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Two distinct functional sites of human interleukin 4 are identified by variants impaired in either receptor binding or receptor activation*

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Interleukin 4 (IL-4) exerts a decisive role in the coordination of protective immune responses against parasites, particularly helminths. A disregulation of IL-4 function is possibly involved in the genesis of allergic disease states. The search for important amino acid residues in human IL-4 by mutational analysis of charged invariant amino acid positions identified two distinct functional sites in the 4-helix-bundle protein. Site 1 was marked by amino acid substitutions of the glutamic acid at position 9 in helix A and arginine at position 88 in helix C. Exchanges at both positions led to IL-4 variants deficient in binding to the extracellular domain of the IL-4 receptor (IL-4R_{ex}). In parallel, up to 1000-fold increased concentrations of this type of variant were required to induce T-cell proliferation and B-cell CD23 expression. Site 2 was marked by amino acid exchanges in helix D at positions 121, 124 and 125 (arginine, tyrosine and serine respectively in the wild-type). IL-4 variants affected at site 2 exhibited partial agonist activity during T-cell proliferation; however, they still bound with high affinity to IL-4R_{ex}. [The generation of an IL-4 antagonist by replacing tyrosine 124 with aspartic acid has been described before by Kruse *et al.* (1992) (*EMBO J.*, 11, 3237-3244)]. These findings indicate that IL-4 functions by binding IL-4R_{ex} via site 1 which is constituted by residues on helices A and C. They further suggest that the association of a second, still undefined receptor protein with site 2 in helix D activates the receptor system and generates a transmembrane signal.

Key words: drug design/partial agonists/receptor signalling

Introduction

Interleukin 4 (IL-4) represents a typical cytokine which triggers and coordinates the immune response against certain parasites by causing the growth and differentiation of a variety of cells (for review see Paul, 1991; Romagnani, 1992; Boulay and Paul, 1992; Scott, 1993). Mast cells, a T-cell subset and thymocytes can synthesize IL-4. However, the primary cellular source of IL-4 after helminth infection is unknown. In cell cultures the responses to IL-4 are manifold; they include notably the induction of IgE (e.g. Romagnani, 1991), MHCII (e.g. Noelle *et al.*, 1984) and CD23 (e.g. Kikutani, 1986) in B-cells, the induction of V-CAM in capillary endothelial cells (Schleimer *et al.*, 1992) or the expression of 15-lipoxygenase in monocytes (Conrad *et al.*, 1992). In addition, IL-4 promotes DNA synthesis and short-term proliferation of T-cells (Hu-Li *et al.*, 1987), thymocytes (Bárcena *et al.*, 1991), fibroblasts (Monroe *et al.*, 1988) and capillary endothelial cells (Toi *et al.*, 1991). In whole animals the loss of the IL-4 gene (Kühn *et al.*, 1991; Kopf *et al.*, 1993) or the neutralization of IL-4 activity (Urban *et al.*, 1991) abolishes the generation of type 2 helper T-cells (T_H2-cells) and the synthesis of IgE. A pathophysiological role of IL-4 is indicated in transgenic mice where the overexpression of IL-4 leads to allergic-like inflammatory disease states (Tepper *et al.*, 1990). Transfected tumour cells expressing IL-4 are rejected *in vivo* by recruited host granulocytes and macrophages (Tepper *et al.*, 1989). For these reasons IL-4 itself as well as IL-4 antagonists may be suitable for a number of therapeutic applications.

IL-4 receptors have been identified on a variety of cell types and cell lines (for review see Beckmann *et al.*, 1992). Typically, IL-4 binding sites occur with a frequency of 300-3000 per cell and exhibit an apparently homogeneous dissociation constant, K_D, of 50-100 pM. Different cells and cellular responses, however, show large differences in sensitivity to IL-4. Concentrations effecting half-maximal responses (EC_{50} values) were reported to range from 0.5 to 200 pM (e.g. Garrone *et al.*, 1991; Rigley *et al.*, 1991; Kruse *et al.*, 1992). The IL-4 activities are mediated by a receptor system for which up to now only a single protein has been identified. In human cells, this IL-4 binding protein consists of an extracellular domain of 207 amino acid residues, a 24 residue transmembrane segment and an unusually large intracellular domain of 569 amino acid residues (Galizzi *et al.*, 1990; Idzerda *et al.*, 1990). The extracellular domain alone binds IL-4 with a K_D of ~100 pM. As a member of the haematopoietin receptor family (Bazan, 1990) it is composed of two fibronectin type III modules (Bork and Doolittle, 1992). In responsive cell lines and activated T-cells IL-4 induces a specific pattern of protein tyrosine phosphorylation which differs from that produced by IL-3 (Wang *et al.*, 1992) or IL-2 (Izuhara *et al.*, 1993). Deletion analysis indicates that a sequence of amino acids from position 97 to position 137 in the intracellular domain is required for transmission of the growth signal in the Ba/F3 cell line (Harada *et al.*, 1992).

The three-dimensional structure of human IL-4 has recently been determined in solution (Redfield *et al.*, 1991; Powers *et al.*, 1992; Smith *et al.*, 1992; T.Müller, W.Sebald and H.Oschkinat, submitted) and in crystallized form (Walter *et al.*, 1992a; Wlodawer *et al.*, 1992). A 4-helix-bundle structure with an up-up-down-down connectivity has been established. A short β-sheet occurs between two long loops connecting helices A/B and helices C/D. Replacement of cysteines in human IL-4 by threonine residues revealed that the disulfide bridge formed by cysteines at positions 46 and

Table I. Variants of human IL-4 and their functional activities

IL-4 variant	EC_{50} value (nM)	R_{max} (%)	Relative IC_{50}
IL-4	0.14	100	1
H1Q	0.12	97	0.9
D4N	0.13	96	0.4
E9Q	3.1	98	170
E9K	320	101	1400
E26Q	0.09	95	0.9
K37Q	0.07	98	0.7
E41Q	0.19	98	0.7
E43Q	0.07	105	0.7
R47Q	0.17	98	1.0
R53Q	0.13	98	8.0
H59Q	0.76	98	1.9
K61Q	0.1	99	1.5
R85Q	0.07	98	1.5
R88Q	2.5	90	210
R88D	400	>69	2900
E103Q	0.35	104	0.8
E114Q	0.09	96	0.7
K117Q	0.07	99	0.7
M120D	12		63
R121D	0.13	31	1.3
K123D	0.24	96	1.5
S125D	0.14	30	1.4
C127D	1.2	100	5.3
S128D	0.14	95	1.0
Y124D		<0.5	3.0

The number in the designation of the variants indicates the position altered by *in vitro* mutagenesis. The amino acid originally present in IL-4 is indicated by the first letter and the substituted amino acid by the second letter, both in the one-letter code. The locations of the altered positions are indicated in Figure 6. Concentrations of the IL-4 proteins effecting half-maximal proliferation of T-cells (EC_{50} values) and the maximal response obtained at saturation levels in relation to normal IL-4 (R_{max} values) indicate the functional activities of the variants. The receptor affinities of the variants as measured during competitive radioligand binding to Raji cells are listed under relative IC_{50}.

99 is essential for the folding of functional IL-4, whereas the other two disulfide bridges formed by cysteines 24/65 and 3/127 are less important (Kruse *et al.*, 1991). Antagonist or partial agonist variants of human IL-4 were obtained by replacing tyrosine 124 with aspartic acid, glycine, asparagine or lysine (Kruse *et al.*, 1992). Receptor-binding residues had not yet been experimentally identified in human IL-4. Mouse IL-4 variants deficient in receptor binding and/or activation have been reported recently (Morrison and Leder, 1992).

The present results were obtained after a systematic search for functionally important amino acid residues in human IL-4. Remarkably, the mutational analysis produced two types of variants impaired in either receptor binding or receptor activation. The location and functional role of the important amino acid residues identified in human IL-4 suggest similarities with other 'small' cytokines of the 4-helix-bundle protein family, such as granulocyte macrophage colony stimulating factor (GM-CSF) (Diederichs *et al.*, 1991; Walter *et al.*, 1992b) or IL-2 (Bazan, 1992; McKay, 1992; Mott *et al.*, 1992). Interestingly, in contrast to the human growth hormone (hGH), which represents the prototype of a 'large' 4-helix-bundle protein, the antagonism-determining site of human IL-4 is located on helix D and not, as in hGH, on the site formed by helices A and C (DeVos *et al.*, 1992; Fuh *et al.* 1992).

Results

Construction and isolation of IL-4 variants

The IL-4 variants constructed and analysed during the present study are compiled in Table I. The positions to be modified were selected on the basis of the following considerations.

Acidic and basic amino acid side chains are usually exposed at the surface of a protein. Consequently, some of the 14 acidic and 27 basic residues present in human IL-4 might be expected to be involved in receptor binding. A subset of 16 of these charged amino acid residues is invariant or highly conserved in the amino acid sequences of the IL-4 from man, mouse, rat, cow and pig (McKnight *et al.*, 1991; Bailey *et al.*, 1993). They appear, therefore, to be of special and general importance despite the species-specificity of IL-4. Only these evolutionarily conserved positions were selected for mutagenesis. The charged residues were replaced by glutamine and in one case by asparagine.

Another set of variants was generated by replacing residues near tyrosine 124 with aspartic acid. In previous experiments replacements of this residue by other amino acid residues and especially by aspartic acid produced partial agonistic or antagonistic variants (Kruse *et al.*, 1992). Side chains of residues at positions 120, 121, 123, 125, 127 and 128 would be juxtaposed to tyrosine 124 on the surface of a helix. It was therefore investigated whether an aspartyl residue at these positions might generate partial agonism of the protein.

The variants shown in Table I could be expressed in *Escherichia coli* in large amounts as inclusion bodies. They were subjected to a renaturation protocol and highly purified by HPLC (see Kruse *et al.*, 1991). The final yields were similar (0.5–2.0 mg per litre of culture) to that obtained with IL-4. Exceptions were variants M120D and C127D which refolded poorly and could be recovered only in low amounts. Variant M120D still contained major contaminants after the final HPLC purification step.

Identification of IL-4 variants with altered properties during T-cell proliferation

Most of the IL-4 variants in the first group induced the proliferation of activated T-cells similarly to IL-4 with a half-maximal response at a concentration of 70–280 pM (see Table I). Four variants, however, clearly exhibited impaired activity (Figure 1A). Increased concentrations of variants E9Q and R88Q were necessary to stimulate T-cell proliferation. At sufficiently high doses the maximal response obtained was equal to that produced by IL-4. A more drastic change at these positions in variants E9K and R88D increased the EC_{50} >1000-fold. Nevertheless, at saturating concentrations of variant E9K the maximal IL-4 response was produced. Accordingly, these variants modified at positions 9 or 88 are pure 'EC_{50} variants'. Repeatedly, the activity of variant R88D decreased at concentrations above 1 μM. Therefore, the maximal response could not be established in the T-cell system. Such a decrease in activity was observed also with IL-4 and the other EC_{50} variants, although only at concentrations above 10 μM (not shown). It has not yet been investigated whether this represents the inhibitory activity of a contaminant or an self-antagonistic activity of the IL-4 proteins. The latter has been observed with hGH (Fuh *et al.*, 1992).

Among the second group of IL-4 mutant proteins with substitutions near position 124, two 'R_{max} variants' were found exhibiting partial agonist activity (Figure 1B). Variants

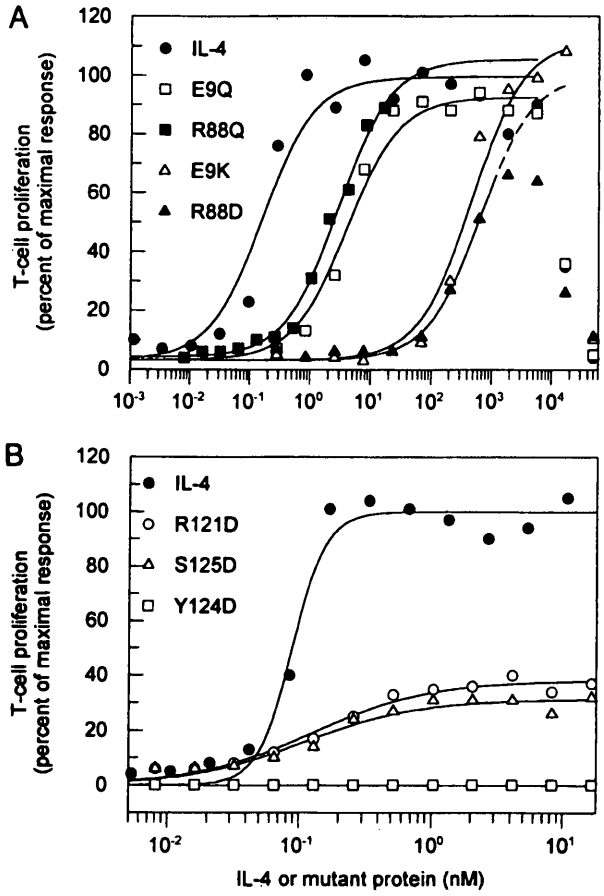

Fig. 1. Proliferation of activated T-cells (PHA blasts) in response to increasing doses of IL-4 (●) and (A) EC_{50} variants E9Q (□), R88Q (■), E9K (△) and R88D (▲), or (B) R_{max} variants R121D (○), Y124D (□) and S125D (△).

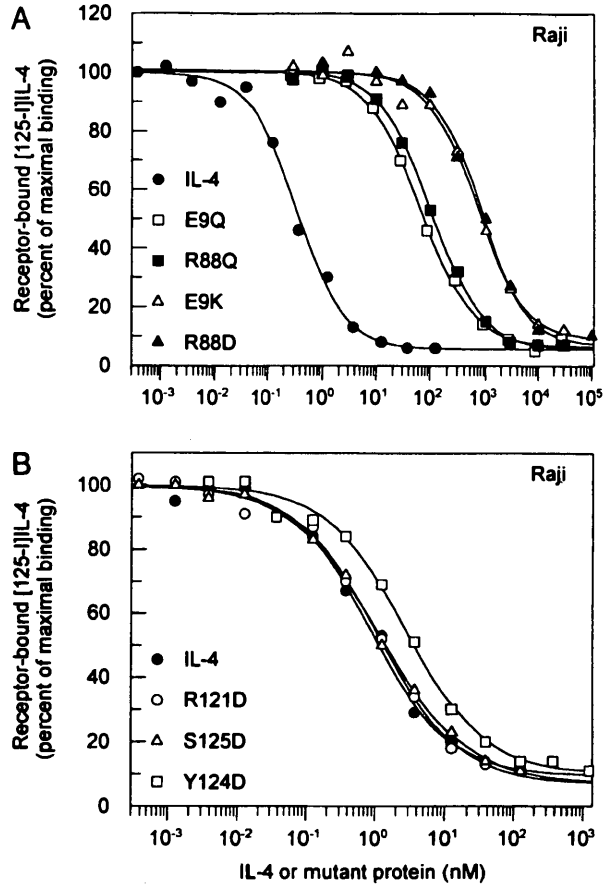

Fig. 2. Competition for binding to the IL-4 receptor of Raji cells between radiolabelled IL-4 and unlabelled IL-4 (●) or (A) EC_{50} variants E9Q (□), R88Q (■), E9K (△) and R88D (▲), or (B) R_{max} variants R121D (○), Y124D (□) and S125D (△).

R121D and S125D stimulated T-cell proliferation with EC_{50} values of 130 and 140 pM respectively, similar to that of IL-4; the maximal response, R_{max}, however, was only 30% of the maximal IL-4 response. These variants are therefore defective in receptor activation. In this respect R121D and S125D resemble variants Y124G, Y124N and Y124K which have been studied previously (Kruse *et al.*, 1992). The R_{max} variants R121D and S125D are also partial antagonists. IL-4-stimulated T-cell proliferation could be inhibited by these variants down to a value corresponding to their maximal partial agonist activities. The competitive inhibitor constants, K_i, were 300–600 pM, not much higher than the EC_{50} values of their partial agonist activities (data not shown). These results support the conclusion that these variants have a reduced signalling activity. Remarkably, the drastic substitutions generated in variants K123D and S128D did not measurably change the functional properties of these proteins under our experimental conditions. The variant C127D exhibited a modest increase in the EC_{50} value. The significance of this remains unclear. The same applies for the slightly reduced R_{max} value and the 100-fold increased EC_{50} value of variant M120D. Considering the poor yields after renaturation and purification, structural instability and major folding defects appear to be possible for C127D and especially for the M120D variant (see Discussion).

Receptor binding of IL-4 variants studied with the receptor of Raji cells

The affinity of the IL-4 variants for the IL-4 receptor present in the Raji lymphoma cell line was analysed by competitive radioligand binding at 4°C employing 0.2–0.5 nM [^{125}I]IL-4 as shown in Figure 2. Variants altered at positons 9 and 88 exhibited much higher K_D values than did IL-4 (see also Table I). The relative IC_{50} values (i.e. IC_{50} variant/IC_{50} IL-4) of E9Q and R88Q were ~200, those of variants E9K and R88D were ~2000. The increase in K_D of the individual variants is similar with E9K and R88D or ~10-fold larger with E9Q and R88Q than the increase in the EC_{50} values. Remarkably, the R_{max} variants R121D, S125D (see Table I and Figure 2B) and Y124D (Kruse *et al.*, 1992) bind to the IL-4 receptor of Raji cells with a K_D comparable to that of normal IL-4. The inefficient receptor activation is not reflected by an altered apparent K_D. While it is unknown whether Raji cells express a functional IL-4 receptor, previous experiments employing primary human T-cells and B-cells have also established that the highly deficient R_{max} variant Y124D binds to the receptor of these cells with a K_D of ~300 pM. This value is 3-fold larger than the K_D of IL-4, 100 pM (Kruse *et al.*, 1992), which has been determined not only in primary cells but also in a variety of IL-4-responsive cell lines (see Beckmann *et al.*, 1992).

Dose–response measurements of EC_{50} variants during a highly sensitive B-cell assay

Human IL-4 induces the expression of CD23 (FcεII) in purified B-cells at EC_{50} values of 2–10 pM (Kruse *et al.*, 1992). This B-cell response is ~50 times more sensitive than the proliferative response of T-cells (see above). The induction of CD23 on B-cells possibly requires a very low occupancy of the IL-4 receptor. It is also possible that a different receptor system exists in B-cells; this is supported by the observation that IL-4-induced expression of CD23 on human B-cells is regulated independently from that of IgM (Rigley *et al.*, 1991). It was therefore interesting to examine how far the EC_{50} variants show a similar increase in EC_{50} during the B-cell assay and during the T-cell response described above.

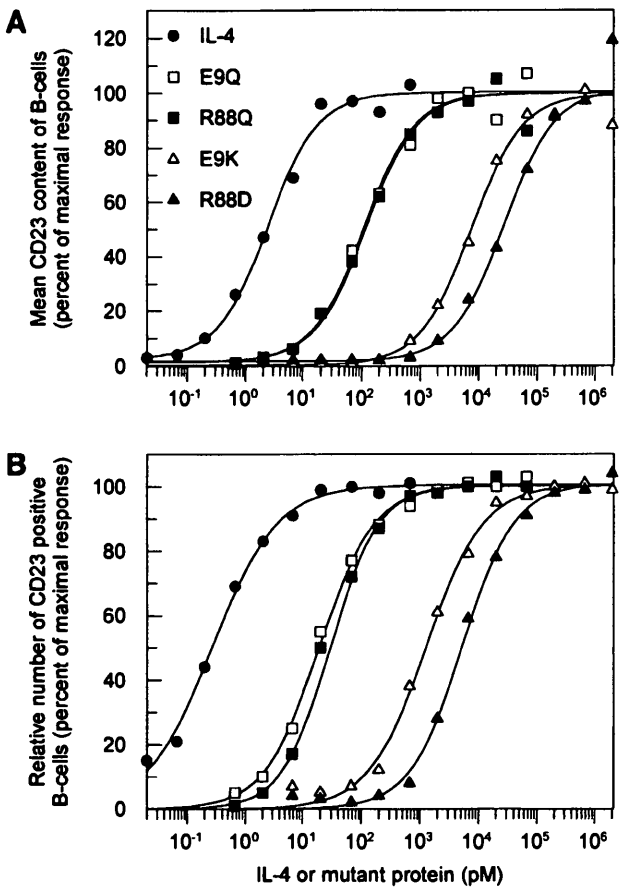

Fig. 3. Induction of the low affinity Fcε receptor (CD23) on B-cells in response to increasing doses of IL-4 (●) or variants E9Q (□), R88Q (■), E9K (△) and R88D (▲). (A) Mean CD23 content of B-cells. (B) Number of CD23 positive B-cells as percent of maximal response.

The experiment presented in Figure 3 yielded EC_{50} values of 2.7 pM for the IL-4-dependent induction of the mean CD23 content, and 0.3 pM for the induction of the percentage of CD23 positive cells, in accordance with previously described experiments (Kruse *et al.*, 1992). The variants E9Q and R88Q produced a half-maximal response only at 30–50 times higher concentrations (see also Table II). The concentrations of E9K or R88D necessary for half-maximal CD23 induction were 2000–10 000 times higher than those required for IL-4. As statistically evaluated and compiled in Table II, these relative EC_{50} values are remarkably similar to those observed during the T-cell assay, despite the fact that the absolute EC_{50} values for the responses of the two cellular systems differ by about two orders of magnitude. These observations give further support to the view that the same IL-4 receptor system operates in B- and T-cells at least as far as the ligand binding and receptor activation is concerned.

Receptor binding of IL-4 variants as studied with the isolated extracellular domain of the IL-4 receptor

The extracellular domain of the human IL-4 receptor ($IL\text{-}4R_{ex}$) comprising amino acid residues 1–207 of the mature receptor protein was expressed in CHO cells and highly purified by affinity chromatography on IL-4–Sepharose. A solid-phase assay employing streptavidin-coated wells of a microtitre plate and immobilized biotinylated $IL\text{-}4R_{ex}$ was developed in order to analyse the binding properties of IL-4 variants. Such a system is more convenient to handle than whole cells. Furthermore, the properties of immobilized $IL\text{-}4R_{ex}$ can be compared with those of the functional receptor. Analysis of purified $IL\text{-}4R_{ex}$ using chemical crosslinking and gel chromatography revealed no oligomeric forms of the protein (S.Arnold and W.Sebald, unpublished observation). Thus, $IL\text{-}4R_{ex}$ becomes immobilized probably as a monomer, and a 1:1 interaction of IL-4 protein and the receptor can be

Table II. Quantitative evaluation of altered characteristics in IL-4 EC_{50} variants and R_{max} variants

Protein	T-cell proliferation			B-cell C23 induction				Competitive radioligand binding			
	Relative EC_{50}		R_{max} (%)	Mean CD23 content		% $CD23^+$ cells		Raji cells		$IL\text{-}4R_{ex}$	
				Relative EC_{50}				Relative IC_{50}			
IL-4	1	(1.9)	107 ± 6	1	(1.6)	1	(2.5)	1	(1.3)	1	(1.3)
E9Q	22	(1.5)	98 ± 7	35	(1.3)	48	(1.7)	170	(1.9)	410	(1.3)
R88Q	18	(1.4)	90 ± 9	28	(1.6)	38	(3)	210	(2.2)	1400	(2)
E9K	2300	(1.3)	101 ± 9	2000	(1.2)	3 200	(1.7)	1400	(2.6)	1700	(2)
R88D	2900	(2.6)	>69 ± 7	7600	(1.3)	11 000	(1.9)	2900	(2)	4800	(2.2)
R121D	0.7	(1.5)	31 ± 19					1.3	(1.6)	0.6	(1.7)
Y124D			<0.5					3	(1.7)	0.7	(1.5)
S125D	0.7	(1.5)	30 ± 19					1.4	(1.5)	1.5	(1.5)

The EC_{50} and IC_{50} values are distributed log normal (see Materials and methods). The numbers in brackets are the standard deviations (in multiples of the mean values) calculated from log EC_{50} ± log SD or log IC_{50} ± log SD.

studied without interference with oligomerization and/or activation steps. This would permit the residues modified in the EC_{50} and R_{max} variants to be assigned specifically to binding functions associated with the cloned IL-4 receptor protein.

Saturation binding of [^{125}I]IL-4 at room temperature is described in Figure 4. Half-maximal binding is observed at 100 pM IL-4. Nonspecific binding determined in the presence of 500 nM unlabelled IL-4 is $<10\%$. The K_D value observed with immobilized IL-4R_{ex} is remarkably similar to the K_D values of 50–100 pM found for the binding of IL-4 to whole Raji cells, B-cells or T-cells at 4°C (e.g. Kruse *et al.*, 1992).

Competitive radioligand binding experiments revealed (Figure 5A) that relative to IL-4 the EC_{50} variants E9Q and R88Q bound to IL-4R_{ex} with a 410-fold and 1400-fold increased K_D respectively. The relative K_Ds of E9K and R88D were slightly higher. These results clearly indicate that a defective interaction with the extracellular domain of the known IL-4 receptor protein causes the increased EC_{50} values of this group of variants.

The binding properties of R_{max} variants R121D, S125D and Y124D (Figure 5B) clearly differ from those of the EC_{50} variants described above. They bind to immobilized IL-4R_{ex} with a K_D similar to that of normal IL-4. Remarkably, the inefficient receptor activation is not reflected by an altered interaction between this type of IL-4 variant and the isolated receptor protein.

A quantitative evaluation and a comparison of the altered responses of EC_{50} variants (and R_{max} variants) are presented in Table II. The highly deficient variants E9K and R88D exhibit a loss of function that is largely the same during both the biological and the binding assays. The standard deviations shown in brackets reveal no significant differences between the experimental data. The moderately deficient variants, E9Q and R88Q, however, appear to be similarly altered only during the biological T- and B-cell assays whereas the binding affinity to Raji cells or IL-4R_{ex} is clearly reduced more. This statistically significant difference remains unexplained. Similar effects have been observed before in the IL-2 system where variants deficient in binding to the α subunit of the receptor (Tac) have normal EC_{50} values during T-cell proliferation (e.g. Weigel *et al.*, 1989; Zurawski and Zurawski, 1989). Considering the different experimental set-ups during competitive radioligand binding and during the biological response measurements, a more complex effect of amino acid substitutions during the cell culture experiments appears to be possible. For example, the IL-4 system in B- and T-cells is probably not in thermodynamic equilibrium, since the receptor off-rates are lower than the internalization rates for the ligand–receptor complex (Galizzi *et al.*, 1989; Liang *et al.*, 1992). Consequently, the biological responses are largely determined by the receptor on-rate. It can be visualized that the on- and off-rates are altered to a different extent in the E9Q and R88Q variants.

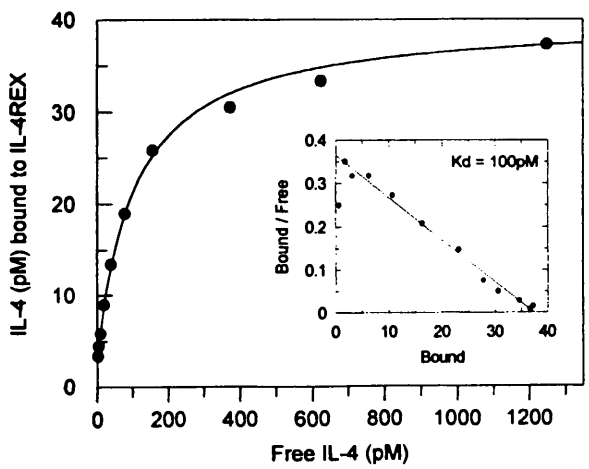

Fig. 4. Saturation binding of [^{125}I]IL-4 to the immobilized extracellular domain of the IL-4 receptor (IL-4R_{ex}). The inset provides the Scatchard transformation of the experimental data.

Discussion

The altered biological activities and the receptor binding properties of the IL-4 variants identified during the present study indicate that human IL-4 provides functionally distinct sites for interactions with receptor proteins. One of these sites determines the signalling efficacy of IL-4, since amino acid exchanges lead to the partial agonistic R_{max} variants. The other site determines the binding to IL-4R_{ex} and accordingly variants affected at this site show increased EC_{50} values. The 'signalling site' is provided by an area around the side chains of arginine 121, tyrosine 124 and serine 125 in helix D. The 'binding site' for IL-4R_{ex} is

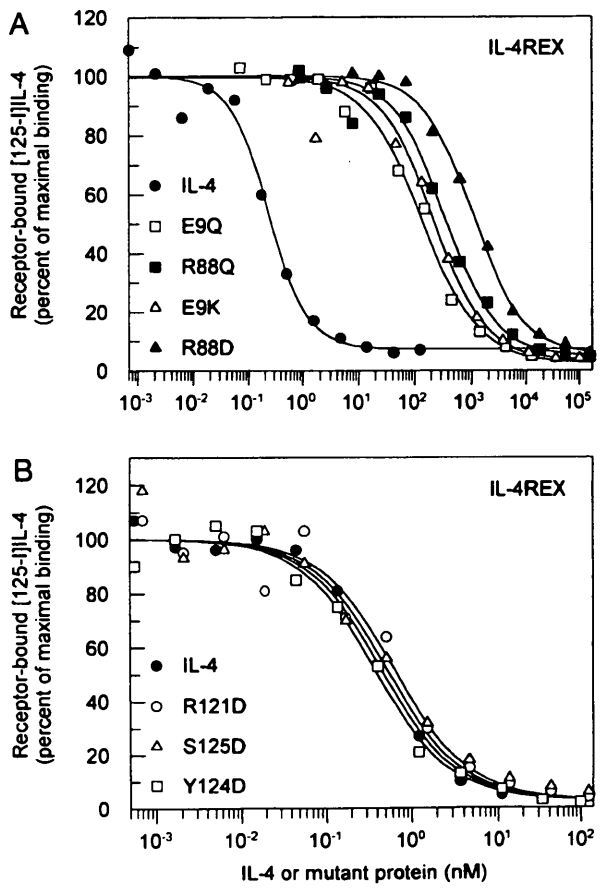

Fig. 5. Competition for binding to isolated IL-4R_{ex} between radiolabelled IL-4 and unlabelled IL-4 (●) or (A) EC_{50} variants E9Q (□), E9K (△), R88Q (■) and R88D (▲) or (B) R_{max} variants R121D (○), Y124D (□) and S125D (△).

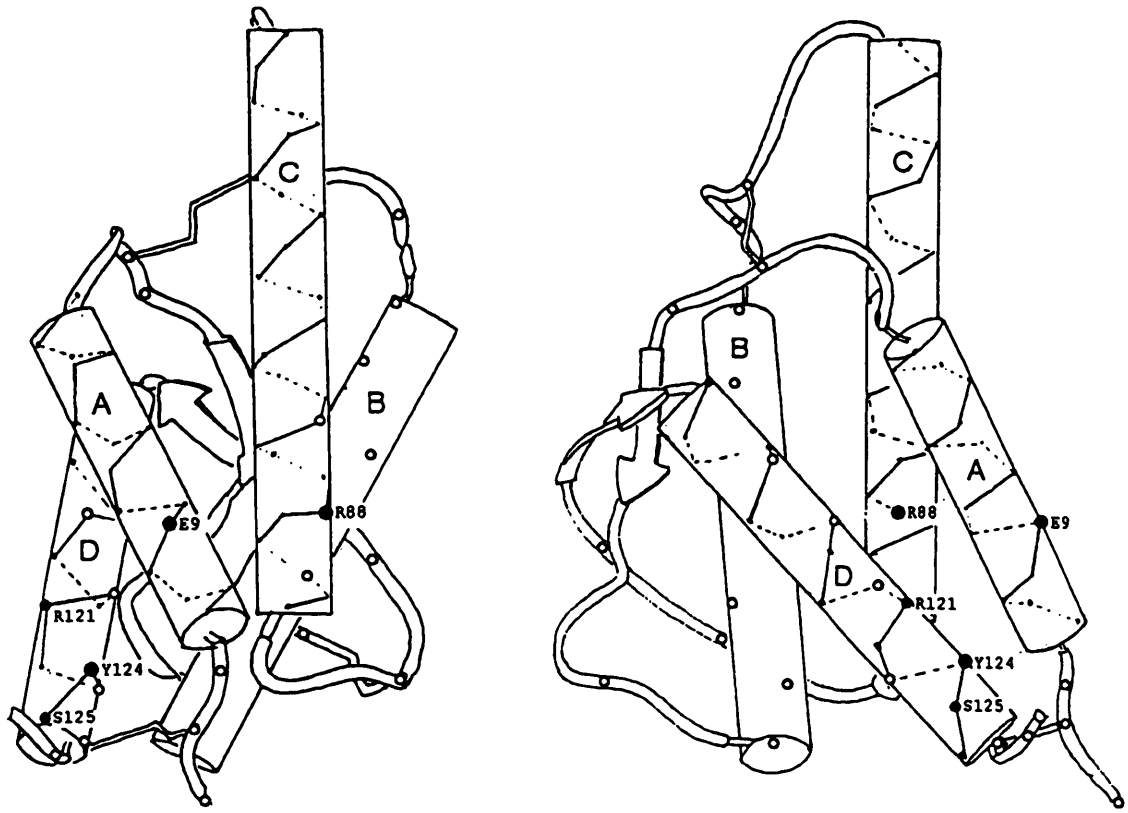

Fig. 6. Schematic drawings of the three-dimensional structure of human IL-4 showing views of helices A and C (**left**) and helices D and A (**right**). Closed circles indicate the approximate locations of 'binding residues' glutamic acid 9 (E9) and arginine 88 (R88) as well as of 'signalling residues' acid arginine 121 (R121), tyrosine 124 (Y124) and serine 125 (S125). (Arginine 88 is located at the back of the protein in the D-A view.) Open circles designate the other positions which so far have been altered by amino acid subsitutions [see Table I and Kruse *et al.*, (1991).]

marked by glutamic acid 9 in helix A and arginine 88 in helix C. The location of these residues is depicted in the two schematical views of human IL-4 in Figure 6. The established three-dimensional structure of human IL-4 shows that these amino acid side chains are exposed completely or, in the case of tyrosine 124, partially at the surface of the protein (e.g. T.Müller, W.Sebald and H.Oschkinat, submitted). Furthermore, the 'binding residues' at positions 9 and 88 are juxtaposed, as are the 'signalling residues' at positions 121, 124 and 125. The functional sites marked by the mutational analysis are therefore compatible with the topology and location of the involved residues. Amino acid substitutions at other positions which have been analysed in the present and a previous study (Kruse *et al.*, 1992) did not produce major functional alterations. A few substitutions interfered with the folding and/or stability of the protein. These negative results do not exclude the possibility that residues at these positions are of functional importance. The replacements performed up to now with only one single amino acid residue may be too insignificant to cause measurable functional defects.

Several lines of evidence support the view that the amino acid substitutions causing the functionally deficient IL-4 variants discussed above do not produce major alterations in the protein fold. (i) The R_{max} variants bind with largely unchanged affinity for IL-4R_{ex}. Thus, the alterations in helix D apparently remain essentially confined to the 'signalling site', and they leave the 'binding site' intact (see also T.Müller, W.Sebald and H.Oschkinat, submitted). (ii) The EC_{50} variants as well as the R_{max} variants are renatured and purified from inclusion bodies in yields similar to IL-4. (iii) When reanalysed by HPLC, the protein is eluted as a symmetrical peak at the IL-4 retention time. (iv) Furthermore, certain monoclonal antibodies against human IL-4, which are sensitive to structural perturbations, bind with normal affinity to these variants (Reusch *et al.*, unpublished observations).

Our conclusions are at variance with some of the interpretations deduced from the study of mouse IL-4 mutant proteins (Morrison and Leder, 1992). Mouse IL-4 variants were generated by alanine-scanning mutagenesis and expressed in CHO cells as fusion proteins with alkaline phosphatase. The mouse variant E12A modified at glutamic acid 12 (which corresponds to human IL-4 glutamic acid 9) exhibited a >100-fold reduced bioactivity and receptor binding affinity. It was suggested that this residue is important for structural integrity. A series of mouse IL-4 variants with modifications in helix D (L104A, D106A, F107A and L111A) each lost both binding and biological function. It was suggested that helix D was part of the receptor binding domain of mouse IL-4 and, more specifically, it was proposed that the side chains of leucine 104, aspartic acid 106, phenylalanine 107 and leucine 111 contributed most to the binding interactions in this region. Perhaps some of these interpretations were premature. It is reasonable to assume that the three-dimensional structures of mouse and human IL-4 are largely comparable, despite species-specificity in receptor binding and bioactivity.

According to the now available structural data on human IL-4, the side chains of the invariant leucine 104, phenylalanine 107 and leucine 111 are integral constitutents of the hydrophobic core and consequently would not be available for receptor interactions. On the other hand, despite the determinants for species-specificity in the first 16 amino acid residues of mouse IL-4, it is quite possible that the invariant side chain of glutamic acid 12 functions in receptor binding also in mouse IL-4. A definite decision, however, requires a structural analysis of these mutant proteins. Interestingly, a deletion of the last C-terminal amino acids of mouse IL-4 generates a variant highly deficient in bioactivity but retaining normal receptor-binding affinity. The deleted residues can be aligned to lysine 123 and also tyrosine 124 and serine 125 of human IL-4 functioning in receptor activation. Thus, a 'signalling site' appears to be present also at the corresponding positions in the helix D of mouse IL-4.

Human GM-CSF and human IL-2 are folded remarkably similarly to human IL-4 despite the fact that the amino acid sequences are only vaguely related. All three proteins consist of ~130 amino acid residues. The 4-helix-bundle structures show the same up-up-down-down connectivity ('double-overhand' topology), and the long AB and CD loops are connected by a short β-sheet. Parts of their three-dimensional structures are nearly superimposable (Wlodawer *et al.*, 1992; T.Müller, W.Sebald and H.Oschkinat, submitted). Some variability exists in the length of the N-terminal end of helix C which is shorter in GM-CSF, and which appears to be shortened also in equine, porcine and mouse IL-4 (Bailey *et al.*, 1993; E.V.Vandergrifft and D.H.Morokov, EMBL database accession no. L06010). A specific feature of the GM-CSF and IL-2 structures is a short helical segment after helix A on the head of the helix bundle, which is not seen in human IL-4. It is also interesting to note that the dimeric human M-CSF, which represents another 4-helix-bundle protein, is connected via the heads of the two protein molecules (Pandit *et al.* 1992).

Mutational analysis of human IL-2 revealed that aspartic acid 20 functions in IL-2 binding to the medium- and high-affinity receptor (Weigel *et al.*, 1989; Sauvé *et al.*, 1991), probably by interacting with the β subunit of the IL-2 receptor (IL-2Rβ). Similar results have been reported for variants of mouse IL-2 with substitutions of aspartic acid 34 (Zurawski *et al.*, 1990), which corresponds to human IL-2 aspartic acid 20. In human GM-CSF, glutamic acid 21 determines high-affinity binding to the GM-CSF receptor (Lopez *et al.*, 1992; Shanafelt and Kastelein, 1992). Because low-affinity binding to the GM-CSF receptor α subunit is not affected in glutamic acid 21 variants, it was reasonable to conclude that this residue interacts directly with the GM-CSF receptor β subunit (GM-CSFRβ). Glutamic acid 9 of human IL-4, which has been shown to determine binding to IL-4R_{ex} in the present study, is located at the equivalent position to GM-CSF glutamic acid 21 or human IL-2 aspartic acid 20 (Wlodawer *et al.*, 1992). On the basis of superficial similarities between the amino acid sequences the functional equivalence of each of these positions has been postulated before (Shanafelt *et al.*, 1991). The present findings support the view that an acidic side chain in helix A functions at least in the 'small' helical cytokines in binding to a receptor subunit.

Helix C of IL-2 or GM-CSF has not been studied at the level of single amino acid replacements. Kaushansky (1992) identified the sequence between residues 78 and 87 in helix C of human GM-CSF as the receptor recognition region. Glutamine 86 is located near glutamic acid 21 in the three-dimensional structure of GM-CSF, like the side chains of arginine 88 and glutamic acid 9 in human IL-4. Thus, a 'binding site' including amino acid side chains from helices A and C may be common to these cytokines. It is tempting to speculate that the receptor subunit binding to this site also exerts a common function in the various receptor systems. This may hold true especially for the extracellular domain which binds the ligand. The finding that chimeric receptors constructed from the intra- and extracellular domains of the IL-3Rβ and IL-2Rβ subunits induce a tyrosine phosphorylation pattern determined by the extracellular ligand-binding domain (Chiba *et al.*, 1993) supports this assumption. A common functional role would imply that the extracellular domain of the IL-4 receptor (IL-4R_{ex}) is equivalent to the complex of that of GM-CSFRα and GM-CSFRβ and that of IL-2Rα and IL-2Rβ. The separated β subunits of these two receptor systems *per se* have no or only very low binding affinity for the ligands. The IL-2Rα subunit binds to amino acid residues (arginine 38 and phenylalanine 42) in the small helix at the head of human IL-2 (Weigel *et al.*, 1989; Sauvé *et al.*, 1991). It has been postulated that the GM-CSFRα subunit binds to helix D of GM-CSF (Kastelein and Shanafelt, 1993) but no experimental data that confirm this conclusion have been presented. If the functional and structural equivalence of the system holds true, GM-CSFRα should bind to the head of GM-CSF.

The receptor subunit binding to the side chains at positions 121, 124 and 125 in helix D of human IL-4 remains still undefined. In principle, it could be a second molecule of IL-4R_{ex} or even a second contact site within IL-4R_{ex} after an allosteric conformational change. Up to now it has not been possible to establish IL-4-dependent dimerization of the IL-4 receptor or of IL-4R_{ex} (S.Arnold *et al.*, unpublished observation). No second binding site to IL-4R_{ex} was detected in the EC_{50} variants described in the present experiments. Thus, the association with a second, hitherto unknown IL-4 receptor subunit appears to be possible. It is conceivable that the formation of such a heterodimer (or a higher aggregate of the dimer) represents the signalling event which triggers the aggregation of the intracellular cytosolic domains (Ullrich and Schlesinger, 1990). Such a situation would correspond to a model of the IL-2 system which has recently been formulated (for review see Voss *et al.*, 1993). An IL-2Rγ subunit has been identified which appears to interact directly with IL-2 after a contact with the complex of the β plus γ subunits (or α plus β plus γ subunits) has been established. This interaction with the γ subunit probably involves glutamine 141 in helix D of mouse IL-2. Variants affected at this position exhibit partial agonist activity (Imler and Zurawski, 1992). They are similar in this respect to the R_{max} variants of human IL-4.

The results obtained with human IL-4 correspond to findings with hGH as far as the existence of two functionally distinct sites for receptor (hGH-BP) interactions are concerned. Surprisingly, however, they differ with respect to the functional role of these sites. Growth hormone binds two molecules of hGH-BP in an obligatory, sequential two-step mechanism (Cunningham *et al.*, 1991). The first hGH-BP binds to site 1 of hGH provided mainly by residues on

helix D, and by residues on one side of helix A and in the loop region between helices A and B. This first interaction of hGH with hGH-BP has a K_D of ~200 pM. The 1:1 complex between hGH site 1 and hGH-BP then binds in a subsequent step a second hGH-BP molecule via site 2. Site 2 comprises residues on helices A and C (DeVos *et al.*, 1992) and from the loop between helices B and C. It is the 'signalling site' and determines the efficacy and agonist activity of hGH. Accordingly, an antagonistic hGH variant (G120R) could be constructed by abolishing binding to site 2 (Fuh *et al.*, 1992). This blocks the receptor activation process at the level of the inactive 1:1 complex. Similarly, the receptor system was shifted to the inactive 1:1 complex by a large excess (>1 μM) of hGH. In human IL-4 the location of the 'binding site' and the 'signalling site' appears to be reversed. If a sequential binding mechanism exists also in the human IL-4 receptor systems then the first step would be an interaction via helices A and C of IL-4 and the subsequent step would be an interaction via helix D. Such a mechanism would, of course, have important implications for the rational design of high affinity IL-4 antagonists.

Materials and methods

Mutagenesis and isolation of IL-4 variants

Mutagenesis of the IL-4 cDNA was performed either according to the gapped duplex method (e.g. Kruse *et al.*, 1991) or by means of recombinant polymerase chain reaction employing two primers complementary for parts of their 5′ and 3′ ends (Higuchi, 1990). The manipulated IL-4 cDNA was expressed in *E.coli* and the IL-4 variant protein was renatured and highly purified as described by Kruse *et al.* (1992). Protein concentration was determined by measuring absorbance at 280 nm.

During some experiments, variants E9Q, E9K, R88Q and R88D were concentrated to 400 μM (6 mg protein/ml) by the following protocol. After HPLC, the protein was frozen in an aluminium block at −70°C and freeze-dried under high vacuum overnight. About 1 mg of the dry, cotton-like protein was dissolved in 0.1 ml water. The pH was adjusted to 8 by addition of 20 μl of 1 M Tris−acetate, pH 8. The solution was dialysed extensively against 20 mM Tris−HCl, 1 mM EDTA, pH 8. Protein was diluted to 6 mg/ml with the same buffer. The solution was frozen in aliquots at −20°C.

Biological assays

DNA synthesis in peripheral blood cells [largely T-cells (PHA blasts)] was determined in duplicate by incorporation of [^{3}H]thymidine. EC_{50} values were calculated from the data obtained in at least three different experiments. The EC_{50} values were distributed log normal. Standard deviations (SD) were calculated from log EC_{50} ± log SD. SD was 1.9-fold for different preparations of IL-4, and 2.3-fold of the mean EC_{50} for all variants except E9Q, E9K, R88Q, R88D, M120D and C127D. The SD of the R_{max} values for IL-4 was ±9; that for all variants except R121D and S125D was ±13. Induction of CD23 in purified B-cells was analysed by fluorescence-activated cell sorting (FACScan, Becton-Dickinson) (Kruse *et al.*, 1992).

Competitive radioligand binding

Iodination of recombinant human IL-4 and binding experiments employing Raji cells were performed at least three times in duplicate as described elsewhere (Kruse *et al.*, 1992). The relative IC_{50} values were distributed log normal. Standard deviations (SD) were calculated from log IC_{50} ± log SD. SD was 1.3-fold for measurements with IL-4, and 2-fold of the mean IC_{50} for all variants except E9Q, E9K, R88Q, R88D, M120D and C127D.

The recombinant extracellular domain of the IL-4 receptor ($IL\text{-}4R_{ex}$) was isolated from the supernatants of a CHO cell line stably transfected with cDNA encoding residues −25 to 207 of the human IL-4 receptor (S.Arnold and W.Sebald, unpublished; e.g. Mosley *et al.*, 1989). The $IL\text{-}4R_{ex}$ was highly purified by affinity adsorbtion to an IL-4−Sepharose 4B matrix and by subsequent chromatography on Superose 12 (Pharmacia). The purified $IL\text{-}4R_{ex}$ was reacted with a 5-fold molar excess of sulfo-NHS-LC-biotin (Pierce), and the mixture was desalted by a passage over BioGel P6 (Bio-Rad). The biotinylated $IL\text{-}4R_{ex}$ was immobilized on streptavidin-coated microtitre plates. Saturation binding of [^{125}I]IL-4 (1.1×10^6 c.p.m./pmol) was performed in 0.1 ml phosphate buffered basal salt, pH 7.4, plus 1% BSA at ambient temperature for 1 h. Competitive radioligand binding assays containing 0.2−0.5 nM [^{125}I]IL-4 plus serial dilutions of IL-4 variants in the same medium were incubated as above. Bound ^{125}I radioactivity was determined after three washes with PBS.

Statistical evaluations

All measurements were evaluated by means of the program GraFit (Erithacus Software) using the equation $y = a/(1 + [X/I]exps) + back$ (IC_{50} − 4 parameter logistic). A series of dose−response curves from one experiment was normalized by the following procedures. The mean values of maximal responses ($\bar{y}$ range) and background ($\overline{back}$) were calculated and substituted for 'a' and 'back' in the above equation. The slope factor, 's', was assumed to be 1. This yielded the equation: $y = \bar{y}/(1 + [X/I]1) + \overline{back}$.

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