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Identification of three adjacent amino acids of interleukin-2 receptor β chain which control the affinity and the specificity of the interaction with interleukin-2

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The β chain of the interleukin-2 (IL-2) receptor (IL-2Rβ) and the interleukin-3 (IL-3) binding protein AIC2A are members of the family of cytokine receptors, which also includes the receptors for growth hormone (GHR) and prolactin. A four amino acid sequence of AIC2A has recently been shown to be critical for IL-3 binding. We analyze here the function of the analogous sequence of human IL-2Rβ and identify three amino acids, Ser132, His133 and Tyr134, which play a critical role in IL-2 binding. We show that some mutant IL-2 proteins with substitutions of a critical Asp residue in the N-terminal α-helix bind the mutant IL-2Rβ receptor with a higher affinity than the wild-type receptor. This suggests that the critical Asp34 in the ligand and the sequence Ser-His-Tyr (positions 132-134) in the receptor interact directly. On the double barrel β-stranded structural model of cytokine receptors, the residues important for ligand binding in IL-2Rβ, AIC2A and GHR map to strikingly similar locations within a barrel, with the interesting difference that it is the N-terminal barrel for GHR and the C-terminal barrel for IL-2Rβ and AIC2A.

Key words: cytokine receptor family/hormone binding/structure-function

Introduction

Interleukin-2 (IL-2), an important immunoregulatory cytokine, exerts its effects by binding and activating specific receptors on the surface of target cells (see Smith, 1989 and Waldmann, 1991 for reviews). The interleukin-2 receptor (IL-2R) is composed of at least two subunits. The α chain (IL-2Rα) binds IL-2 with low affinity (K_d = 10^{-8} M; see Waldmann, 1991 for references). The β chain of the receptor (IL-2Rβ) binds IL-2 with intermediate affinity (K_d = 10^{-9} M) when expressed on the surface of lymphoid cells (Hatakeyama *et al.*, 1989a), but not when expressed on the surface of fibroblasts (Tsudo *et al.*, 1990). However, co-expression of the α and β chains of the receptor on fibroblasts reconstitutes a higher affinity IL-2R (K_d = 10^{-10} M; Tsudo *et al.*, 1990; Minamoto *et al.*, 1990). These results suggest that IL-2Rβ is associated with another receptor component on lymphoid cells that helps IL-2Rβ to bind IL-2. Co-expression of the α and β chains on the surface of lymphoid cells results in the formation of the high affinity IL-2R characteristic of activated T cells (K_d = 10^{-11} M; Smith, 1989; Hatakeyama *et al.*, 1989a).

Structure-function studies have identified several residues of IL-2 that are involved in specific interaction with IL-2Rα (Sauvé *et al.*, 1991; Zurawski,S., Doyle,E., Vega,F. and Zurawski,G., in preparation). Furthermore, the residue Asp20 in hIL-2 and the analogous Asp34 in mIL-2 have been shown to control the binding to IL-2Rβ (Collins *et al.*, 1988; Zurawski and Zurawski, 1989; Zurawski *et al.*, 1990). Finally, substitutions of Gln141 in mIL-2 results in molecules which bind with reduced affinity to IL-2Rβ expressed on lymphoid cells, but bind normally to IL-2Rβ co-expressed with IL-2Rα on fibroblasts, suggesting that these latter mutant proteins might be deficient in interaction with a third receptor component. Interestingly, these mutant proteins are partial agonists, being unable to activate the receptor even at saturating concentrations (Zurawski *et al.*, 1990; Imler and Zurawski, 1992).

Comparative sequence analysis has shown that IL-2Rβ belongs to a new family of cytokine receptors (Bazan *et al.*, 1990; Cosman *et al.*, 1990; Patthy, 1990). Among the members of this family, the mouse IL-3 binding protein AIC2A and the common β subunit (AIC2B) of the mouse granulocyte macrophage colony stimulating factor and IL-5 receptors have 91% amino acid identity, yet only AIC2A binds IL-3 (Gorman *et al.*, 1990; Itoh *et al.*, 1990; Devos *et al.*, 1991; Kitamura *et al.*, 1991; Shanafelt *et al.*, 1991; Takaki *et al.*, 1991). This observation has facilitated the mapping of residues of AIC2A that are critical for IL-3 binding (Wang *et al.*, 1992). In an attempt to unveil a possible functional significance to the predicted structural relationship between members of the cytokine receptor family, we have investigated the function of residues of human IL-2Rβ (hIL-2Rβ) corresponding to the critical binding sequence in AIC2A. We demonstrate here that the location of sequences important for ligand binding has been conserved between AIC2A and hIL-2Rβ. Furthermore, we show that mutation of this sequence changes the specificity of the receptor, suggesting a direct interaction between this sequence of hIL-2Rβ and a critical Asp residue in the N-terminal α-helix of IL-2.

Results

Identification in IL-2Rβ of a sequence critical for IL-2 binding

Sequence alignment of members of the cytokine receptor family shows that a number of residues are conserved (Bazan, 1990; Cosman *et al.*, 1990; Patthy, 1990; Figure 1). Wang *et al.* (1992) identified in the IL-3 binding protein AIC2A a four amino acid sequence, IPKY, which is important for ligand binding and which is located between two blocks of conserved amino acids (positions 335-339 and 350-356 in AIC2A, corresponding to positions 122-126 and 139-145 in hIL-2Rβ; see Bazan, 1990). We

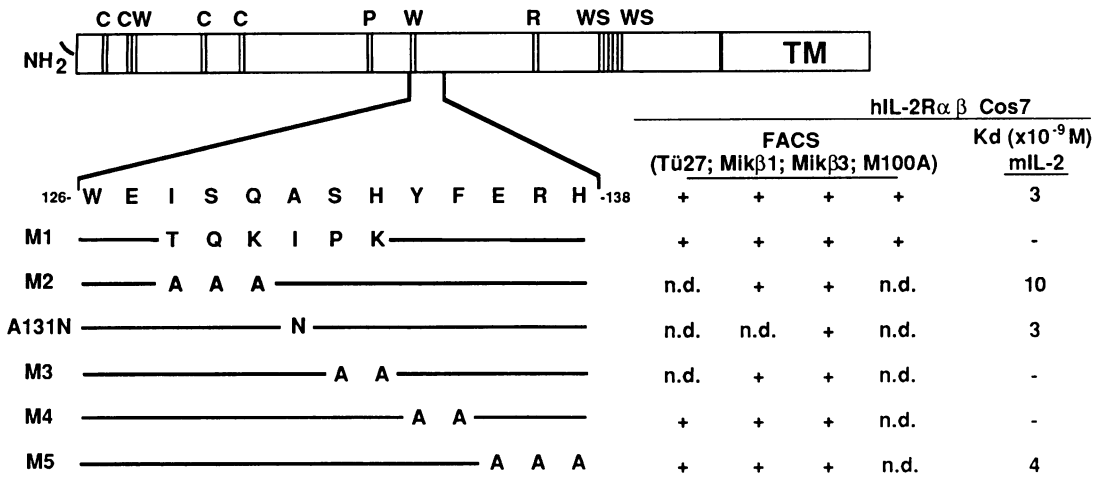

Fig. 1. Identification of residues important for IL-2 binding using alanine substitution scanning (Cunningham and Wells, 1989). **Top:** schematic representation of the extracellular and transmembrane (TM) domains of hIL-2Rβ. A number of residues conserved in most members of the cytokine receptor family are indicated. **Bottom:** The hIL-2Rβ sequence corresponding to the proposed B′−C′ loop in the C domain in the structural model of the receptor (Bazan, 1990) is shown, as are the sequence changes in the hIL-2Rβ mutants. Wild-type and mutant hIL-2Rβ were co-transfected with hIL-2Rα in Cos7 cells and analyzed by FACScan with the anti-IL-2Rβ monoclonal antibodies TU27 (Takeshita *et al.*, 1989), Mikβ1 and Mikβ3 (Tsudo *et al.*, 1989) and M100A (Endogen). Receptor binding assays were performed as described in Materials and methods. +: with normalization to the level of hIL-2Rα expressed, the fluorescence intensity of the transfectants was similar to that of the wild-type hIL-2Rβ transfected cells (see Materials and methods); n.d.: not done; −: no specific binding detected under the conditions used.

therefore decided to mutate hIL-2Rβ, starting from the highly conserved tryptophan (W) residue at position 126 (Figure 1). We chose to work with the human IL-2Rβ, for which monoclonal antibodies are available (see below), in order to be able to check the cell surface expression of mutant IL-2Rβ. The mutated coding regions were transferred to an expression vector and the function of the expressed proteins was studied upon transient transfection in Cos7 cells. Since IL-2Rβ by itself binds IL-2 only very poorly (Hatakeyama *et al.*, 1989; Tsudo *et al.*, 1990), the cells were co-transfected with an expression vector encoding hIL-2Rα. The transfected cells were analyzed by flow cytometry on a FACScan using a panel of monoclonal antibodies to define hIL-2Rβ expression, and by receptor binding assays under high affinity conditions. Figure 1 shows that the wild-type and all the mutant receptors were expressed at similar levels on the surface of the transfected Cos7 cells. More qualitatively, stable BAF3 clones expressing hIL-2Rβ or the mutant M1, which has the most extensive sequence alteration, were analyzed with serial dilutions of the four monoclonal antibodies; they showed identical decreases of staining after FACS analysis (data not shown). However, when IL-2 binding was studied, dramatic differences were observed between wild-type IL-2Rβ and some of the mutant receptors. Replacement of residues 128−133 of hIL-2Rβ by their counterparts in AIC2A totally abolished IL-2 binding (Figure 1, mutant M1). The alanine-scanning mutants (Cunningham and Wells, 1989; Figure 1, mutants M2 to M5) revealed that the integrity of the SHYF sequence (residues 132−135) is essential for IL-2 binding and that the adjacent sequences are relatively unimportant.

We constructed point mutations to define the importance of the individual amino acids in the crucial 132−135 region. Table I shows that substitution of Ser132, His133 and Tyr134 had the most dramatic effects on IL-2 binding, while substitution of Phe135 had only a minor effect. Although the relatively conservative Ser to Ala change at position 132 has only a minor effect, the Ser to Val change greatly disrupted IL-2 binding.

Table I. Dissociation constants for binding of mIL-2 and mIL-2 mutant proteins with substitutions at position 34 to receptors containing mutant hIL-2Rβ

IL-2Rβ:	K_d[a] (× 10^{-9} M)			
	mIL-2	D34H	D34G	D34K
wild type	3	82	88	−[b]
A131N	3	81	n.d.[c]	n.d.
S132A	11	13	39	n.d.
S132V	63	5	13	n.d.
H133A	36	6	31	36
H133D	53	11	14	37
H133K	−	8	17	n.d.
Y134A	53	51	n.d.	n.d.
F135A	10	47	30	n.d.

[a]Cos7 cells were co-transfected with expression vectors for hIL-2Rα and the different versions of hIL-2Rβ. Receptor binding assays were performed 3 days later in the presence of 50 pM [^{125}I]hIL-2, as described in Materials and methods.
[b]No specific binding was detected.
[c]Not determined.

The SHY sequence is involved in IL-2 binding

Our assay to test the activity of the IL-2Rβ mutants involved binding of IL-2 to an IL-2Rαβ complex. This raises the possibility that the SHY sequence may play a critical role in mediating the interaction between the α and β chains, rather than the interaction between IL-2 and IL-2Rβ. To test this possibility, we introduced some of the mutant IL-2Rβ expression plasmids into lymphoid cell lines, in which IL-2 binding is known to occur in the absence of IL-2Rα expression (Hatakeyama *et al.*, 1989a). We first introduced wild-type IL-2Rβ and the H133A and H133D mutant receptors into the mouse IL-3 dependent proB BAF3 cell line (Palacios and Steinmetz, 1985). This cell line becomes IL-2 responsive upon transfection with IL-2Rβ cDNA (Hatakeyama *et al.*, 1989b). FACScan analysis revealed that

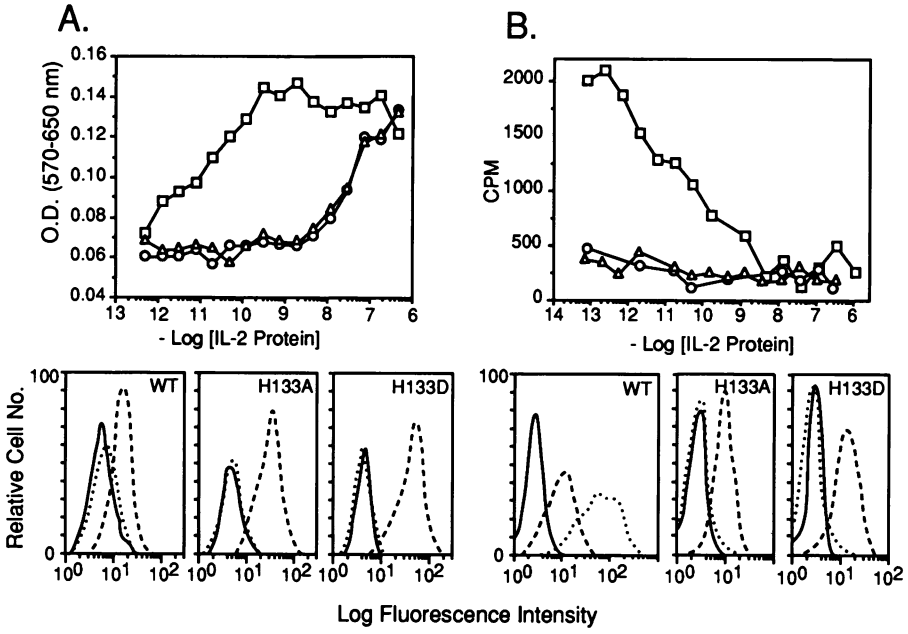

Fig. 2. IL-2Rβ molecules with substitutions of His133 show functional defects even in the absence of IL-2Rα expression. (**A**) BAF3 clones transfected with wild-type or mutant IL-2Rβ cDNAs were established. Staining with the Mikβ1 (dashed line) and 7D4 (anti-mIL-2Rα; Malek *et al.*, 1983; dotted line) monoclonal antibodies and FACScan analysis confirm that these cells express hIL-2Rβ but not mIL-2Rα (bottom panel). The proliferative response to hIL-2 of the transfectants was analyzed using an MTT colorimetric assay and the dose−response curves of typical clones expressing IL-2Rβ wild-type (□) and the mutants H133A (○) and H133D (△) are shown in the top panel. (**B**) Jurkat cells transfected with expression vectors encoding IL-2Rβ or the mutants H133A and H133D were stained with the Mikβ1 (dashed line) and BB10 (anti-hIL-2Rα; J.Wijdenes, unpublished; dotted line) monoclonal antibodies and analyzed by flow cytometry (bottom panel) and cold hIL-2 competition assay. Under high affinity conditions (50 pM [^{125}I]hIL-2), specific IL-2 binding could only be detected with the wild-type transfectant (□) but not with the clones expressing the IL-2Rβ mutants H133A (○) or H133D (△).

the transformants expressed hIL-2Rβ, but not mIL-2Rα (Figure 2A, bottom panel). The ability of hIL-2 to stimulate the growth of these clones was then studied, and the BAF3 clones with His133 mutant receptors were found to be dramatically less responsive to hIL-2 (~1000-fold; Figure 2A, top panel). Since it was difficult to perform receptor binding assays with these cells, we transfected expression vectors encoding wild-type hIL-2Rβ or the H133A and H133D mutant receptors into human Jurkat T cells. While high affinity binding could be detected on the Jurkat clone expressing the wild-type receptor, no specific binding could be detected on the clones expressing the mutant IL-2Rβ receptors (Figure 2B, top panel). FACScan analysis showed that the wild-type IL-2Rβ clone also expressed hIL-2Rα (Figure 2B, bottom panel). However, neither the parental Jurkat cells (not shown) nor the clones expressing hIL-2Rβ H133A or H133D receptors expressed hIL-2Rα. The introduction of wild-type IL-2Rβ in these Jurkat cells therefore somehow induced the synthesis of IL-2Rα. Since the Jurkat derivatives we used (ATCC CRL 8163) produce IL-2, it is probable that in the wild-type receptor clone IL-2Rα was induced via an autocrine loop. Together, the data presented in Figure 2 using BAF3 and the Jurkat clones show that mutation of His133 has a dramatic effect on IL-2 binding even in the absence of IL-2Rα, and strongly suggest that the mutations of the SHY sequence affect the interaction of IL-2Rβ with IL-2. Finally, we cross-linked [^{125}I]hIL-2 to Cos7 cells transfected with expression vectors for IL-2Rα, IL-2Rα and IL-2Rβ, or IL-2Rα and IL-2Rβ H133A. A major labelled protein of ~70 kDa representing the IL-2−IL-2Rα complex was detected in all the transfectants (Figure 3). To ensure maximal cell surface expression the IL-2Rβ

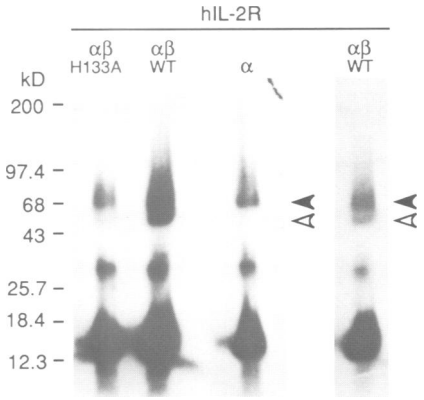

Fig. 3. Chemical cross-linking of [^{125}I]hIL-2 to wild type or mutant IL-2Rs expressed on the surface of Cos7 cells. Cos7 cells transfected with IL-2Rα alone, or IL-2Rα and truncated versions of either wild type or the H133A IL-2Rβ mutant were incubated with 10 nM [^{125}I]IL-2 at 4°C for 2 h and then cross-linked. The cell lysates were analyzed by SDS−PAGE. Complexes of [^{125}I]IL-2 and IL-2Rα (filled arrow) and [^{125}I]IL-2 and truncated IL-2Rβ (open arrow) are indicated. The band of approximate molecular weight 30 kDa is not specific since it could not be competed away by the addition of a 200-fold excess of unlabelled hIL-2 (not shown). The band migrating at ~15 kDa is [^{125}I]IL-2. The right panel shows a shorter exposure of the middle lane in the left panel.

constructs used in the Cos7 cells experiments had a deletion of their cytoplasmic domain (see Materials and methods). The resulting molecules were 28 kDa smaller than native IL-2Rβ and IL-2−IL-2Rβ complexes migrated more quickly (with an apparent size of 60 kDa) than IL-2−IL-2Rα complexes on PAGE. Figure 3 shows that the H133A

mutation primarily affected the formation of the IL-2–IL-2Rβ complex.

Mutation of the SHY sequence changes the specificity of IL-2Rβ

IL-2 structure–function studies have shown that mutation of a critical Asp residue in the N-terminal α-helix (position 20 in hIL-2; 34 in mIL-2) affects IL-2 binding to IL-2Rβ, but not to IL-2Rα (Collins *et al.*, 1988; Zurawski and Zurawski, 1989; Zurawski *et al.*, 1990). It has been proposed that this acidic residue contacts a specific residue on IL-2Rβ through ionic interaction (Collins *et al.*, 1988). Using transiently transfected Cos7 cells, we looked at the binding specificities of the IL-2Rβ mutant receptors for mIL-2 mutant proteins with substitutions of Asp34 (Figure 4; Table I). We found that mutant IL-2Rβ with substitutions of Ser132, His133 and Tyr134 had lost their preference for an acidic side chain at position 34 in the ligand. This could in some cases (i.e. Y134A) be explained by the fact that the residual binding measured was due to IL-2Rα. However, some mutant IL-2Rβs (S132V, H133A, H133D and H133K) bound the mutant mIL-2 proteins with a higher affinity than they bound mIL-2 (Figure 4). For instance, combination in a receptor binding assay of the mutations S132V or H133A in IL-2Rβ with the mutation D34H in the ligand resulted in a binding affinity similar to that measured with the wild-type receptor and ligand (Table I). These data indicate a direct interaction between the critical Asp34 in the ligand and residues at positions 132–134 in the receptor. Although we examined combinations of a spectrum of different mutations in the ligand and in the receptor, the nature of the chemical complementarity between the ligand and the receptor remains unclear (Table I).

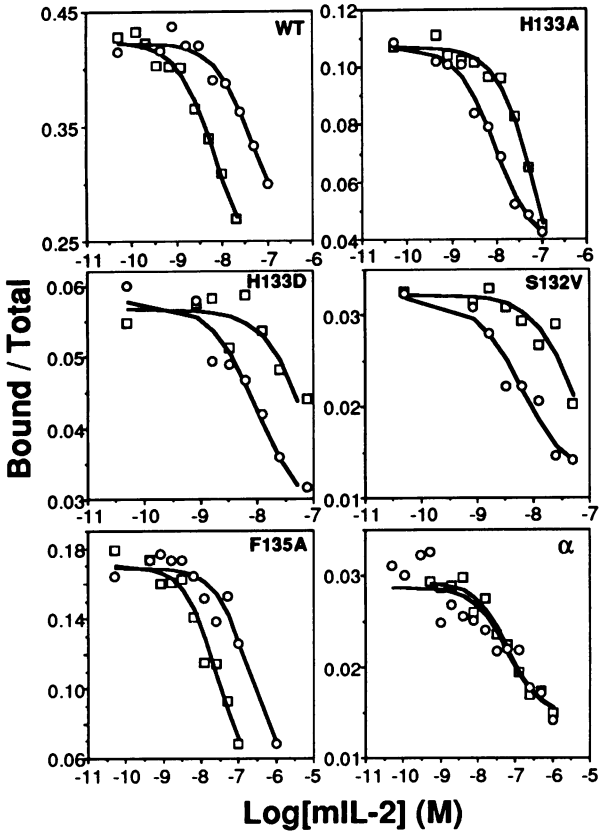

Fig. 4. Mutations of Ser132 and His133 in hIL-2Rβ affect the specificity of the interaction with IL-2. Cos7 cells were transfected with hIL-2Rα either alone (bottom right panel) or together with hIL-2Rβ wild type (WT) or hIL-2Rβ containing the mutations H133A, H133D, S132V or F135A. Receptor binding assays were performed 3 days later, and the ability of increasing concentrations of mIL-2 (□) or the mIL-2 mutant protein D34H (○) to compete for the binding of 50 pM [^{125}I]hIL-2 was compared.

Mutations in the extracellular domain of hIL-2Rβ affect the signalling ability of some mIL-2 mutants

In order to study the effects of the IL-2Rβ mutations on signalling, BAF3 clones expressing mIL-2Rα and wild-type or mutant hIL-2Rβ were established. Since activity of IL-2 on BAF3 clones expressing mutant IL-2Rβ receptors was low (Figure 2A), we established clones expressing mIL-2Rα to increase the affinity of the receptors. This allowed us to study the maximal response of the clones to saturating concentrations of IL-2 proteins. We tested by proliferation assays the signalling ability of members of the three classes of IL-2 mutants that have previously been identified (Zurawski *et al.*, 1990; see Introduction). Y59A and F56V, mutant proteins which bind IL-2Rα with reduced affinities (Zurawski *et al.*, in preparation) showed no defect in stimulating maximal proliferative responses of the BAF3 clones expressing wild-type or mutant receptors (Figure 5). Furthermore, mutations of IL-2Rβ did not grossly affect the difference between mIL-2 and mIL-2 F56V in their affinities for the receptor (Table II). The D34G, D34H and D34K IL-2 proteins fully stimulated BAF3 clones bearing the wild type receptor, but showed lower maximal responses on the clones bearing the mutant receptors (Figure 5). When the receptor binding properties of one of these IL-2 mutants, D34H, were examined, it was found that it bound receptors with H133A and H133D IL-2Rβ with a slightly lower affinity than mIL-2 (Table II). This is in contrast to the results obtained using transiently transfected Cos7 cells (Figure 4) and could be due to interaction of hIL-2Rβ with cell-type specific components or to the presence of endogenous mIL-2Rβ in the BAF3 clones. We could indeed detect a significant amount (3- to 5-fold less in HT2 cells) of mIL-2Rβ mRNA in our clones (data not shown). The likelihood of these cells expressing functional mIL-2Rβ is further supported by the observation that transfection of BAF3 cells with IL-2Rα cDNA is sufficient to make them IL-2 responsive (Collins *et al.*, 1990). Finally, the mIL-2 mutant proteins Q141K, Q141V and Q141D, which bind normally to IL-2Rαβ expressed on fibroblasts but have a signalling defect and bind with reduced affinity to IL-2Rβ and IL-2Rαβ expressed on lymphoid cells (Zurawski *et al.*, 1990), were also affected by the IL-2Rβ mutations; Q141K and Q141V have a potency of ~30% on BAF3 cells bearing wild-type receptors, but were inactive on cells bearing the mutant IL-2Rβ receptors (Figure 5). Table II shows that a mutant mIL-2 protein with a substitution at position 141 bound the wild-type receptor with an affinity similar to mIL-2, but had a strong binding defect to receptors with mutant IL-2Rβ.

Discussion

We have identified in hIL-2Rβ a sequence of three amino acids that is important for interaction with IL-2. The fact that hIL-2Rβ is a member of the cytokine receptor family

played an important role in this study. The monoclonal antibody AIC2, raised against IL-3 responsive cells, allowed the expression cloning of two homologous cDNAs, AIC2A and AIC2B, which belong to the cytokine receptor family (Itoh *et al.*, 1990; Gorman *et al.*, 1990). Despite their high percentage of identity at the amino acid level (91%), only AIC2A can bind IL-3. This observation permitted Wang *et al.* (1992) to identify residues of AIC2A likely to interact with IL-3. Mutations at Tyr340 and Asp422 were shown to reduce the affinity of AIC2A for IL-3. More strikingly, mutation of the sequence IPKY (residues 367–370) in the corresponding MAYSF sequence in AIC2B completely abolished IL-3 binding. On the double barrel model of the cytokine receptors proposed by Bazan (1990), this critical sequence motif is located in the loop between the B′ and C′ β-strands (Figure 6). In this study, we mutagenized the analogous residues in hIL-2Rβ. Our results are surprisingly analogous to those obtained with AIC2A, and show that Ser132, His133 and Tyr134 are required for IL-2 binding. A possible explanation of the phenotypes of the mutant proteins is that they have altered folding. A number of observations argue against this possibility. First, the mutant receptors are expressed at levels comparable to the wild-type receptor, whereas poor expression can be correlated with an unstable or misfolded protein (see Bass *et al.*, 1991; Miyazaki *et al.*, 1991; Rosakis-Adcock and Kelly, 1991; Wang *et al.*, 1992). Secondly, four monoclonal antibodies were found to bind the IL-2Rβ mutant M1 in a fashion similar to the wild-type receptor, indicating that their binding sites were not affected by this severe mutation (Figure 1 and data not shown). Thirdly, some IL-2Rβ mutants, although affected in their interaction with mIL-2, bind mIL-2 mutant

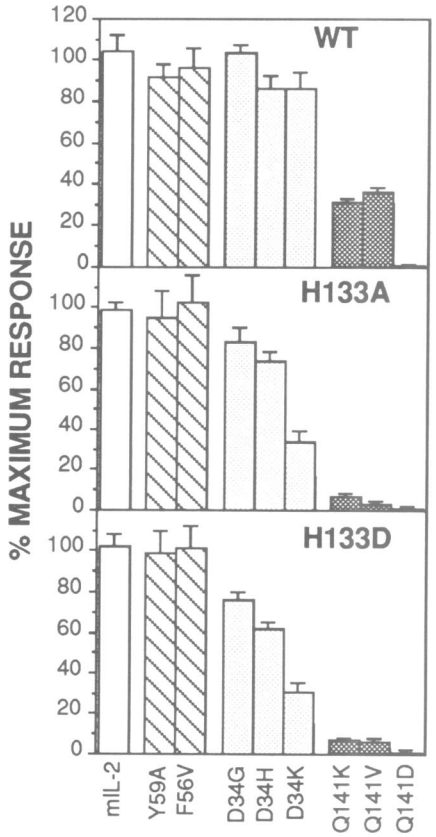

Fig. 5. Mutation of His133 in hIL-2Rβ affects the signalling ability of certain mIL-2 mutants. BAF3 transfectants expressing mIL-2Rα and hIL-2Rβ wild type, or mIL-2Rα and the hIL-2Rβ mutants H133A and H133D were established and characterized by FACScan analysis. Representative clones were exposed to saturating levels of mIL-2 wild type and mutant proteins for 24 h and analyzed by the MTT colorimetric assay to determine their maximal proliferative responses. Each experiment was in quadruplicate and the standard error is shown. The Y59A and F56V proteins (hatched bars) are specifically defective in interaction with IL-2Rα (Zurawski *et al.*, in preparation). The mIL-2 D34G, H and K proteins (lightly shaded bars) are specifically defective in interaction with IL-2Rβ (Zurawski and Zurawski, 1989; Zurawski *et al.*, 1990). The mIL-2 Q141K, V and D proteins (darkly shaded bars) bind with normal affinity to IL-2Rβ when it is expressed on fibroblasts but not when it is expressed on lymphoid cells (Zurawski *et al.*, 1990; Imler and Zurawski, 1992).

Table II. Dissociation constants for binding of members of the three classes of mIL-2 mutant proteins to receptors containing mutant hIL-2Rβ

IL-2Rβ:	K_d[a] (× 10^{-10} M)			
	mIL-2	F56V	D34H	Q141V
wild type	3	360	300	4
H133A	11	390	36	87
H133D	21	1176	33	231

[a]Stable BAF3 clones expressing mIL-2Rα and the different versions of hIL-2Rβ were established. Receptor binding assays were performed as described in Materials and methods, in the presence of 10 pM [^{125}I]hIL-2. The number of high affinity binding sites estimated by the LIGAND program were 3170 (wild type); 5120 (H133A) and 8960 (H133D). FACS analysis showed that the level of mIL-2Rα expressed was similar within 10% for the three clones.

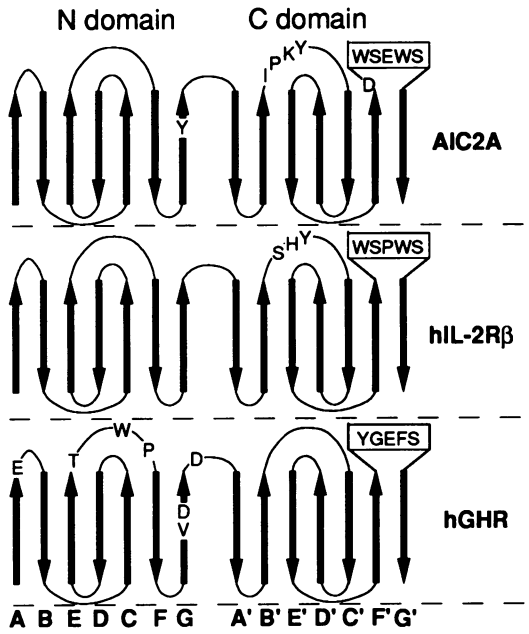

Fig. 6. Location of residues important for ligand binding in three members of the cytokine receptor family upon the β-stranded structure proposed by Bazan (1990). This model proposes two linked immunoglobulin-like domains, N and C, which form barrel structures and pack with their axes at an angle to create a V-shaped crevice containing the putative ligand binding site (Bazan, 1990). AIC2A has two repeats of this structure and only the second, membrane-proximal, repeat is shown. The critical WSXWS box present in most of the cytokine receptors, and the corresponding YGEFS sequence in the growth hormone receptor in the F′–G′ loop is boxed. Mutation of only residues Trp104 and Pro106 (W and P in the E–F loop) of hGHR caused a >10-fold reduction in binding to GH (Bass *et al.*, 1991).

proteins with substitutions at Asp34 with high affinity (Table I). Fourthly, the other function of the receptor, signalling, was not affected when wild-type IL-2 was studied (Figures 2 and 5), whereas mutations disrupting the structure of the molecule might be expected to prevent the receptor from altering its conformation to signal (see Fukunaga *et al.*, 1991; Miyazaki *et al.*, 1991). Interestingly, however, the mIL-2 mutants with substitutions of Asp34 could not signal as efficiently as mIL-2 on cells with mutant IL-2Rβ, although they bound the mutant receptor better than the wild-type receptor. This extends the findings obtained with the Gln141 substitution mIL-2 proteins (Zurawski *et al.*, 1990; Imler and Zurawski, 1992), namely that receptor binding and receptor activation can be dissociated in the IL-2 system.

A short sequence critical for ligand binding is therefore located at a similar position in hIL-2Rβ and AIC2A, a component of the IL-3 receptor. It will be interesting to see if other members of the cytokine receptor family (Bazan, 1990; Cosman *et al.*, 1990; Patthy, 1990) have ligand binding sequences at analogous positions. While this work was in progress, an extensive study of another member of the cytokine receptor family, the growth hormone receptor (GHR), was published (Bass *et al.*, 1991). Several important residues for growth hormone (GH) binding were identified in the proposed N domain of the cytokine receptor structure proposed by Bazan (1990) (Figure 6). However, none of the mutations tested in the proposed C domain, including some in the B′−C′ loop, decreased the affinity of the receptor for GH. Although GHR, IL-2Rβ and AIC2A belong to the same family of receptors, it appears that their hormone binding determinants are not similarly located. It is, however, noteworthy that the two most critical residues of GHR (resulting in >10-fold reduction in affinity for GH), Trp104 and Pro106, are in the E−F loop of the cytokine receptor model (Figure 6). In this model, the E−F and B′−C′ loops are mirror images and form both sides of a V shaped 'cytokine cradle' (Bazan, 1990). It may be significant that, unlike hIL-2Rβ and AIC2A, GHR does not have a consensus WSXWS motif, which in hIL-2Rβ has also been shown to be important for ligand binding (Miyazaki *et al.*, 1991).

A critical aspect of structure−function studies is the distinction between indirect structural effects, i.e. disrupting the conformation of the molecule, and direct functional effects, i.e. disrupting an active site in the molecule. In the absence of a defined structure of the ligand−receptor complex, positive identification of interacting residues can only be made based on information gathered on both molecules involved. In such experiments, it would be expected that the effect of a mutation in the ligand and the effect of a mutation in the receptor would be additive, except in the case where the two mutations affect areas that interact directly. Such an approach has been used to identify two serine residues of the β-adrenergic receptor involved in interactions with hydroxyl groups from the aromatic ring of catechol agonists (Strader *et al.*, 1989) and also to determine the residues involved in the electrostatic interactions determining the specificity of the cAMP-dependent protein kinase (Gibbs and Zoller, 1991). In the case of IL-2, extensive structure−function studies have been performed. A number of residues have been identified as critical for IL-2Rα binding (Sauvé *et al.*, 1991; Zurawski and Zurawski, 1989; Zurawski *et al.*, in preparation), but only one residue, Asp20 in human and Asp34 in mouse IL-2, has been identified as critical for IL-2Rβ binding (Collins *et al.*, 1988; Zurawski *et al.*, 1990). This suggests a more restricted interaction between IL-2Rβ and IL-2 than between IL-2Rα and IL-2. Our data, using both transiently transfected Cos7 cells and stable cell lines show that mutation of His133 in hIL-2Rβ abolishes the strict requirement for an acidic side chain at position 34 in mIL-2. Moreover, on transiently transfected Cos7 cells, hIL-2Rβ mutants with substitutions at Ser132 or His133 bound mutant mIL-2 molecules with substitutions at position 34 with a higher affinity than they bound mIL-2. These data strongly suggest that the SHY sequence on the hIL-2R receptor interacts directly with the Asp residue in the N-terminal α-helix of IL-2. More precise measurements of the interaction between the mutant IL-2Rβ receptors and mutant IL-2 molecules with substitutions at position 34, using stable transformants or ideally soluble receptors in a cell-free assay, could help define the chemical nature of the interaction between IL-2 and IL-2Rβ in the absence of a crystal structure of the complex.

Structure−function studies of mIL-2 have also shown that replacement of Gln141 in the C-terminal α-helix results in partial agonists which interact normally with IL-2Rαβ on fibroblasts, but which have a binding defect for the receptor expressed on lymphocytes (Zurawski *et al.*, 1990; Imler and Zurawski, 1992). This suggested that these mutants were deficient in interaction with a third, uncharacterized 'γ' component of IL-2R (Saragovi and Malek, 1990; Sharon *et al.*, 1990; Saito *et al.*, 1991). We show here that the Q141V mIL-2 protein, although it binds wild-type hIL-2Rβ at an affinity similar to mIL-2, has a strong binding defect to hIL-2Rβ with substitutions at His133 (Table II). This is accompanied by an increased signalling defect (Figure 5). Preliminary experiments show that a binding defect is also observed on the same mutant receptors transiently expressed on Cos7 cells (data not shown), and therefore the binding defect on BAF3 cells cannot be explained by the presence of molecules associating with IL-2Rβ. This result suggests that the action of the Gln141 residue occurs via IL-2Rβ. This action may be via a second ligand binding site that is revealed by disruption of the SHY sequence and that is subject to modulation by cell-type specific receptor components. Miyazaki *et al.* (1991) indeed identified another sequence in hIL-2Rβ at positions 194−198 (WSPWS, Figures 1 and 6), which is important for IL-2 binding. Alternatively, local structural changes might be associated with both the substitutions at position 141 in mIL-2 and at position 133 in hIL-2Rβ, which, when combined, result in the much reduced affinity between these two molecules. More experiments are required to clarify the molecular basis of the phenotype of the Gln141 mutant proteins. The kind of approach described here could provide some useful insights; however, it is clear that the action of both the Asp34 and Gln141 residues on IL-2Rβ can be influenced by the presence of uncharacterized cell-type specific receptor components.

Materials and methods

Generation of hIL-2Rβ mutants

Since definition of the level of expression of the mutant receptors was critical for this study, we chose to work with human IL-2Rβ, for which a number of monoclonal antibodies have been obtained. A truncated version of hIL-2Rβ cDNA (from nucleotide 1 to the *Nco*I restriction site at position 946, corresponding to amino acids 1−246) was subcloned into M13, and oligonucleotide-mediated mutagenesis was performed on uracil-containing

single-stranded DNA (Kunkel *et al.*, 1987). The mutated cDNAs were then sequenced and cloned into the SRα promoter-based expression vector pME18S (K.Maruyama, unpublished). Full length versions of the mutants were reconstructed in pME18S utilizing the unique *Bcl*I (position 923) and the *Bam*HI (position 2054) restriction sites of the hIL-2Rβ cDNA.

Cell transfections and antibody binding analysis

Cos7 monkey fibroblasts were transfected with 5 μg of expression vector pCD (Okayama and Berg, 1983) containing the cDNA of hIL-2Rα, and 15 μg of pME18S encoding wild-type or mutant hIL-2Rβ by electroporation at 960 μF and 230 V. These conditions limited the amount of low affinity IL-2Rα at the surface of the transfected cells and favored the formation of higher affinity receptors (not shown). Each mutant receptor was transfected at least twice. For the stable transfectants, the neomycin resistance gene under the control of the SV40 promoter (Wang *et al.*, 1992) was added to the expression vectors. 2×10^7 cells mixed with 20 μg of plasmid DNA were electroporated at 960 μF and 400 or 250 V for mouse BAF3 or human Jurkat cells, respectively. Transfectants were selected with neomycin G418 (1 mg/ml) and analyzed by flow cytometry.

For flow cytometric analysis of expression, the cells were incubated for 30 min at 4°C with anti-receptor antibodies in phosphate-buffered saline (PBS), washed twice and incubated for another 30 min with fluorescein isothiocyanate-conjugated antibodies. The stained cells were then analyzed on a FACScan (Becton Dickinson). For the transiently transfected Cos7 cells, in order to take into account the variation of transfection efficiency in the comparison of expression levels between wild-type and mutant IL-2Rβ, a normalization to the level of IL-2Rα expressed was done: the percentage of cells positive for IL-2Rβ was divided by the percentage of cells positive for IL-2Rα and multiplied by the percentage of cells positive for IL-2Rα in the wild-type IL-2Rβ transformants. 5–10% of the transfected cells generally expressed the receptor, and variation between transfectants for IL-2Rα did not exceed 30%.

Proliferation and receptor binding assays

Cell proliferation was measured by MTT colorimetric assays as described (Zurawski and Zurawski, 1989). IL-2 binding was determined at 4°C by cold ligand competition assay (Zurawski *et al.*, 1990) over a period of 3 h and the dissociation constants were calculated using the LIGAND program (Munson, 1983). The concentration of [^{125}I]hIL-2 used was 50 pM for the transiently transfected Cos7 cells and 10 pM for the BAF3 clones. Each experiment was performed in triplicate. For the transiently transfected Cos7 cells, when the standard error was >50% of the K_d value, the experiment was repeated at least once. For the stable BAF3 clones, the standard error was <25% of the K_d value.

Chemical cross-linking

Chemical cross-linking was performed as described (Kitamura *et al.*, 1991). Briefly, cells incubated with 10 nM [^{125}I]hIL-2 at 4°C for 2 h were collected by centrifugation and resuspended in PBS containing 200 μM disuccinimidyl suberate (Pierce). The cells were then solubilized in 1% Triton X-100 and analyzed on an SDS/4–20% gradient polyacrylamide gel (Daiichi Chemicals) followed by autoradiography.

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