estimated by densitometer tracings of Coomassie Brilliant Blue R-250 stained protein gels using lysozyme as a standard.

p55 receptor binding assay

^{125}I-Labeled hIL-2 (Met0, Ala125, 780 Ci/mmol, Amersham) at 1 nM was the labeled ligand and mIL-2 proteins prepared as described above were the competing ligands and were used over a range of 24 pM to 100 μM. Assays were in 200 μl HBSS (Gibco) and 0.1% BSA (UltraPure, Boehringer Mannheim) and 10^6 A22 cells. Incubation was at 4°C for 90 min in Macrowell tube strips in a 96-chamber strip holder (Skatron) attached to a rotator (model 151, Scientific Industries) or a Micro-shakerII (Dynatech) for sufficient agitation to maintain the cells in suspension. To separate bound ligand from free ligand the reaction was overlaid onto 150 μl of 84% silicone, 16% paraffin oil in 400 μl conical bottom tubes and centrifuged for 2 min at 4°C (Eppendorf 5413). The pellets were cut off with a razor blade and analyzed in a Cobra 5010 gamma counter (Packard). The receptor-bearing A22 cells were a stable cell line isolated from L cells co-transfected with pCDSRα-mouse IL-2 p55 receptor cDNA (Yokota *et al.*, 1987; Miller *et al.*, 1985) and pSV2Neo (Southern and Berg, 1982) by the calcium phosphate method (Graham and van der Eb, 1973; Tsui *et al.*, 1982) with selection for neomycin G418. By indirect fluorescence staining with the 7D4 monoclonal antibody and FACS analysis, A22 cells displayed staining that was 44% relative to that of HT2 cells which bear ~150 000 p55 receptors per cell (Robb *et al.*, 1984).

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