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Partial agonist/antagonist mouse interleukin-2 proteins indicate that a third component of the receptor complex functions in signal transduction

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Some mouse interleukin-2 (mIL-2) proteins with substitutions at residue Gln141 are unable to trigger a maximal biological response. The Asp141 protein induces the lowest maximal response. The Asp141 protein can weakly antagonize the biological activity of mIL-2 and strongly antagonizes the biological activity of active mIL-2 mutant proteins that have defects in interactions with the high affinity receptor. Residue 141 mutant proteins bind with reduced affinity to T cells expressing the high affinity IL-2 receptor, yet bind normally to transfected fibroblasts expressing only the α and β chains of the receptor. These results suggest that a third receptor component is important for both binding and signal transduction.

Key words: binding/mutagenesis/structure-function/T-cell

Introduction

IL-2 is a protein hormone that is required for the proliferation and differentiation of various cells of the immune system (Smith, 1989). The proliferation of activated T lymphocytes is elicited by the binding of IL-2 to specific high-affinity ($K_d \sim 10^{-11}$ M) cell surface receptors (IL-2R; Smith, 1989). The receptor is complex and consists of at least two polypeptides called IL-2Rα/p55 and IL-2Rβ/p75 (Smith, 1989). Alone, IL-2Rα binds IL-2 with low affinity ($K_d \sim 10^{-8}$ M; Leonard *et al.*, 1984; Nikaido *et al.*, 1984; Miller *et al.*, 1985). IL-2Rα is not necessary for signal transduction since there are IL-2 responsive cells that have no IL-2Rα (Bich-Thuy *et al.*, 1987) and some mIL-2 mutant proteins that bind normally to IL-2Rα are biologically inactive (Zurawski and Zurawski, 1989). When expressed on the surface of lymphoid cells that are deficient in IL-2Rα, IL-2Rβ facilitates intermediate affinity ($K_d \sim 10^{-9}$ M) binding of IL-2 (Hatakeyama *et al.*, 1989a). The failure of nonlymphoid cells expressing IL-2Rβ to bind IL-2 suggests that an additional receptor component or components may be required for intermediate affinity IL-2 binding (Hatakeyama *et al.*, 1989a). Recent biochemical studies have identified two additional polypeptides associated with the IL-2R$\alpha\beta$ receptor present in IL-2R (Saragovi and Malek, 1990). It is likely that one or both of these polypeptides associate with IL-2Rβ to form the intermediate affinity receptor. We define this receptor as IL-2R$\beta\gamma$ where γ refers to a component (or components) of IL-2R that interacts with IL-2Rβ to facilitate this intermediate affinity binding of IL-2. The intracellular domain of IL-2Rβ is directly involved in signal transduction since certain mutant forms of IL-2Rβ expressed on some lymphoid cells are defective in signaling yet facilitate normal intermediate affinity IL-2 binding (Hatakeyama *et al.*, 1989b).

One major goal of studying the molecular nature of the IL-2-IL-2R interaction is to determine if the binding and activation events can be separated. Altered forms of IL-2 which occupy the receptor but fail to elicit signal transduction should be IL-2 antagonists and may be useful immunosuppressive agents. Structure-function studies have identified residues on both the human (hIL-2; Collins *et al.*, 1988; Weigel *et al.*, 1989) and mouse (mIL-2; Zurawski and Zurawaki, 1989) IL-2 proteins which interact specifically with either IL-2Rα or IL-2R$\beta\gamma$. In a systematic deletion and substitution mutagenesis study of the N-terminal region of mIL-2 (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988, 1989), we found that certain mIL-2 proteins with substitutions of Asp34 were defective in biological activity, yet bound normally to IL-2Rα. In a similar study of the C-terminal region of mIL-2 (Zurawski and Zurawski, 1988 and unpublished), we found that certain mIL-2 proteins with substitutions of Gln141 were also defective in biological activity, yet bound normally to IL-2Rα. The mutant phenotype of proteins with substitutions at Gln141 is similar to hIL-2 proteins with 1-3 residue deletions in the region analogous to the mIL-2 Gln141 position (Collins *et al.*, 1988).

This article shows that most mIL-2 proteins with substitutions at Gln141 are defective in binding to IL-2R$\beta\gamma$ but bind normally to IL-2Rα and IL-2R$\alpha\beta$. Certain mIL-2 Gln141 substitution proteins also have deficiencies in receptor activation and antagonize mIL-2 function. We propose that such mIL-2 mutant proteins are specifically defective in interaction facilitated by a third functional component (γ) of IL-2R and that this interaction is required for the signal transduction process. This article also shows that most mIL-2 proteins with substitutions at Asp34 are defective in binding to IL-2R$\beta\gamma$ and IL-2R$\alpha\beta$, yet bind normally to IL-2Rα. We propose that Asp34 substitution proteins, which do not have deficiencies specific to receptor activation and do not antagonize mIL-2 function, are defective in binding associated with IL-2Rβ. These data, therefore, pinpoint two mIL-2 residues that appear to affect specifically interactions with each of two functionally distinct components of the signal transducing IL-2R$\beta\gamma$ complex.

Results

Partial agonist Gln141 substitution proteins

In order to investigate the specific nature of the interaction of Gln141 substitution proteins with IL-2R, cassette mutagenesis was used to obtain plasmids that directed the expression in *Escherichia coli* of all possible mIL-2 Gln141 substitution proteins. The Gln141 substitution proteins were examined for their abilities to sustain the proliferation of various cell lines and to interact with various components

Table I. Properties of Gln141 and Asp34 substitution proteins

		HT2 cells		FD.C/2 cells		MC/9.TM cells		Receptor binding[d]			
		Activity[a]	Plateau[b]/ antagonized[c]	Activity	Plateau	Activity	Plateau	$\alpha\beta\gamma$	α	$\beta\gamma$	$\alpha\beta$
	mIL-2	>20		>20		>20		H	+	+	+
I[e]	Q141N	>20	†	>20		>20		I	+	−	
	Q141M	>20	†	>20		>20		I	+	−	+
	Q141H	>20	†	>20		>20		I	+		
	Q141A	>20	†	>20		>20		I	+		
	Q141S	>20	†	>20		>20		I	+		
	Q141W	>20	††	≤20, ≥1		•[f]		+			
	Q141Y	>20	††	≤20, ≥1		≤20, ≥1	↓	I	+	−	
	Q141R	>20	††	≤20, ≥1		≤20, ≥1	↓	I	+		
	Q141K	>20	↓ ††	≤20, ≥1	↓	≤20, ≥1	↓	I	+	−	+
	Q141E	>20	††	≤20, ≥1		≤20, ≥1	↓	I	+		
	Q141F	>20	††	≤20, ≥1		≤20, ≥1	↓	I	+		
	Q141T	>20	††	≤20, ≥1		≤20, ≥1	↓	I	+		
III	Q141L	<1	↓ ††	<1	↓	<1	↓↓	I	+		
	Q141V	<1	↓ ††	<1	↓	<1	↓	I	+		
	Q141I	<1	↓ ††	<1	↓	<1	↓↓	I	+		
	Q141D	<1	↓↓ •	<1	↓	<1	↓↓	I	+	−	+
IV	Q141C	<<1	•	<<1		<<1	↓	L	↓		
	Q141P	<<1	•	<<1		<<1	↓	L	↓		
	Q141G	<<1	↓ •	<<1	↓	<<1	↓↓	L	↓		
	Δ52−56	500	††	20		10		I	↓	+	↓
	il24GP	>20	††	•	•	•	•	I	↓		
	[g]D34E	>20		•	•	•	•		+	+	+
	[h]D34Q	>20	††	•	•	•	•	L	+		−
	[h]D34S	>20	††	•	•	•	•	L	+		
	[g]D34T	>20	††	•	•	•	•		+	−	−
	[h]D34I	>20	††	•	•	•	•	L	+		
	[g]D34G	>20	††	•	•	•	•		+	−	−
	[g]D34V	≤20, ≥1	††	•	•	•	•		+	−	−
	[h]D34P	≤20, ≥1	††	•	•	•	•	L	+		
	[g]D34W	<<1	•	<<1	•	<<1		L	+	−	−
	[h]D34R	<<1	•	•	•	•	•	L	+		−

[a]Biological activities were normalized for amount of mIL-2 mutant protein (Zurawski and Zurawski, 1988, 1989) and are expressed as a percentage relative to that of mIL-2. [b]Plateau refers to the maximal response observed for mIL-2; ↓ indicates a response in the range 50−90% of maximal, ↓↓ indicates a response of <50% of maximal; no symbol indicates a maximal response. [c]Antagonized refers to biological assays in the presence of 0.2 μM *mIL-2.Asp141*; † indicates 20 to 50-fold decreased biological activity; †† indicates >250-fold decreased biological activity; no symbol indicates <10-fold decreased biological activity. [d]For $\alpha\beta\gamma$, H, I and L indicate high, intermediate and low affinities as specified in Figure 3. For binding to α, + indicates affinity within 5-fold of that observed for mIL-2 and ↓ indicates affinity <10-fold that of mIL-2. Data for binding to $\beta\gamma$ and $\alpha\beta$ are from Figures 3 and 4; no symbols means not determined, + indicates affinity within 2-fold of that observed for mIL-2, − indicates affinity <10-fold that of mIL-2. [e]Mutant proteins are described in the single letter code. Q141N to Q141S are class I Gln141 substitution proteins; Q141W to Q141T are class II; Q141L to Q141D are class III; Q141C to Q141G are class IV; others are from Zurawski and Zurawski, 1988, 1989. •[f] indicates not determined. [g]D34 substitution proteins have been described previously (Zurawski and Zurawski, 1989). [h]D34 substitution proteins from this work.

of IL-2R. The biological activities of the mIL-2 Gln141 substitution proteins were determined on the mouse T cell line HT2 (Zurawski *et al.*, 1986), the myeloid progenitor FD.C/2 cell line (Le Gros *et al.*, 1985), and a laboratory subline (MC/9.TM) of the immature mast cell line MC/9 (Nabel *et al.*, 1981). Our findings permitted the Gln141 substitution proteins to be grouped into four classes (Table I). Relative to mIL-2, the class I proteins had near-normal biological activities on all three cell lines. The class II proteins had near-normal biological activities on HT2 cells and reduced activities (between 1% and 20%) on the other cell lines. The class III and IV proteins had low biological activities (<1%) on all cell lines. The class IV proteins generally had very low biological activities (≤0.1%) on all cell lines and, unlike the other classes, had defects in IL-2Rα binding (see below).

Even at high concentrations, several mIL-2 Gln141 substitution proteins gave a suboptimal maximal response in HT2 bioassays (Figure 1 and Table I). This property is characteristic of partial agonists (Black, 1989) and is unlike that of all other mIL-2 mutant proteins (including Asp34 substitution proteins) thus far analyzed (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988, 1989 and unpublished). Partial agonism was most pronounced when the proteins were assayed on MC/9.TM cells. All class II mIL-2 Gln141 substitution proteins gave suboptimal maximal responses (90%−50% of the maximum) on MC/9.TM cells, but usually gave maximal responses on HT2 and FD.C/2 cells (Table I). Class III mIL-2 Gln141 substitution proteins gave suboptimal maximal responses (<50%) on HT2, FD.C/2 and MC/9.TM cells (Table I). In other biological systems, different maximal responses by various cell types

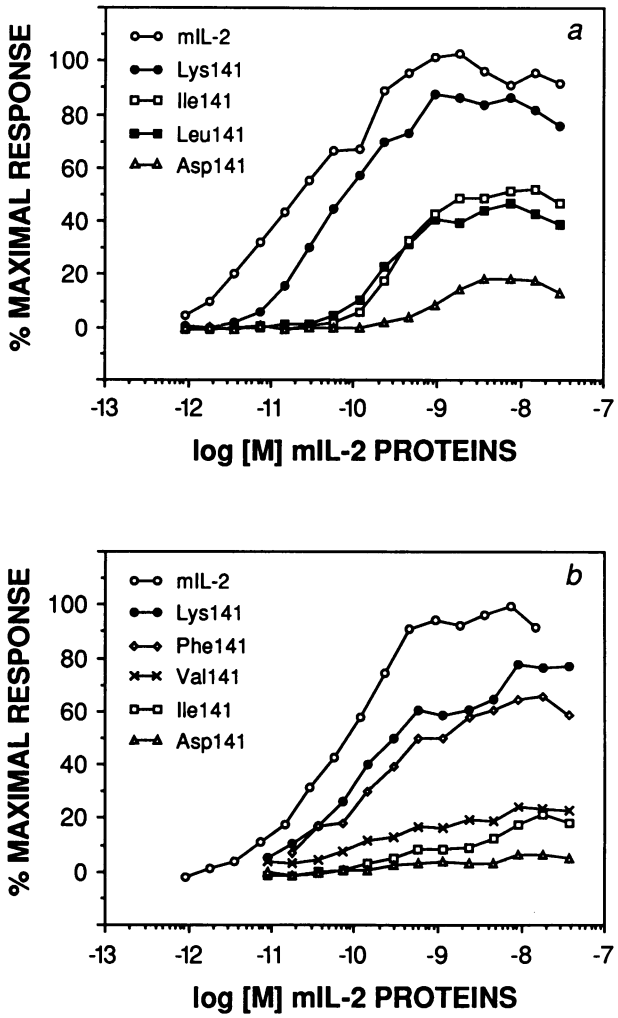

Fig. 1. Partial agonism by mIL-2 Gln141 substitution proteins. **A.** Response of HT2 cells. mIL-2 proteins added (listed from maximum to minimum response) were: mIL-2, *Lys141*, *Ile141*, *Leu141*, *Asp141*. **B.** Response of MC/9.TM cells. mIL-2 proteins added (listed from maximum to minimum response) were: mIL-2, *Lys141*, *Phe141*, *Val141*, *Ile141*, *Asp141*. 100% maximal response refers to maximum OD (570−650 nm) observed with mIL-2.

to partial agonism is associated with differences in cellular receptor number and/or receptor type (Black, 1989). Preliminary examination of the IL-2R composition of the FD.C/2 and MC/9.TM cells by FACSCAN analysis (Zurawski and Zurawski, 1989) revealed reduced levels of IL-2Rα on both cell types compared with that present on HT2 cells (unpublished).

The above data show that the side chain at residue Gln141 is crucial for the activation of IL-2R. Gln141 is conserved in hIL-2 (Taniguchi *et al.*, 1983) and is located in the C-terminal α-helical region (Brandhuber *et al.*, 1987). In a study of semi-synthetic analogues of hIL-2 (Landgraf *et al.*, 1989), certain changes of the stability and amphiphilicity of the C-terminal α-helix also resulted in proteins with partial agonist properties. However, this study (Landgraf *et al.*, 1989) was limited by the low biological activity of the semi-synthetic hIL-2 analogues (~2000-fold less than natural hIL-2) and did not identify the important side chain(s) on the C-terminal α-helix.

Antagonism by mIL-2.Asp141 protein

Partial agonism by structural analogues of a natural ligand is indicative of defective activation of the receptor (Black, 1989). Such partial agonists should antagonize the action of the natural ligand by competing for receptor binding sites. The *mIL-2.Asp141* protein, which had the lowest maximal response (Figure 1), was tested for antagonism of mIL-2 action in biological assays. Figure 2a shows that the biological activity of mIL-2 assayed on HT2 cells was slightly reduced by the presence of 0.2 μM *mIL-2.Asp141*. The concentration of *mIL-2.Asp141* present in this assay represented a 5×10^4 molar excess over the amount of mIL-2 required for a maximal response. If antagonism by *mIL-2.Asp141* reflects competition with mIL-2 for receptor binding sites, then biologically active mutant mIL-2 proteins with reduced receptor binding should be antagonized by *mIL-2.Asp141* to a greater extent than the slight antagonism observed against mIL-2. Previously characterized biologically active proteins such as *mIL-2.i124GlyPro* (Zurawski and Zurawski, 1989) and *mIL-2.Δ52−56* (Zurawski and Zurawski, 1988) which have defects in interaction with IL-2Rα had a ~1000-fold reduced biological activity when assayed on HT2 cells in the presence of 0.2 μM *mIL-2.Asp141* (Figure 2 and Table I). In other work (unpublished results and below), we have shown that *mIL-2.Asp141* strongly antagonizes all known biologically active mIL-2 proteins that have defects in IL-2R interaction.

Antagonist bioassays reveal defects in biologically active mIL-2 Gln141 and Asp34 substitution proteins

All biologically active mIL-2 Gln141 substitution proteins were more susceptible than mIL-2 to antagonism by *mIL-2.Asp141* (Table I and Figure 2C and D). The biological activities of all class I mIL-2 Gln141 substitution proteins were 20 to 50-fold reduced when assayed on HT2 cells in the presence of *mIL-2.Asp141* (Table I and Figure 2C). In similar assays, the biological activities of all class II and class III Gln141 substitution proteins were >250-fold reduced by *mIL-2.Asp141* antagonism (Table I and Figure 2D). The sensitivity of the mIL-2 Gln141 substitution proteins to antagonism by *mIL-2.Asp141* suggests that they have defects in binding to IL-2R. Indeed, as shown below, all mIL-2 Gln141 substitution proteins (including *mIL-2.Asp141*) had reduced affinities for binding to high affinity IL-2R.

In a previous analysis of substitutions at the important mIL-2 residue Asp34, we noted that all but bulky or basic side chains are acceptable at this position for biological activity (Zurawski and Zurawski, 1989). Table I shows that most Asp34 substitution proteins with near-normal biological activities were strongly antagonized by *mIL-2.Asp141*. This suggests that such Asp34 substitution proteins have defects in binding to IL-2R which were previously unrecognized. The receptor binding analysis described below confirmed this suggestion.

Gln141 is crucial for binding associated with IL-2Rγ

Figure 3A and Table I show that class I, II and III mIL-2 Gln141 substitution proteins bound to high affinity receptors (called IL-2Rαβγ) on HT2 cells at reduced affinities. The reduced binding by these proteins was similar to that of biologically active mIL-2 proteins defective in IL-2Rα interaction (e.g. *mIL-2.Δ52−56*, Figure 3A). To determine whether the reduced affinities for IL-2Rαβγ involved altered

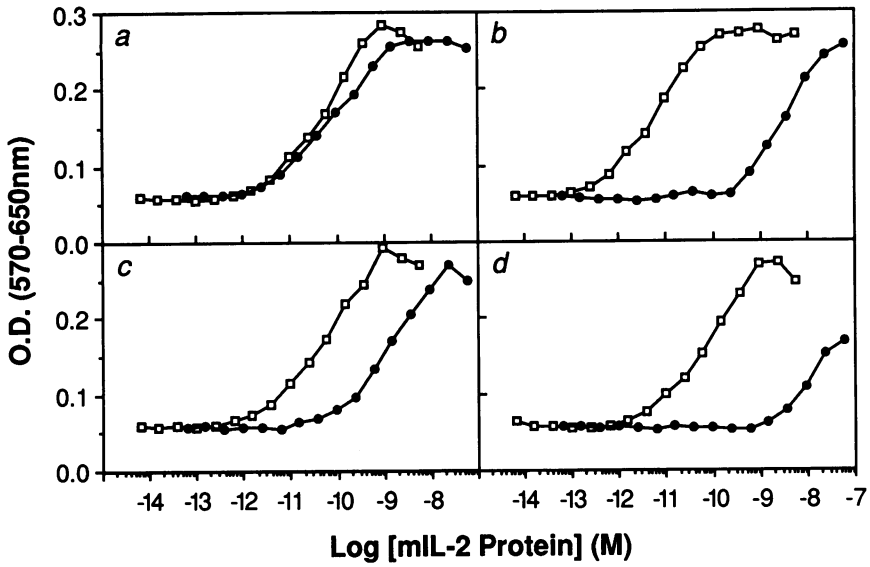

Fig. 2. Antagonist properties of *mIL-2.Asp141*. Biological activity of various proteins were determined on HT2 cells in the presence (filled circles) and absence (open squares) of 0.2 μM *mIL-2.Asp141*. **A**, mIL-2; **B**, i124GlyPro; **C**, *Met141*; **D**, *Arg141*.

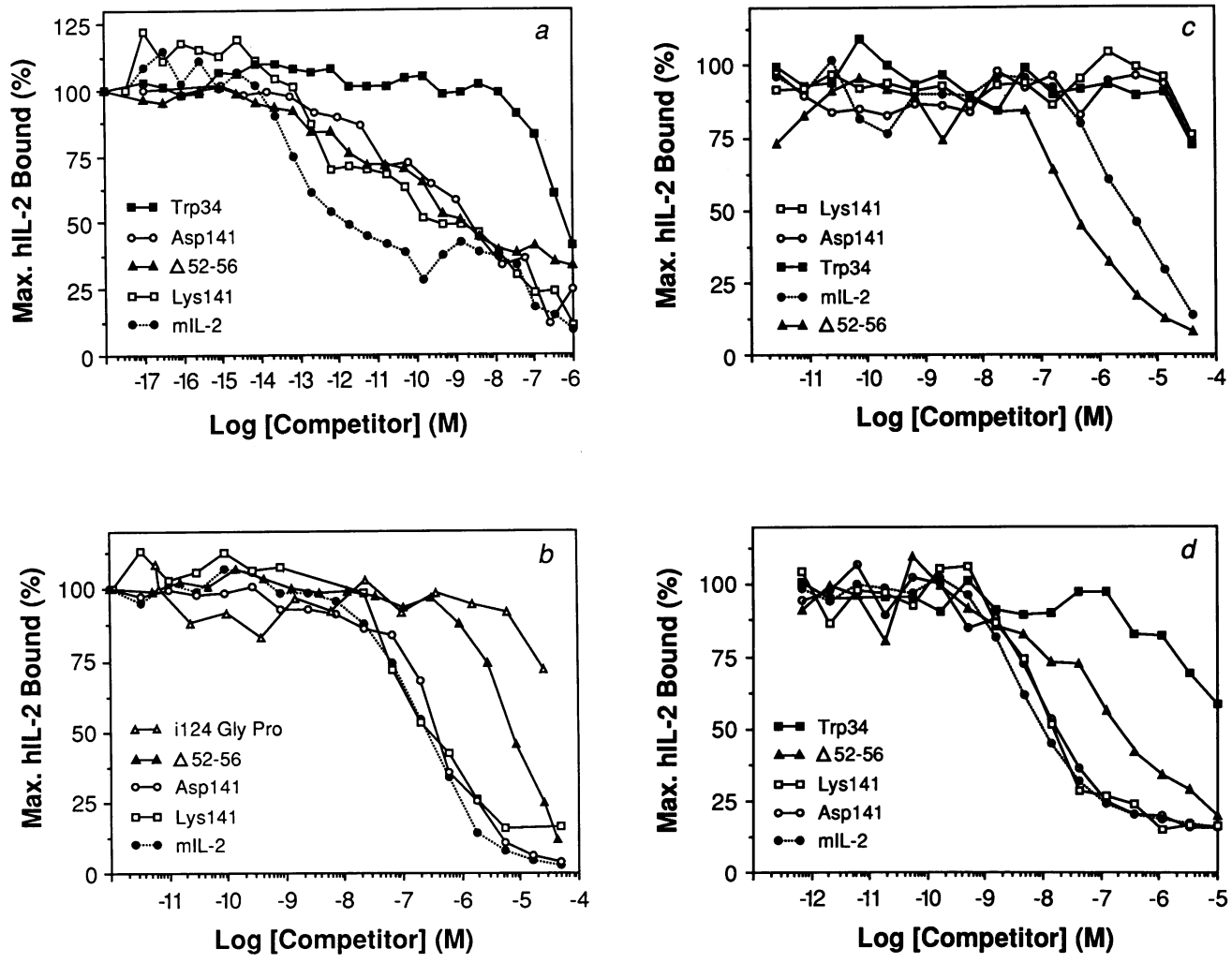

Fig. 3. Competitive receptor binding analysis of mIL-2 mutant proteins. **A**, High affinity IL-2Rαβγ on mouse HT2 cells. **B**, Low affinity mouse IL-2Rα on transfected L cells (Zurawski and Zurawski, 1989). **C**, Intermediate affinity IL-2Rβγ on human YT2C2 cells (Teshigawara *et al.*, 1987). **D**, IL-2Rαβ on Cos 7 cells co-transfected with mouse IL-2Rα and human IL-2Rβ cDNAs. mIL-2 proteins: filled circle and dashed line, mIL-2; filled triangle, *Δ52–56;* filled square, *Trp34*; open circle, *Asp141*; open square, *Lys141*; open triangle, *i124GlyPro.*

recognition to one or more of the IL-2R subunits, the Gln141 substitution proteins were examined for binding to IL-2Rα, IL-2Rβγ and IL-2Rαβ. Binding to IL-2Rα was examined using a mouse p55 cDNA-transfected stable L cell line expressing IL-2Rα. Except for class IV proteins, all Gln141 substitution proteins bound normally to IL-2Rα (Figure 3B and Table I). The three Gln141 class IV substitutions, Cys141, Pro141 and Gly141, are those that are the most likely to cause structural perturbations, via formation of inappropriate disulfides, or disruption of the C-terminal α-helix. Such structural alterations could account for the very low biological activities and reduced IL-2Rα binding of these proteins. Binding to IL-2Rβγ was examined using an IL-2Rα deficient subline (2C2; Teshigawara *et al.*, 1987) of YT human natural killer cells. The *mIL-2.Δ52−56* protein, which is biologically active and defective in binding to IL-2Rα (Table I and Figure 3B), bound to YT2C2 cells with a 5-fold higher affinity than mIL-2 (Figure 3C). This increased affinity, which was identical to the 5-fold higher biological activity of this protein on HT2 cells (Table I), confirmed that YT2C2 cells are deficient in IL-2Rα. This increased affinity for IL-2Rβγ contrasted with the expected decreased affinity of *mIL-2.Δ52−56* for IL-2Rα and IL-2Rαβ (Figure 3B and D). Representatives of class I, II and III Gln141 substitution proteins all had >50-fold lower affinities than mIL-2 for IL-2Rβγ (Figure 3C and Table I). Binding to IL-2Rαβ was examined using Cos 7 cells transiently co-transfected with expression plasmids encoding mouse IL-2Rα and human IL-2Rβ. On such cells, IL-2Rα and IL-2Rβ form a complex that has a higher affinity for IL-2 than of IL-2Rα alone (Figure 3B and D). Representatives of class I, II and III Gln141 substitution proteins all had affinities similar to mIL-2 for IL-2Rαβ (Figure 3D and Table I). These data show that the class I, II and III mIL-2 Gln141 substitution proteins have normal binding to IL-2Rα and IL-2Rαβ, but are defective in binding to IL-2Rαβγ and IL-2Rβγ. Thus, the mIL-2 Gln141 substitution proteins must be deficient in some aspect of recognition associated with IL-2Rγ. Since several of the mIL-2 Gln141 substitution proteins are also defective in receptor activation, it is likely that binding associated with IL-2γ is crucial for receptor activation.

The Gln141 side chain appears to have mutationally distinguishable roles in both binding and activation associated with IL-2Rγ. The requirement of a Gln side chain at the 141 position is very specific for binding associated with IL-2Rγ. The native Gln side chain is the only acceptable side chain at the 141 position for high affinity binding to IL-2Rαβγ or for low level antagonism by *mIL-2.Asp141* in the HT2 antagonist bioassay (Table I). In hIL-2, the role of the analogous Gln126 residue has been subjected to limited analysis. The single hIL-2 Gln126−Glu substitution protein examined had normal biological activity (Ju *et al.*, 1987). Substitutions that conferred partial agonist/antagonist properties in mIL-2 were not analyzed (Ju *et al.*, 1987). Preliminary studies of such substitutions at hIL-2 residue Gln126 suggest that this is a critical residue, and that it has a role similar to mIL-2 residue Gln141 (unpublished). These data reveal the potential pitfalls that can result from studies based on limited substitution analysis designed simply to change the charge properties of potential surface residues (Weigel *et al.*, 1989; Ju *et al.*, 1987).

The requirement for a Gln side chain at position 141 for receptor mediated activation is apparently not as rigid as the requirement for binding. Many mIL-2 Gln141 substitution proteins were fully proficient in activating HT2 cells (Table I). However, several such proteins that were fully proficient in activation of HT2 cells were defective in activation of other cell types (Table I). It is therefore possible that, when these proteins are examined in other IL-2 assays where receptor number/type and signaling responses differ, all mIL-2 Gln141 substitution proteins may prove to have activation defects.

The importance of examining mutant mIL-2 proteins in various biological assays is further illustrated by differences in responses to the class II Gln141 substitution proteins by the three cell lines examined. These proteins, which have reduced affinities for IL-2R, elicited near-normal biological responses from HT2 cells, but gave reduced biological activities on FD.C/2 and MC/9.TM cells (Table I). These and previously reported data on mIL-2 (Zurawski and Zurawski, 1989) and hIL-2 (Weigel *et al.*, 1989) proteins that have near-normal biological activities, yet are defective in binding to IL-2Rα, show that biological assays based upon the commonly used HT2 and CTLL-2 cell lines do not strictly reflect the affinity of the IL-2−IL-2R interaction. Since class I Gln141 substitution proteins had normal biological activity on FD.C/2 and MC/9.TM cells (Table I), assays based on these cells also do not strictly reflect the affinity of the IL-2−IL-2R interaction. The antagonist bioassay on HT2 cells described above is the only biological assay which detected all known defects in interaction of IL-2R with mutant mIL-2 proteins with near-normal biological activities on HT2 cells. This new assay should greatly facilitate future IL-2 structure−function studies as well as permit reappraisal of previous studies which were based on assays that are not totally affinity dependent.

Asp34 is crucial for binding associated with IL-2Rβ

The susceptibility to antagonism by *mIL-2.Asp141* of biologically active proteins with substitutions at the important 34 position (see above and Table I) was a clue that these proteins may be defective in interaction with IL-2R. A previous study (Zurawski and Zurawski, 1989) revealed that basic or bulky substitutions (e.g. Lys, Trp) of Asp34 result in proteins that are biologically inactive, yet bind normally to IL-2Rα. Proteins such as *mIL-2.Trp34* are phenotypically similar to class III mIL-2 Gln141 substitution proteins in that they are also biologically inactive, yet bind normally to IL-2Rα. Figure 3C shows that *mIL-2.Trp34* was also similar to class III Gln141 substitution proteins in being deficient in binding to IL-2Rβγ. Previous studies (Zurawski and Zurawski, 1989) revealed that substitutions other than those with basic or bulky side chains at position 34 resulted in proteins with near-normal biological activity. Like the biologically active class I and II Gln141 substitution proteins, biologically active proteins such as *mIL-2.Val34*, *mIL-2.Thr34* and *mIL-2.Gly34* were also defective in binding to IL-2Rβγ (Figure 4A).

The above data show that substitutions at Asp34 and Gln141 can result in proteins with a similar spectrum of biological and receptor binding properties. This is surprising in that Asp34 and Gln141 are probably on opposite surfaces of mIL-2 (Brandhuber *et al.*, 1987) and might thus be expected to interact with different components of IL-2R. However, one difference between position 34 and position 141 substitution proteins is that the former do not have partial agonist/antagonist properties (Zurawski and Zurawski,

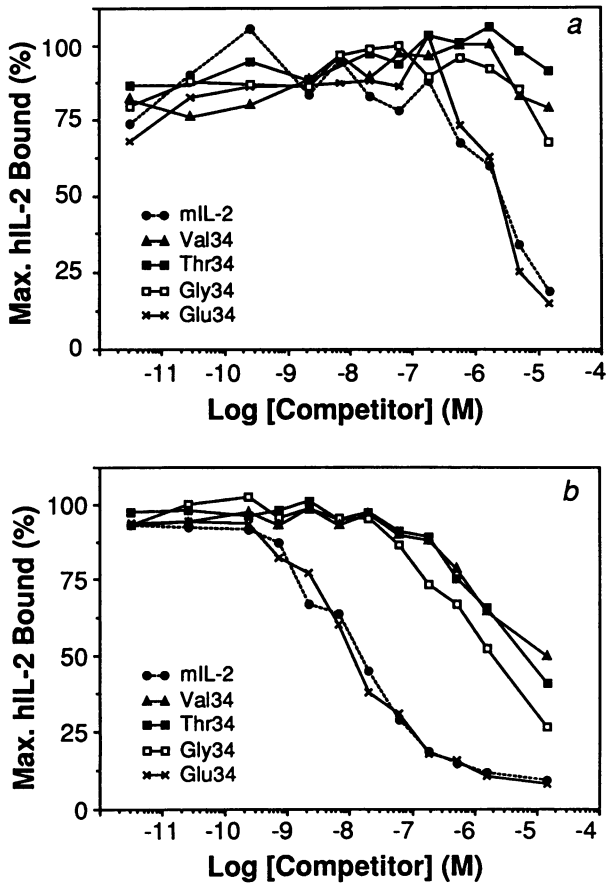

Fig. 4. Competitive receptor binding analysis of mIL-2 Asp34 substitution proteins. **A**, Intermediate affinity IL-2Rβγ on human YT2C2 cells (Teshigawara *et al.*, 1987). **B**, IL-2Rαβ on Cos 7 cells co-transfected with mouse IL-2Rα and human IL-2Rβ cDNAs. mIL-2 proteins: filled circle and dashed line, mIL-2; filled triangle, *Val34*; filled square, *Thr34*; open square, *Gly34*; cross, *Glu34*.

1989). Examination of the biologically inactive *mIL-2.Trp34* and *mIL-2.Arg34* proteins in IL-2Rαβγ and IL-2Rαβ receptor assays revealed properties that were different from the biologically inactive class III Gln141 substitution proteins. The *mIL-2.Trp34* and *mIL-2.Arg34* proteins interacted with receptors on HT2 cells only at concentrations consistent with binding to IL-2Rα alone, while the class III Gln141 substitution proteins clearly interacted with the high affinity IL-2R on such cells, albeit with greatly reduced affinities (Figure 3A). Biologically active and inactive Asp34 substitution proteins had $<1\%$ of normal binding to IL-2Rαβ (Figures 3D and 4B). In contrast, both biologically active and inactive Gln141 substitution proteins had near-normal binding to IL-2Rαβ (Figure 3D and Table I).

The above data show that the Asp34 and Gln141 residues have profoundly different roles in the IL-2−IL-2R interaction. The residue at position 34 appears to be crucial for binding associated with IL-2Rβ while the residue at position 141 is crucial for binding associated with IL-2Rγ. Binding assays for IL-2Rγ or IL-2Rαγ do not exist and we therefore cannot determine if the Asp34 residue is also important for binding associated with IL-2Rγ. The antagonist bioassay (Table I) and receptor binding assays (Figures 3 and 4) for various Asp34 substitution proteins indicated that the side chain requirement at position 34 is not as rigid as the absolute requirement for Gln at position 141. Although all Asp34 substitution proteins have yet to be analyzed, the

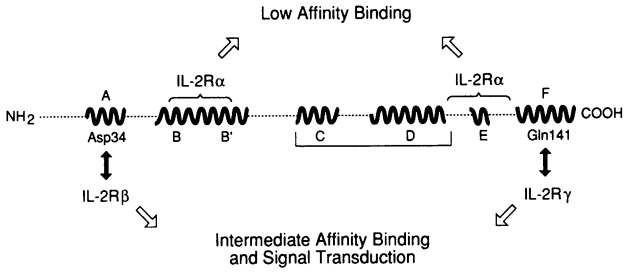

Fig. 5. Model depicting the regions of mIL-2 recognized by the different IL-2R subunits. NH_2 and COOH indicate, respectively, the N and C-termini of the 149 residue mIL-2 protein. The probable locations of α-helices A−F (Brandhuber *et al.*, 1987) are indicated. The straight line bracket indicates the single disulfide linkage. Single arrows and brackets indicate sites or domains of interaction between IL-2 residues and the α, β and γ subunits of IL-2R. Double arrows indicate actions resulting from IL-2−IL-2R subunit interactions. The model is described in detail in the text.

mIL-2.Glu34 protein had identical properties to mIL-2 (Figure 4) while all other Asp34 substitution proteins examined had defects in biological assays or antagonist biological assays (Table I).

Discussion

A model for IL-2−IL-2R interactions

Our data indicate that binding and subsequent signal transduction elicited by the interaction of IL-2 with IL-2R is a process of surprising complexity. In the functional high affinity receptor found on T cells, at least three receptor subunits or components play crucial roles. We have shown that the regions of IL-2 recognized by these components are located on separate parts of the IL-2 structure and that normal interaction with two of these components (IL-2Rβ and IL-2Rγ) are necessary for signal transduction (Figure 5). Some mutant mIL-2 proteins are defective in interaction facilitated by IL-2Rγ and have partial agonist/antagonist properties, yet appear to have normal interaction with IL-2Rβ. This result suggests that the action of IL-2Rγ occurs subsequent to that of IL-2Rβ.

The most likely model for IL-2−IL-2R interaction (Figure 5) is that IL-2 first binds IL-2Rα. IL-2Rα is itself able to bind IL-2 with a low affinity and a very high on−off rate (Smith, 1989), and is present on the T cell surface in ~10-fold molar excess (Smith, 1989). This binding occurs by IL-2Rα interaction with a large surface which, in mIL-2, includes residues in α-helix B and the region included between the end of α-helix D and residue Phe132 at the start of α-helix F (Zurawski and Zurawski, 1989; Figure 5 and unpublished data). The IL-2−IL-2Rα complex then associates with IL-2Rβ. Alternatively, if IL-2Rα is already associated with IL-2Rβ, then the excess IL-2Rα serves to concentrate IL-2 at the cell surface as rapidly associating/dissociating IL-2−IL-2Rα. Such exchanges occur until IL-2 binds to IL-2Rα that is associated with IL-2Rβ. IL-2Rβ is itself unable to bind IL-2, yet together with IL-2Rα binds IL-2 with a higher affinity than IL-2Rα alone (Hatakeyama *et al.*, 1989a). Asp34 on the N-terminal α-helix A and presumably other residues interact with IL-2Rβ to decrease the high IL-2−IL-2Rα dissociation rate. IL-2 residues, which in mIL-2 include Gln141 on the C-terminal α-helix F, then interact with IL-2Rγ and conformational or chemical

changes resulting from the interaction with IL-2Rγ or IL-2Rβγ initiate signal transduction.

The ability of some cell types deficient in IL-2Rα to respond to IL-2R[5], together with the inability of IL-2Rβ alone to bind IL-2 (Hatakeyama *et al.*, 1989a), suggests that IL-2Rγ is already associated with IL-2Rβ prior to binding of IL-2. Receptor mediated activation is independent of binding to IL-2Rα or binding associated with IL-2Rβ and is dependent on binding associated with IL-2Rγ. Two schemes could explain the observation that IL-2Rβ is physically required for cellular activation (Hatakeyama *et al.*, 1989b). Firstly, IL-2Rγ and IL-2Rβ may associate directly such that binding and activation associated with IL-2Rγ is dependent on this interaction. Secondly, IL-2Rγ may interact allosterically with IL-2Rβ such that this interaction exposes a new domain on IL-2Rβ. This new domain could bind IL-2 residues including Gln141 and binding facilitated by both IL-2Rβ domains is required for activation. Resolution of the precise nature of the interaction of IL-2Rγ with IL-2Rβ would be facilitated by cDNA cloning of mRNAs for further IL-2R associated proteins (Saragovi and Malek, 1990). Such cDNA(s) should allow reconstitution of IL-2Rβγ on fibroblasts and therefore permit mutational analysis of IL-2Rβ−IL-2Rγ interaction.

Potential pharmaceutical applications for mutant IL-2 proteins

The activation defective mIL-2 Gln41 substitution proteins could be prototypes for novel types of pharmaceutically useful hIL-2 analogues. Analogues of *mIL-2.Asp141* with increased affinity for IL-2R might function as immunosupressive agents for organ transplant recipients and patients with autoimmune diseases. Partial agonist hIL-2 analogues of some mIL-2 Gln141 substitution proteins could also have potential in immunotherapy. Data presented here show that it is possible to engineer IL-2 proteins that are biologically active on some IL-2 responsive cells, yet are inactive on others (Table I). High dosage hIL-2 therapy is effective against some cancer types (Rosenberg *et al.*, 1985). This therapy depends on the activation of killer cell precursors and is accompanied by side effects that result from the release of tumor necrosis factor and perhaps other pyrogenic lymphokines by activated lymphocytes (Meir *et al.*, 1989). The work presented here suggests that it may be possible to engineer partial agonist hIL-2 proteins that elicit a subset of normal IL-2 responses that (for example) includes activation of killer cell precursors yet excludes activation of lymphokine production by lymphocytes.

Materials and methods

Molecular and biochemical techniques

Materials and protocols for recombinant DNA work, including synthesis and cloning of synthetic DNA, DNA sequencing, expression and characterization of mutant mIL-2 proteins and partial purification of mutant mIL-2 proteins have been described previously (Zurawski and Zurawski, 1988, 1989).

Biological assays

MTT colorimetric biological assays were done as described previously (Zurawski *et al.*, 1986; Zurawski and Zurawski, 1988) except that IL-2 responsive FD.C/2 and MC/9.TM cells were used at 4000 cells per 100 μl. Antagonist bioassays were done in the presence of 0.2 μM *mIL-2.Asp141*.

Receptor binding assays

Receptor binding analyses were done as described (Zurawski and Zurawski, 1989) except that dibutyl phtalate/dioctyl phtalate (3:2) was used to separate bound from free ligand. [^{125}I]hIL-2 was used at 1 nM for low affinity binding and at 40 pM for intermediate and high affinity binding. For IL-2Rαβ binding, Cos 7 cells were co-transfected by the DEAE−dextran/chloroquine method with pCDSRα-mouse IL-2Rα (Zurawski and Zurawski, 1989) and pME18S (K.Maruyama, unpublished) containing human IL-2Rβ cDNA (Hatakeyama *et al.*, 1989a) with a deletion of the cytoplasmic domain.

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