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Structure of interleukin 4 mutant E9A suggests polar steering in receptor-complex formation

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Interleukin 4 (IL-4) is a pleiotropic cytokine which induces T-cell differentiation and class switching of B cells. It therefore plays a central role in the development of allergies and asthma. An IL-4 variant in which Glu9 was mutated to alanine shows an 800-fold drop in binding affinity towards its high-affinity receptor chain. As shown by surface plasmon resonance measurements, this mostly arises from a decreased association rate. Here, the crystal structure of this mutant is reported. It reveals that the protein has a virtually identical structure to the wild type, showing that the unusual behaviour of the mutated protein is not a consequence of misfolding. The possibility that polar interactions in the encounter complex have a steering effect is discussed.

1. Introduction

Interleukin 4 (IL-4) belongs to the haematopoietic cytokines and regulates central events in the early phase of a Th2-dominated immune response. Stimulation of naive T cells by IL-4 leads to the production of IL-4 itself as well as other cytokines and immunoglobulin class switching of B cells towards IgE production. This process, which is the natural defence mechanism against parasite infections in mucosal tissue and skin, plays a pathophysiological role in the development of type I allergies and asthma and makes the IL-4 system a prime target for the design of small-molecule drugs and the development of second-generation therapeutic proteins (Reinemer *et al.*, 2000).

The responses mediated by IL-4 are generated by the heterodimerization of transmembrane receptor chains in a strictly sequential manner. In a first step, IL-4 binds to the extracellular part of its high-affinity receptor α-chain (IL-4Rα). This primary event is followed by the recruitment of a second receptor chain with lower affinity. This low-affinity binding receptor can be the common γ chain (γ_c), yielding the so-called type I receptor complex, or the IL-13Ra1 chain (Callard *et al.*, 1997), resulting in a type II receptor complex. γ_c is also part of the receptor complexes of IL-2, IL-7, IL-9 and IL-15 (Sugamura *et al.*, 1996), and IL-13Ra1 is also recognized by IL-13. The cross-linking of receptor ectodomains by the ligand leads to association of tyrosine kinases of the Jak family to the cytoplasmic parts of the receptors and activation of the Jak/Stat pathway (Leonard & O'Shea, 1998).

Structurally, IL-4 belongs to the group of short-chain four-helix-bundle cytokines (Mott & Campbell, 1995). This protein family shows the typical 'up–up–down–down' topology of α-helices, which makes long connecting loops necessary between helices running in the same direction. The understanding of molecular recognition between IL-4 and IL-4Rα has greatly been increased by analyses of the three-dimensional structures of IL-4 (Wlodawer *et al.*, 1992; Walter *et al.*, 1992; Smith *et al.*, 1992; Powers *et al.*, 1992) and recently of the complex between IL-4 and the extracellular part of the IL-4Rα chain (IL-4BP; Hage *et al.*, 1999). IL-4BP consists of two fibronectin type III domains which are oriented nearly perpendicular to each other and are connected by a short six-amino-acid linker. Three distinct structural clusters of interaction were identified which are located around the polar ligand residues Glu9, Arg88 and Arg85.

Numerous mutants of the human IL-4 protein have been generated and characterized in order to identify residues important for receptor binding (Wang *et al.*, 1997). From these studies, it was deduced that the highest energetic contributions upon binding of the receptor α chain come from Glu9 and Arg88, with a lesser contribution from Arg85. The finding that charged residues are central to binding is in strong contrast to the situation on the interface between another well studied four-helix-bundle cytokine, human growth hormone, and its receptor, which is structurally and functionally dominated by hydrophobic residues (de Vos *et al.*, 1992).

Here, we present the crystal structure of IL-4 variant Glu9→Ala (IL-4[E9A]) and the

functional analysis of this mutant protein in a biosensor assay.

2. Materials and methods

IL-4[E9A] was expressed in *Escherichia coli* as inclusion bodies. After cell lysis and washing, the protein was refolded and purified to homogeneity by chromatographic means as described in Kruse *et al.* (1993).

Protein–protein interaction analysis was performed by surface plasmon resonance using the BIAcore system (Pharmacia) as described in Shen *et al.* (1996). Briefly, IL4-BP was biotinylated and immobilized on a streptavidin-coated sensor chip. Protein samples were applied in HBS buffer (10 m*M* HEPES pH 7.4, 150 m*M* NaCl, 3.4 m*M* EDTA, 0.005% surfactant P20) at concentrations of 2.5–1000 n*M*. Wild-type (wt) IL-4 was used as a positive control. Sensograms were recorded at a flow rate of 50 µl min^{-1} and evaluated with the BIA evaluation software on the basis of equimolar association.

Crystals of the IL-4[E9A] variant were obtained at 293 K in hanging drops composed of equal volumes of reservoir (100 m*M* sodium acetate pH 5.4, 63% satured ammonium sulfate) and protein solution (10 mg ml^{-1} in water). Diffraction data from a single crystal (Table 1) were collected on a MAR300 image plate mounted on a Rigaku RU-200BH rotating-anode generator at 100 K using a solution containing 30% glucose, 60% satured ammonium sulfate in 100 m*M* sodium acetate at pH 5.4 as a cryoprotectant. Data processing was performed with *DENZO*/*SCALEPACK* (Otwinowski & Minor, 1997).

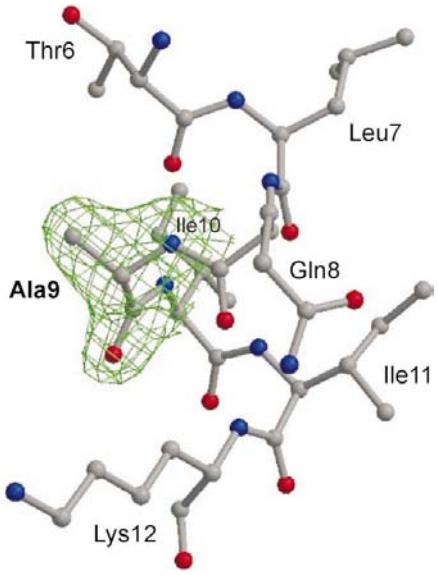

Figure 1
Section of helix *A*, residues 6–12. The $2F_o - F_c$ simulated-annealing omit map density around the mutated residue Ala9 is contoured at 1σ.

Initial phases were obtained from the model of wild-type IL-4 (Wlodawer *et al.*, 1992). A single round of rigid-body refinement followed by simulated annealing and energy minimization was performed with *CNS* (Brunger *et al.*, 1998) to remove model bias. Resulting $2F_o - F_c$, $F_o - F_c$ and simulated-annealing electron-density maps clearly showed an alanine at the mutated position (Fig. 1). The model was improved in iterative rounds of manual rebuilding using *O* (Jones *et al.*, 1991), incorporation of 145 water molecules and maximum-likelihood refinement with *CNS*.

Figures were generated using *Molscript* (Kraulis, 1991), *Bobscript* (Esnouf, 1999) and *Raster3D* (Merritt & Bacon, 1997).

Table 1
Data-processing and refinement statistics of IL-4[E9A].

(*a*) Crystals and data processing; values as defined in *SCALEPACK* (Otwinowski & Minor, 1997). Values in parentheses are for the highest resolution shell.

Space group	$P4_12_12$
Unit-cell parameters (Å)	$a = b = 91.05$, $c = 45.60$
Resolution (Å)	20–2.05 (2.10–2.05)
No. of collected reflections	61298 (4531)
No. of unique reflections	12421 (795)
Completeness (%)	99.2 (98.8)
Multiplicity	5.0 (4.6)
Intensities $[I/\sigma(I)]$	34.8 (7.7)
R_{merge} (%)	3.4 (16.8)

(*b*) Refinement statistics as defined in *CNS* (Brunger *et al.*, 1998).

R_{cryst} (%)	22.5
R_{free} (%)	24.7
R.m.s.d. bonds (Å)	0.006
R.m.s.d. angles (°)	1.20
R.m.s.d. *B*, bonded main-chain atoms (target)	1.68 (1.5)
R.m.s.d. *B*, bonded side-chain atoms (target)	2.38 (2.0)
Average *B* value (Å^2)	32.5
Coordinate error (cross-validated σ_A) (Å)	0.18

3. Results and discussion

The BIA2000 surface plasmon resonance system (Pharmacia) was used to determine association and dissociation rate constants for the formation of the complex between IL-4[E9A] and immobilized IL-4BP. The association rate of IL-4[E9A] was found to be seriously decreased; we measured a value of $2.8 \times 10^4\ M^{-1}\ s^{-1}$, which is approximately 500-fold lower than that for wt IL-4 ($1.3 \times 10^7\ M^{-1}\ s^{-1}$; Wang *et al.*, 1997). On the other hand, the dissociation rate was only weakly altered, with a measured value of $3.6 \times 10^{-3}\ s^{-1}$ compared with $2.1 \times 10^{-3}\ s^{-1}$ for the wild type. The resulting dissociation constant K_d for IL-4[E9A] is 128 n*M* (0.16 n*M* for the wild type). Such severe effects on association rates have not been detected with any other alanine variant of charged side chains on IL-4. Interestingly, a mutation of Glu9 to Gln has quite a different effect: the association rate decreases by only a factor of 1.5, while the dissociation rate is increased by a factor of 120 (Wang *et al.*, 1997). This shows that association is not determined by electrostatic steering by this particular side chain. In the case of IL4[E9A], the surprising observation that the loss of three well defined hydrogen bonds (Fig. 2) which are formed by Glu9 in

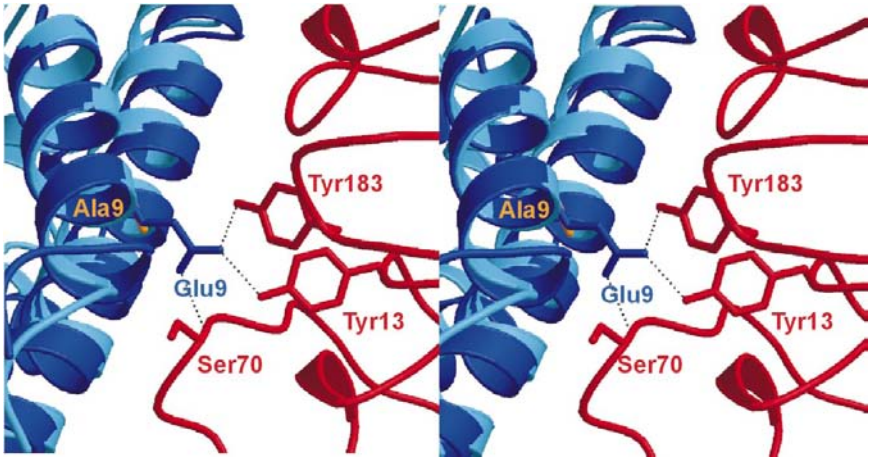

Figure 2
Polar interactions of the Glu9 side chain of wt-IL4 (dark blue) in the complex with IL-4BP (red, coordinates taken from PDB entry 1iar). Three well defined hydrogen bonds (dotted lines) are lost when the glutamic acid side chain is mutated to alanine. The structure of the free E9A mutant is superimposed onto the wt-IL4 (light blue) with the mutated Ala marked in orange.

the complex with the receptor (Hage *et al.*, 1999) affects the association rate but not the dissociation rate urged us to solve the crystal structure of this mutant.

The structure of IL4[E9A] was refined at 2.05 Å resolution to an R factor of 22.5% ($R_{\rm free}$ = 24.7%). All residues of the polypeptide chain could be traced and are located in allowed regions of the Ramachandran plot (Collaborative Computational Project, Number 4, 1994). Residues 37 and 38 located in a flexible loop region are poorly defined. Details of data processing and refinement are compiled in Table 1.

The overall structure of the mutant protein is almost identical to the wild-type form (Wlodawer *et al.*, 1992). The r.m.s. deviation for positions of all C^{α} atoms is 0.37 Å. Except for the mutation at position 9 and some conformational differences in poorly defined and mobile surface side chains or terminal residues, no significant differences exist between the two structures.

The absence of structural perturbation in the binding epitope together with the results from the functional analyses suggest that processes during association of IL-4 and its receptor are affected. As can be seen from the binding behaviour of the two described IL-4 variants, these are controlled by polar but not by ionic interactions, since the removal of the charge in variant E9Q has only a minor effect on the association rate. Furthermore, association seems to require only one of the two O atoms of the glutamic acid side chain to be present.

We therefore propose that during and/or after formation of the encounter complex, polar interactions (hydrogen bonds) between Glu9 O^{ε} (or Gln9 O^{ε} in E9Q) and the receptor lead to stabilization of the encounter complex. The effect of these hydrogen bonds is rather a steering during complex formation than a stabilization of the final tight complex, because the dissociation rate of the complex is not severely altered in IL-4[E9A]. The exchange of one of the O atoms in the side chain of Glu9 for a hydrogen donor as in the amide group of IL-4[E9Q] would then allow the described steering interactions during the encounter (when ligand and receptor residues are still relatively far apart from each other), but seems to evoke repulsive forces in the tight complex, as can be seen by the increased dissociation rate for this mutant.

For a deeper understanding of the process of complex formation, which is important for the design of antagonistic or superagonistic compounds, we will have to await the crystal structure of free IL-4BP.

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