The EMBO Journal vol.11 no.9 pp.3237-3244, 1992

Conversion of human interleukin-4 into a high affinity antagonist by a single amino acid replacement

N.Kruse, H.-P.Tony[1] and W.Sebald[2]

Theodor-Boveri-Institut für Biowissenschaften (Biozentrum) der Universität Würzburg, Physiologische Chemie II, Am Hubland, D-8700 Würzburg and [1]Medizinische Poliklinik der Universität, Klinikstr. 6-8, 8700 Würzburg, Germany

[2]Corresponding author

Communicated by H.Michel

Interleukin-4 (IL-4) represents a prototypic lymphokine (for a recent review see Paul, 1991). It promotes differentiation of B-cells and the proliferation of T- and B-cell, and other cell types of the lymphoid system. An antagonist of human IL-4 was discovered during the studies presented here after Tyr124 of the recombinant protein had been substituted by an aspartic acid residue. This IL-4 variant, Y124D, bound with high affinity to the IL-4 receptor (K_D = 310 pM), but retained no detectable proliferative activity for T-cells and inhibited IL-4-dependent T-cell proliferation competitively (K_i = 620 pM). The loss of efficacy in variant Y124D was estimated to be >100-fold on the basis of a weak partial agonist activity for the very sensitive induction of CD23 positive B-cells. The subsitution of Tyr124 by either phenylalanine, histidine, asparagine, lysine or glycine resulted in partial agonist variants with unaltered receptor binding affinity and relatively small deficiencies in efficacy. These results demonstrate that high affinity binding and signal generation can be uncoupled efficiently in a ligand of a receptor belonging to the recently identified hematopoietin receptor family. In addition we show for the first time, that a powerful antagonist acting on the IL-4 receptor system can be derived from the IL-4 protein.

Key words: drug design/partial agonists/receptor signalling

Introduction

Interleukin-4 (IL-4) represents a typical immunoregulatory lymphokine (for review see Paul and Ohara, 1987; Finkelman *et al.*, 1990; Paul, 1991). It is produced mainly by activated T-cells and mast cells and has a wide range of biological activities on various cell types of the lymphoid system. B-cells are stimulated *in vitro* to express class II major histocompatibility complex (MHC II) molecules, the IgE low affinity receptor (FcεRII, CD23) and immunoglobulins class E and G1. DNA synthesis is induced in activated B-cells, as well as in mature T-cells, foetal thymocytes, mast cells and others.

Mice treated with neutralizing antibodies directed against IL-4 or against the IL-4 receptor (Finkelman *et al.*, 1990; Urban,Jr *et al.*, 1991), as well as mice bearing an inactivated IL-4 gene (Kühn *et al.*, 1991), are unable to produce IgE and show reduced serum levels of IgG1 after challenge with the relevant stimuli. This indicates a physiological role of IL-4 at least in the regulation of IgE levels, most likely by acting as an isotype switching factor. Consequently, IL-4 may exert a pathophysiological role in the generation of disease states, as for example hyper-IgE syndrome or IgE-mediated allergic conditions (see Finkelman *et al.*, 1990; Tepper *et al.*, 1990).

IL-4 function is mediated by its binding to plasma membrane receptors occurring at the relatively small numbers of 150-2500 molecules per target cell (Cabrillat *et al.*, 1987; Park *et al.*, 1987). A single dissociation constant of ~100 pM has been determined for this interaction. The IL-4 receptor probably comprises only a single polypeptide chain of ~800 amino acid residues as suggested by chemical crosslinking experiments using ^{125}I labelled IL-4 (Mosley *et al.*, 1989; Galizzi *et al.*, 1990; Harada *et al.*, 1990; Idzerda *et al.*, 1990). Binding to IL-4 *in vitro* is not influenced by structural elements apart from the extracellular portion of the receptor, since similar dissociation constants were found for association with IL-4 with the entire receptor and only the ligand binding domain in the case of both the mouse and the human protein (Maliszewski *et al.*, 1990; Garrone *et al.*, 1991). At present it is unclear if a second protein aggregates with the receptor after IL-4-induced activation. The IL-4 receptor is evolutionarily related to the large family of hematopoietin receptors, including receptors (or receptor subunits) for interleukins-2, 3, 4, 5, 6 and 7, for GM- and G-CSF and also for erythropoietin, growth hormone and prolactine (Bazan, 1990a,b; Cosman *et al.*, 1990). All these proteins show particular homologies in the amino acid sequence of their extracellular ligand binding domains.

NMR studies revealed a three-dimensional structure of human IL-4 comprising a four-helix bundle motif with an up-up-down-down connectivity (Redfield *et al.*, 1991) similar to both human and porcine growth hormone (Abdel-Meguid *et al.*, 1987; De Vos *et al.*, 1992). Other ligands binding to members of the haematopoietin receptor family also appear to be helically organized proteins (see Bazan, 1990a,b). The crystal structure of human IL-2 shows a four-helix bundle (Brandhuber *et al.*, 1987) albeit with a connectivity different from IL-4. The amino acid sequences of these ligands display no clear homologies. A puzzling similarity, however, is apparent in the pattern of amino acid residues forming one surface of a putative amphipathic helix at the C-terminus of these proteins (Sanderson *et al.*, 1988; see Bazan, 1990b). Sequence positions involved in receptor binding have been identified in several ligands of the haematopoietin receptor family, e.g. in IL-2 (Collins *et al.*, 1988; Weigel *et al.*, 1989; Zurawski and Zurawski, 1989), growth hormone (Cunningham and Wells, 1991; Cunningham *et al.*, 1991), GM-CSF and IL-5 (Shanafelt *et al.*, 1991). Amino acid residues involved in signal generation, however, have been more difficult to identify. In order to address this issue, contributions of particular

amino acids to receptor affinity and biological activity, respectively, have to be distinguishable. As yet, uncoupling of receptor binding from biological effects has been observed to some extent only in the case of certain mutant derivatives of both human and murine IL-2 (Liang *et al.*, 1988; Zurawski *et al.*, 1990).

Recently we have generated a set of mutant variants of human IL-4 with single substitutions at the positions of all cysteinyl and aromatic residues (Kruse *et al.*, 1991). One of these variants, Y124D, was completely inactive even at micromolar concentrations during a T-cell proliferation assay. Subsequently, we realized that variant Y124D efficiently inhibits IL-4 induced T-cell proliferation. This promoted the generation of IL-4 variants in which Tyr124 was substituted by residues differing in size, polarity or charge, respectively. The receptor binding affinity of these isolated mutant proteins was determined, as well as their biological activities in two *in vitro* assays differing in sensitivity by nearly two orders of magnitude. The results of these experiments indicated that the nature of the side chain at position 124 of human IL-4 dramatically affects signal generation and biological activity, while the influence on receptor binding is marginal.

The demonstration of the feasibility to obtain an efficient antagonist of the IL-4 receptor system has several important implications. First, inhibitory IL-4 variants represent potential drugs helpful for example in the treatment of IgE-mediated diseases (see e.g. Finkelman *et al.*, 1990; Paul, 1991) similar to therapeutic monoclonal antibodies against IL-4 or to the soluble extracellular IL-4 receptor domain (Maliszewski *et al.*, 1990; Garrone *et al.*, 1991). Secondly, antagonistic ligands may prove valuable tools for the investigation of IL-4 receptor-mediated signal transducing mechanisms. Thirdly, considering the homologies and similarities mentioned above, it is an intriguing possibility that similar antagonistic variants will be found for other ligands of this receptor family (see also Zurawski *et al.*, 1990).

Results

Reduced efficacy of IL-4 variants in T-cell proliferation

Recombinant human IL-4 promoted DNA synthesis in prestimulated T-cells with a half-maximal response at a concentration (EC_{50}) of 230 pM (Figure 1). Substitution of Tyr124 by aspartic acid near the C-terminus of the 129-residue Il-4 resulted in the mutant protein Y124D, which had no measurable activity in the T-cell proliferation assay (see also Kuruse *et al.*, 1991). Substitution of Tyr124 by a series of other amino acid residues caused less pronounced alterations. As the mutant protein Y124F had the same bioactivity as wild type IL-4 (see Table I for statistical evaluations), the hydroxyl group of Tyr124 is not essential. His124 caused a small but significant reduction of the maximal response. Asparagine, lysine or glycine at this position reduced the potency to 29–13% of wild type IL-4. A comparison of mutant proteins Y124N and Y124D clearly demonstrated that the introduction of a negative charge caused the most dramatic effect on IL-4 activity. Remarkably, the half-maximal response of the residual activity (EC_{50}) of the mutant proteins remained within the same concentration range (130–230 pM) as found for wild type IL-4. This is a typical characteristic of partial agonists for which binding and signal generation (efficacy) are

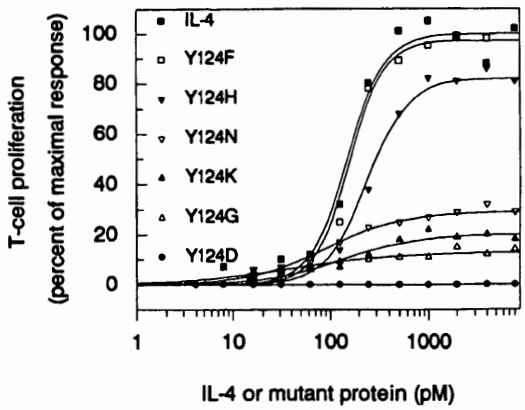

Fig. 1. Proliferation of prestimulated T-cells (PHA blasts) in response to increasing concentrations of IL-4 (■) and mutant proteins Y124F (□), Y124H (▼), Y124N (▽), Y124K (▲), Y124G (△) and Y124D (●).

partially uncoupled (Ruffolo,Jr, 1982; Black, 1989). The unaltered EC_{50} values for the variants further suggest that the response is limited by receptor occupancy (Ruffolo,Jr, 1982). Mutant proteins affected in receptor binding (see e.g. Collins *et al.*, 1988; Weigel *et al.*, 1989; Zurawski and Zurawski, 1989; Cunningham and Wells, 1991; Cunningham *et al.*, 1991; Shanafelt *et al.*, 1991) show a different behaviour such that they produce a maximal response similar to the wild type if applied in sufficient concentrations.

Partial agonism of IL-4 variants in B-cell differentiation

Compared with the T-cell proliferation assay, much lower concentrations of IL-4 were sufficient to stimulate B-cells for the induction of CD23 (FcεRII), the low affinity IgE receptor (Kikutani *et al.*, 1986; DeFrance *et al.*, 1987). After stimulation, the number of CD23 positive cells increased with EC_{50} of ~0.4 pM for IL-4 (Figure 2A) (see also Solari *et al.*, 1989). In this very sensitive assay, mutant protein Y124D behaved as a partial agonist. The number of B-cells that were stimulated by Y124D to express CD23 was up to 45% of that found for wild type IL-4, with an EC_{50} of 210 pM. Very high IL-4 concentrations did not increase the number of CD23 positive cells further, but stimulated higher mean CD23 expression in the responding cells as also determined by FACS analysis. The mean EC_{50} was 5 pM IL-4 in different experiments (see Table I). Mutant protein Y124D induced a maximal response of 8% compared with wild type IL-4 (Figure 2B). Half-maximal response occurred at 310 pM Y124D. These results clearly established that the mutant protein Y124D has a very weak partial agonist activity that was detectable only in the sensitive B-cell assay. The effective concentration for the half-maximal response of 210 pM and 310 pM, however, was in the range of the EC_{50} found for IL-4 and the other mutant proteins in the T-cell system.

Variant Y124G induced a maximal number of CD23 positive B-cells (99%) and a maximal mean CD23 content (77%) similar to IL-4 (= 100%). The EC_{50} for these responses, however, were found to be increased several-fold to ~5 and 40 pM, respectively (Figures 2A and B, see also Table I). Seemingly, these are the properties of a variant defective in receptor binding. However, Y124G shows normal receptor affinity during competitive radioligand binding to B-lymphoma cells and normal EC_{50} during the

Table I. Effective concentration (EC_{50}) and maximal response (R_{max}) of IL-4 and Il-4 variants evaluated from T- and B-cell assays

Protein	T-cell proliferation		B-cell (spleen)			
			Mean CD23 content		Number of CD23 positive cells	
	EC_{50} (pM)	R_{max} (%)	EC_{50} (pM)	R_{max} (%)	EC_{50} (pM)	R_{max} (%)
IL-4	230 (140–390)	100 ± 7	5 (2.5–10)	100 ± 11	0.4 (0.1–1.6)	100 ± 6
Variant Y124F	150 (110–210)	97 ± 6				
Variant Y124H	190 (130–290)	82 ± 5				
Variant Y124N	220 (170–290)	29 ± 7				
Variant Y124K	230 (210–250)	20 ± 4				
Variant Y124G	130 (120–140)	13 ± 4	~40	~77	~5	~99
Variant Y124D		<0.5 ± 0.7	310 (160–620)	8 ± 3	~210 (120–360)	45 ± 6

EC_{50} values were distributed log normal. Numbers in brackets were calculated from log EC_{50} ± log SD.
R_{max} values ± SD were related to R_{max} of IL-4 as 100%.
Approximate values (~) were obtained from a single experiment.

T-cell proliferation assay (see Tables I and II). Thus, the properties of partial agonist Y124G are consistent with an IL-4 receptor system in B-cells operating at low occupancy during CD23 induction. In this case, the EC_{50} values for a partial agonist are expected to increase proportionally to the loss of efficacy (Ruffolo,Jr, 1982).

The CD23 positive cells represented 67% of the total cells analysed by FACS. This percentage remained constant at all saturation levels. Thus, the induction of the number of CD23 positive cells and the induction of mean CD23 content most probably reflected two aspects of the same process in the same cell population. Of two alternatives—either each induced cell immediately develops a full CD23 content or the whole population is first induced to develop a low CD23 content which upon higher stimulation is increased—the second is consistent with the observed data.

Competitive receptor binding

The dose–response curves suggest that the amino acid exchanges at position 124 did not dramatically alter the receptor affinity of the mutant proteins compared with wild type IL-4. This has been corroborated by radioligand receptor binding experiments (Figure 3). IL-4, as well as the mutant proteins studied here, competed efficiently with iodinated IL-4 for the IL-4 receptors present on the B-lymphoma cell line Raji (Cabrillat *et al.*, 1987; Park *et al.*, 1987) as shown in Figure 3 for Y124G and Y124D. The displacement curves of all variants, with the exception of Y124D, were not significantly different from that of IL-4 (see also Table II). Half-maximal displacement of iodinated IL-4 required a 3.4-fold higher concentration of mutant protein Y124D compared with that of IL-4. Corresponding competitive binding studies performed with both PHA-activated T-cells and activated tonsillar B-cells yielded similar relative IC_{50} values (Table II). (Unfortunately, the large numbers of activated B-cells necessary for radioligand binding could not routinely be obtained from human spleen).

The concentration of 1 nM [^{125}I]IL-4 during the competitive binding experiments was ~10-fold higher than the dissociation constant. Thus, the K_D of the competing ligands could not be determined from the IC_{50} (Munson, 1983). The K_D of [^{125}I]IL-4 determined independently (see Materials and methods) was 91 pM for the B-lymphoma cells and 81 pM for the PHA-activated T-cells. Assuming a receptor dissociation constant K_D of ~100 pM for IL-4 (see also Cabrillat *et al.*, 1987; Park *et al.*, 1987), mutant protein Y124D accordingly had a K_D of 310 pM.

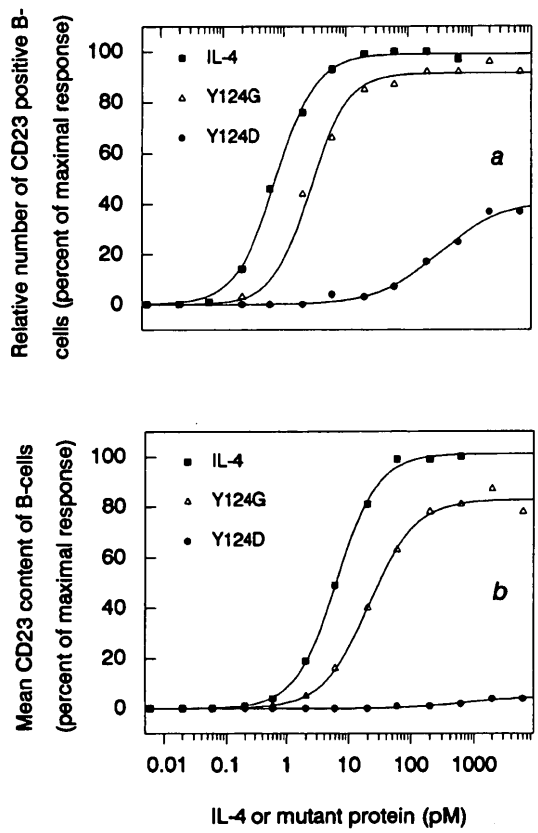

Fig. 2. Induction of the low affinity Fcε receptor (CD23) on B-cells in response to increasing concentrations of IL-4 (■) or mutant proteins Y124G (△) and Y124D (●). (**a**) Number of CD23 positive B-cells as percent of maximal response. (**b**) Mean CD23 content of B-cells.

Activated human B- and T-cells both expose 500–1000 molecules of IL-4 receptor at their surface showing a K_D of ~100 pM in terms of IL-4 binding (see above). Therefore the proliferative response of T-cells appears to closely follow the receptor occupancy. Assuming that in the B-cell assays the biological response is solely mediated by the receptor with a K_D of 100 pM, the induction of CD23 requires the occupancy of only a few percent of the receptors by IL-4. We suggest that only in case of the 'emasculated' (Black, 1989) Y124D mutant protein, the receptor had to be maximally saturated in order to generate a small partial response. It remains unclear, however, if the assay periods

Table II. Competitive radioligand binding (IC_{50}), receptor dissociation constant (K_D) and competitive inhibitor constant (K_i) of IL-4 and IL-4 variants evaluated from B- and T-cell assays

Protein	PHA-activated T-cells		B-cells			T-cell proliferation	B-cell (spleen)	
	IC_{50} (%)	K_D (pM)	Lymphoma (Raji)		Tonsillar	K_i(pM)	mean CD23 content	number of CD23 positive cells
			IC_{50} (%)	K_D (pM)	IC_{50} (%)		K_i (pM)	
IL-4	100 (80–130)	81 ± 7	100 (70–150)	91 ± 5	~100			
Variant Y124F			75 (70–80)					
Variant Y124H			80 (70–90)					
Variant Y124N			110 (100–120)					
Variant Y124K			110 (100–120)					
Variant Y124G			68 (60–80)					
Variant Y124D	320 (230–450)		340 (280–410)		~300	620 (390–990)	810 (620–1050) ~640[a]	1000 (320–3200) ~630[a]

IC_{50} and K_i values were distributed log normal. The IC_{50} of the variants were related to the IC_{50} of IL-4 as 100%. Numbers in brackets were calculated from log IC_{50} ± log SD or log K_i ± log SD.
K_D values ± SD were calculated from Scatchard analyses.
[a]Tonsillar B-cells were used in one experiment.

of several days conventionally used allow for a straight comparison between receptor occupancy and response.

Antagonist activity of IL-4 variant Y124D

The results presented above lead to the prediction that the mutant protein Y124D functions as a pure antagonist in the T-cell system and as a partial antagonist of IL-4 in the much more sensitive B-cell system. This has been actually demonstrated by competition experiments (Figure 4). IL-4-stimulated proliferation of T-cells was competitively inhibited by mutant protein Y124D (Figure 4A). From the apparent EC_{50} in the presence of different concentrations of Y124D an inhibitor constant K_i of 620 pM could be calculated (see Table II). A similar K_i of 810 pM was determined during competititon of IL-4-dependent CD23 induction on B-cells (Figure 4B). Included in Table II is also the inhibitory constant K_i of 1000 pM for the induction of the number of CD23 positive B-cells (Figure 4C). The latter value shows a high variability due to the pronounced partial agonist activity of variant Y124D during this response (45%). For the partial agonists Y124G, Y124N, Y124K and even for Y124H, and incomplete inhibition of IL-4 activity was established in the T-cell proliferation assay down to the level determined by their respective partial agonist activities (see Figure 1). The calculated K_i values were in the range of 200 pM (data not shown).

A comparison of the dose–response curves in Figure 4A, B and C shows that the slopes of the T-cell responses are steeper. The same difference was observed for the experimental results presented in Figures 1 and 2A, and B. The binding curve of [^{125}I]IL-4 to B-lymphoma cells or activated T-cells has a slope with a calculated Hill coefficient of 0.81–1.15 (data not shown) similar to the B-cell responses (0.91–1.4). The Hill coefficient calculated for the slope of the dose–response curve for IL-4-dependent T-cell proliferation is ~2-fold higher (2–2.7). In the competitive inhibition experiments, the slope was reduced ~2-fold in the presence of antagonist Y124D, both in the T-cell (1.3–1.5) and in the B-cell (0.5–0.6) assays. The significance of these differences is still unclear. They might also indicate specific aspects of receptor activation, but we cannot presently rule out an origin from experimental conditions.

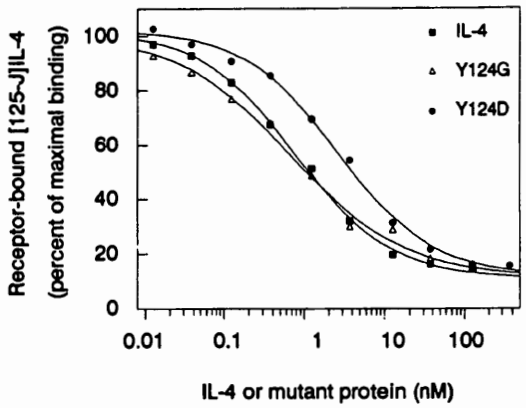

Fig. 3. Competition for binding to the IL-4 receptor of Raji cells between radiolabelled IL-4 and IL-4 (■) or mutant proteins Y124G (△) or Y124D (●).

Discussion

The results presented here indicate conclusively that Tyr124 of IL-4 is of crucial importance for the potency of the lymphokine for signal generation, but not for the binding of IL-4 to its receptor. Whereas biological activity was severely impaired in several substitution mutants at position 124 (see below), binding of this variant ligands to IL-4 receptor was not at all or only marginally affected. As compiled in Tables I and II, this is shown first by radioligand receptor binding experiments using the IL-4 receptor of the B-lymphoma cell line Raji (Park *et al.*, 1987), or of both activated T- and B-cells, secondly, by determining the EC_{50} of the partial agonist activities in both the B-cell and the T-cell assay and thirdly, by determining the competitive inhibitor constants, K_i, in both cellular systems.

The impairment of biological activity, i.e. the loss of efficacy of IL-4, strongly depends on the nature of the side chain introduced at position 124. In the T-cell assay, efficacy is reduced ~5-fold with side chains of asparagine, lysine and glycine, respectively, and >100-fold with an aspartyl side chain (see Table I). Interestingly, the loss of efficacy appears to be less pronounced during the B-cell differentiation assays. This, however, corresponds to the

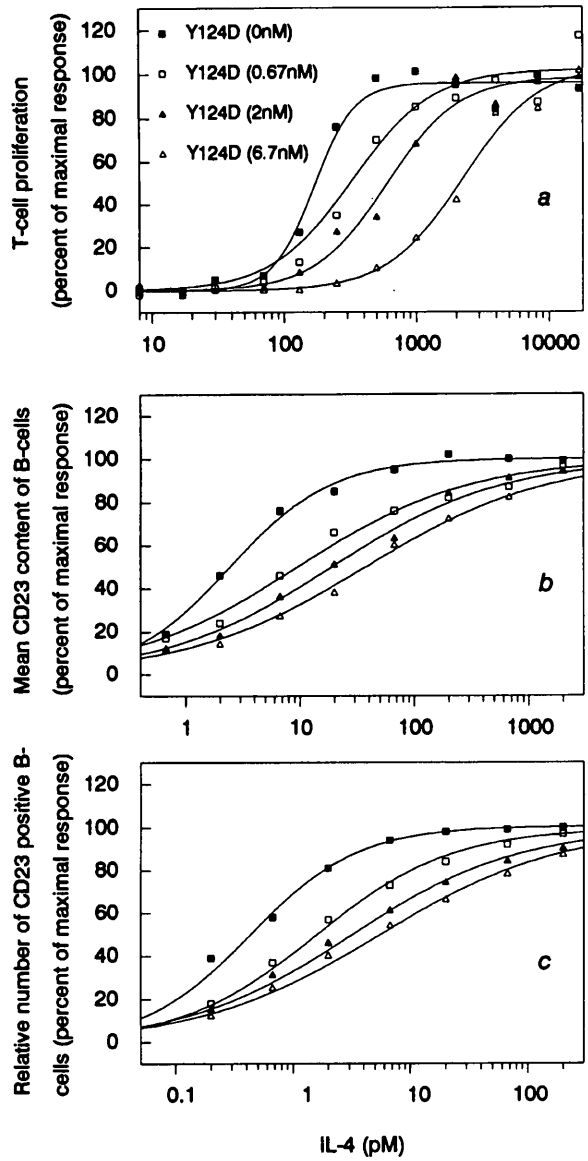

Fig. 4. Competitive inhibition of IL-4-dependent T-cell proliferation. (a) IL-4-induced CD23 content of B-cells (b) and IL-4-induced number of CD23 positive B-cells (c) by mutant protein Y124D at concentrations of 0 nm (■), 0.67 nM (□), 2 nM (▲), and 6.7 nM (△). The results in (B) and (C) are corrected by subtracting partial agonist activities of variant Y124D.

~40-fold lower EC_{50} of IL-4 for the mean CD23 induction or the 500-fold lower EC_{50} for induction of CD23 positive cells when compared with the T-cell response.

These apparent discrepancies in IL-4 efficacy can be explained in terms of receptor occupancy. The EC_{50} of ~200 pM for T-cell proliferation is similar to the IL-4 receptor dissociation constant. Accordingly, this response may be limited by the number of activated receptors. The B-cell response appears to be limited by a signalling step following receptor activation. This step then would determine the EC_{50} of 5 pM (0.4 pM) in these processes, which correspond to a receptor occupancy of ~5% (mean CD23 content) or 0.4% (number of CD23 positive cells). The calculation and its underlying assumptions are detailed in Materials and methods. The lower efficacy of the IL-4 variants can thus be partially compensated for by higher receptor saturation. In case of mutant protein Y124D maximal response is 8% (45%) with an EC_{50} or 310 pM (210 pM) for mean CD23 induction (CD23 positive cells). According to these data, the loss of efficacy is 125- to 300-fold. During the T-cell proliferation assays, the mean error of the control was 0.5% of the maximal response of IL-4. The responses of variant Y124D were within the mean error of the control. Thus, the efficacy of Y124D is at least 200-fold lower than that of IL-4 in this system. Although these calculations are rough estimates derived from long term assays, measuring complex proliferation and differentiation processes, at least they should provide information concerning the upper limit. (An alternative explanation of the results involving a very small subpopulation of receptors, e.g. 20–50 molecules/cell, with a very low dissociation constant in the range of 1 pM in the B-cells appears rather unlikely, since the antagonist Y124D inhibits with the same inhibitory constant of 620–1000 pM both B- and T-cells.)

The identification of a whole panel of partial agonists suggests that the residue at position 124 is directly involved in signal generating interactions. The amino acid exchange in mutant protein Y124D, however, produces an unexpectedly large effect. Thus, additional conformation effects appear to be possible in this case. It may be relevant in this respect that variant Y124D does not easily crystallize (T.Müller and W.Sebald, unpublished observation) in contrast to IL-4 (Cook *et al.*, 1991). It remains to be established if further residues in the IL-4 molecule, together with Tyr124, form a site distinct from a IL-4 receptor binding site, which is involved in signal generation. According to the available structural information (Redfield *et al.*, 1991; see also Diederichs *et al.*, 1991) residues at the C-terminal end of helix D are close to Tyr124. Cys127 forms a disulfide bond to Cys3. Thus, side chains of helix A may also be in proximity to Tyr124. It is interesting to note that for human GM-CSF, which has the same fold as IL-4, a receptor binding site was postulated consisting of two peptide stretches centered around Arg24 (helix A) and Met79 (helix C) (Diederichs *et al.*, 1991). According to the GM-CSF crystal structure, this binding site would be spatially far away from the C-terminus of helix D.

Tyr124 of human IL-4 is found at the corresponding position of murine IL-4 (Sanderson *et al.*, 1988). It occurs at the end of helix D (Redfield *et al.*, 1991), only six residues away from the C-terminus. It has been noted (Bazan, 1990b; Sanderson, 1988) that this C-terminal segment has features in common with other known ligands of the haematopoietin receptor family. Amino acid substitutions of Glu141 of murine IL-2 (Zurawski *et al.*, 1990), whose position appears to be equivalent to Tyr124 of human IL-4, yielded a series of partial agonists. In particular, murine IL-2 variant Q141D was inactive or only weakly active in proliferation assays using various cell lines. However, high affinity binding was lost and no competitive inhibition of IL-2-dependent proliferation of HT2 cells could be found even at a 5×10^4-fold molar excess of variant Q141D. Variant Q141D of murine IL-2 is a capable antagonist, however, against mutant proteins of IL-2 with receptor-binding defects. mIL-2 variants with substitutions of Glu141 by asparagine (class I), lysine (class II), aspartic acid (class III) and glycine

(class IV), could be grouped into four classes according to their differing partial agonist activities. This is unlike the variants of human IL-4, where substitutions of Tyr124 by asparagine, lysine and glycine produced very similar agonist activities (see Figure 1, Table I). Thus, it remains unclear in how far the generation of partial agonism by an aspartyl side chain at the human IL-4 Y124 and at the murine IL-2 Q141 positions indicates comparable structural requirements.

Two non-overlapping binding sites were identified in human growth hormone (Cunningham and Wells, 1991; Cunningham *et al.*, 1991; De Vos *et al.*, 1992) that are occupied in a sequential manner by two growth hormone receptor molecules. Site 1 comprises the C-terminus of helix D (Cunningham *et al.*, 1991). The equivalent of IL-4 Tyr124 might be human growth hormone Val185, which is located at the end of helix D and which is part of site 1. The biological activity of a growth hormone variant V185D has not yet been assessed. It was postulated (Cunningham *et al.*, 1991) that non-dimerizing variants of growth hormone, which have lost the ability to bind two receptor molecules, should show antagonistic properties.

A natural receptor antagonist was detected in the interleukin 1 system. The human protein (IL-1Ra) shares 19% identical amino acid positions with IL-1α and 26% identical positions with IL-1β and is probably composed of 12 β-strands. It behaves as a pure antagonist in binding with high affinity to the IL-1 receptor (type I) without evoking any detectable response (Eisenberg *et al.*, 1990; Hannum *et al.*, 1990). This indicates that in the IL-1 system receptor binding and receptor activation are completely separable. An efficient uncoupling of both steps also was obtained by single amino acid replacements in IL-1 molecules. IL-1β variants R127G (Gehrke *et al.*, 1990) and D145K (Ju *et al.*, 1991) as well as an IL-1α variant D151Y (Yamayoshi *et al.*, 1990) exhibited little biological activity but bound with a largely unaltered affinity to the receptor. The discontinuous receptor binding site identified in human IL-1β (Labriola-Tompkins *et al.*, 1991) clusters around Arg4 and does not comprise the positions determining receptor activation. This could argue for a structural separation of two independent functional sites in the IL-1 protein. Interestingly, however, significant differences exist among those amino acid residues of IL-1α, IL-1β and Il-1Ra that probably provide the contact points in each receptor–ligand complex. Accordingly, an apparently unaltered receptor affinity of an engineered antagonist might also result from multiple compensating effects and it does not necessarily indicate a structural independence of the two ligand domains that bind or activate the receptor, respectively.

Especially for IL-1α variant D141G pure agonist or partial agonist activities were established in various cellular responses (Yamayoshi *et al.*, 1990), which resemble our results with human IL-4 variant Y124D. Considering the fundamental structural differences between IL-1 and IL-4, as well as between their respective receptors, it is interesting to speculate that the occurrence and the straight forward identification of high affinity antagonists in both systems is correlated with a single chain composition of both the receptors.

Other more complex receptor systems may put more stringent constraints on structural modifications of the ligand. In the multichain IL-2 receptor system the intrinsically high K_D of 70 nM of the β subunit (the homologue of the IL-4 receptor) was found to decrease to 5 pM in the presence of the α subunit (see Ringheim *et al.*, 1991). A decreased K_D of 1.2 nM was observed in YT cells due to the presence of a still poorly defined γ subunit. Interestingly, IL-2 variants affected in α subunit binding showed an apparently unaltered EC_{50} in the biological assays (Weigel *et al.*, 1989; Zurawski and Zurawski, 1989; Ju *et al.*, 1991). For the explanation of this puzzling behaviour a model was recently proposed (Grant *et al.*, 1992) according to which the α subunit contributes several functions to IL-2-mediated signalling through the high affinity IL-2 receptor system. Interestingly, the partial agonist and antagonist variants affected at position Gln141 of murine IL-2 (Zurawski *et al.*, 1990) were described to be deficient in γ subunit interactions. They inhibited efficiently, however, the IL-2 variants defective in α subunit binding. Possibly, the interaction of the γ subunit and the β subunit is critical for receptor activation and any disturbance of this interaction will reduce both receptor binding and receptor activation. This complex subunit interplay in the IL-2 receptor system therefore complicates its analysis, but may also provide special advantages for the engineering of selective agonists (see Zurawski, 1991).

Increasing evidence supports the view that the formation of receptor homodimers or the forming of heterooligomers represents an important step in receptor transmembrane signalling (see Schlessinger, 1988; Williams, 1989; Ullrich and Schlessinger, 1990). This leads to the question how the ligand triggers this aggregation. 'Allosteric receptor oligomerization' (Schlessinger, 1988), proposed as a model for tyrosine kinase receptors, involves two conformations of the extracellular domain. Only the high affinity ligand binding conformation forms oligomers. Bridging of two receptor proteins by a single ligand molecule represents another possible mechanism for oligomerization (Ullrich and Schlessinger, 1990; Cunningham *et al.*, 1991) supported by the recent finding that growth hormone forms a 1:2 complex with the isolated extracellular receptor domain. For the IL-4 receptor, no evidence for the occurrence of dimers or oligomers has as yet been provided. A single class of IL-4 receptor or IL-4 binding protein has been identified so far, exhibiting one homogeneous dissociation constant of ~100 pM. It remains to be established whether IL-4 receptor signalling involves the formation of a homodimer or whether the aggregation with other hitherto unknown proteins is necessary. The availability of antagonist IL-4 variants will open up new ways to address the question of which mechanisms operate during IL-4 receptor signalling.

Materials and methods

Production of IL-4 mutant proteins

Human IL-4 and mutant proteins were produced in *Escherichia coli*, subjected to a renaturation step and highly purified by CM-Sepharose 6B chromatography followed by HPLC (Weigel *et al.*, 1989; Kruse *et al.*, 1991). By means of *in vitro* mutagenesis Tyr124 (Kruse *et al.*, 1991) had been replaced by phenylalanine, histidine, asparagine, lysine, glycine or aspartic acid, respectively, to generate mutant proteins Y124F, Y124H, Y124N, Y124K, Y124G and Y124D. The mutated genes recloned into the expression plasmid were sequenced in both directions (373A DNA Sequencer, Applied Biosystems) to confirm the mutation. Protein concentration was determined by measuring absorbance at 280 nm. It was assumed that 1 mg IL-4/ml (50% Acetonitril and 0.1% trifluoracetic acid) yields an absorbance of 1 at 280 nm ($\epsilon_{280}^{0.1\%} = 1$). For protein measurements of variants deficient in Try124, the absorbance at 280 nm was multiplied by a factor of 1.15.

Slack,J., Beckmann,M.P. and Grabstein,K.H. (1990) *J. Immunol.*, **144**, 3028–3033.
Mosley,B. *et al.* (1989) *Cell*, **59**, 335–348.
Munson,P.J. (1983) *Methods Enzymol.*, **92**, 543–576.
Park,L.S., Friend,D., Sassenfeld,H.M. and Urdal,D.L. (1987) *J. Exp. Med.*, **166**, 476–488.
Paul,W.E. and Ohara,J. (1987) *Annu. Rev. Immunol.*, **5**, 429–459.
Paul,W.E. (1991) *Blood*, **77**, 1859–1870.
Redfield,C., Smith,L.J., Boyd,J., Lawrence,G.M.P., Edwards,R.G., Smith,R.A.G. and Dobson,C.M. (1991) *Biochemistry*, **30**, 11029–11035.
Ringheim,G.E., Freimark,B.D. and Robb,R.J. (1991) *Lymphokine Cytokine Res.*, **10**, 219–224.
Ruffolo,R.R.Jr (1982) *J. Auton. Pharmac.*, **2**, 277–295.
Sanderson,C.J., Campbell,H.D. and Young,I.G. (1988) *Immunol. Rev.*, **102**, 29–50.
Shanafelt,A.B., Miyajima,A., Kitamura,T. and Kastelein,R.A. (1991) *EMBO J.*, **10**, 4105–4112.
Solari,R. *et al.* (1989) *Biochem. J.*, **262**, 897–908.
Schlessinger,J. (1988) *Trends Biochem. Sci.*, **13**, 443–447.
Tepper,R.I., Levinson,D.A., Stanger,B.Z., Campos-Torres,J., Abbas,A.K. and Leder,P. (1990) *Cell*, **62**, 457–467.
Ullrich,A. and Schlessinger,J. (1990) *Cell*, **61**, 203–212.
Urban,J.F.Jr, Katona,I.M., Paul,W.E. and Finkelman,F.D. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 5513–5517.
Weigel,U., Meyer,M. and Sebald,W. (1989) *Eur. J. Biochem.*, **180**, 295–300.
Williams,L.T. (1989) *Science*, **243**, 1564–1570.
Yamayoshi,M., Ohue,M., Kawashima,H., Kotani,H., Iida,M., Kawata,S. and Yamada,M. (1990) *Lymphokine Res.*, **9**, 405–413.
Yokota,T., Otsuka,T., Mosmann,T., Banchereau,J., DeFrance,T., Blanchard,D., De Vries,J.E., Lee,F. and Arai,K.-I. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 5894–5898.
Zurawski,S.M. and Zurawski,G. (1989) *EMBO J.*, **8**, 2583–2590.
Zurawski,S.M., Imler,J.-L. and Zurawski,G. (1990) *EMBO J.*, **9**, 3899–3905.
Zurawski,G. (1991) *Trends Biotechnol.*, **9**, 250–257.

Received on February 4, 1992; revised on May 19, 1992