

Original Article

Conformational flexibility of an anti-IL-13 DARPin[†]

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Abstract

Designed ankyrin repeat proteins (DARPin[®]) are artificial non-immunoglobulin binding proteins with potential applications as therapeutic molecules. DARPin 6G9 binds interleukin-13 with high affinity and blocks the signaling pathway and as such is promising for the treatment of asthma and other atopic diseases. The crystal structures of DARPin 6G9 in the unbound form and in complex with IL-13 were determined at high resolution. The DARPin competes for the same epitope as the IL-13 receptor chain 13R α 1 but does not interfere with the binding of the other receptor chain, IL-4R α . Analysis of multiple copies of the DARPin molecule in the crystal indicates the conformational instability in the N-terminal cap that was predicted from molecular dynamics simulations. Comparison of the DARPin structures in the free state and in complex with IL-13 reveals a concerted movement of the ankyrin repeats upon binding resulted in the opening of the binding site. The induced-fit mode of binding employed by DARPin 6G9 is very unusual for DARPins since they were designed as particularly stable and rigid molecules. This finding shows that DARPins can operate by various binding mechanisms and suggests that some flexibility in the scaffold may be an advantage.

Key words: crystal structure, DARPin, epitope, IL-13, induced fit

Introduction

Interleukin-13 (IL-13) is a cytokine secreted by T helper type 2 (Th2) cells and associated primarily with the induction of airway disease as well as dermal fibrosis and atopic dermatitis (Zhu *et al.*, 1999; Wills-Karp, 2004). Elevated IL-13 levels have been detected in the airways and sputum of patients with asthma (Saha *et al.*, 2008). IL-13 signaling is mediated via the heterodimeric IL-13R α 1/IL-4R α complex shared by IL-4 (Wills-Karp, 2004). IL-13 also binds to a second receptor, IL13R α 2, that functions primarily as a decoy receptor with a role in TGF β production and fibrosis (Fichtner-Feigl *et al.*, 2006). Therefore, therapeutic agents that can inhibit binding of IL-13 to either of its receptors may be useful for intervening in all IL-13 mediated pathologies. Anti-IL-13 neutralizing antibodies have demonstrated the therapeutic

potential for the treatment of asthma and other atopic diseases (Mitchell *et al.*, 2010).

To overcome certain limitations of antibodies and to broaden the therapeutic choice, alternative non-immunoglobulin scaffolds have been employed to produce binding proteins with desired properties (Gebauer and Skerra, 2009). Designed ankyrin repeat proteins (DARPins[®]) are very promising in offering some advantages over antibodies for target binding in drug discovery and drug development (Binz *et al.*, 2004; Stump and Amstutz, 2007; Tamaskovic *et al.*, 2012; Plückthun, 2015). The ankyrin elementary module is composed of 33 amino acids folded into a β -turn followed by two antiparallel α -helices and a loop connected to the next repeat. Several repeats, typically two or three, are stacked together through

hydrophobic interactions and are capped on both sides with the N- and C-terminal repeats that present a hydrophilic surface to the outside. DARPin have both the convex and concave surfaces suitable for binding different types of targets. Target recognition is achieved by randomization of several positions of potential interaction residues within each repeat.

In collaboration with Molecular Partners AG, DARPin 6G9 was developed to bind human IL-13 with high affinity and block signaling through its receptor IL-13R α 1/IL-4R α . DARPin 6G9 binds human IL-13 with a K_D in the picomolar range and exhibits cross-reactivity towards cynomolgus monkey IL-13 (Jacobs *et al.*, 2014). The crystal structure of DARPin 6G9 alone and in complex with cyno IL-13 was determined to support the DARPin design and to enable analysis of conformational changes upon binding IL-13. Comparison of the structures indicated an induced-fit mode of DARPin binding to the target which is unusual for this class of proteins.

Materials and Methods

Proteins

Cynomolgus monkey IL-13 with the N-terminal SUMO tag was expressed in *Escherichia coli* strain BL21 (DE3). It was purified by affinity chromatography using a HisTrap column (GE Healthcare) equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole). The protein was eluted using a gradient of Buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole). The expression SUMO tag was cleaved by the SUMO protease (Lifesensors). The protein was further purified by size-exclusion chromatography using a Superdex 200 column (GE Healthcare) in 20 mM Tris, pH 7.4, 100 mM NaCl.

The numbering of residues throughout this report follows the scheme utilized in the other PDB depositions (LaPorte *et al.*, 2008; Teplyakov *et al.*, 2009) so that Residue 1 corresponds to Residue 20 of UniProtKB entry Q0PW92 (IL-13 from *Macaca fascicularis*). The full sequence of cyno IL-13 (Residues 1–132) corresponds to Residues 15–146 of human IL-13 (UniProtKB entry P35225).

DARPin 6G9 with the N-terminal 6xHis tag was expressed in *E. coli* strain BL21 (DE3) and purified by affinity chromatography using a HisTrap column (GE Healthcare) equilibrated with Buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM imidazole). The protein was eluted using a gradient of Buffer B (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 250 mM imidazole). The pooled fractions were diluted 1:10 with 20 mM Bis-Tris, pH 6.8, and further purified by ion exchange chromatography using a HiTrap Q FF column (GE Healthcare) equilibrated with Buffer A (20 mM Bis-Tris, pH 6.8, 10 mM NaCl). The protein was eluted using a gradient of Buffer B (20 mM Bis-Tris, pH 6.8, 600 mM NaCl). The protein was further purified by ion exchange chromatography using a Mono Q column (GE Healthcare) equilibrated with Buffer A (20 mM Tris, pH 7.4). The protein was eluted using a gradient of Buffer B (20 mM Tris, pH 7.4, 1 M NaCl).

Crystallization

IL-13 and DARPin 6G9 were concentrated to 20 mg/mL in 20 mM Tris pH 7.4, 50 mM NaCl. The complex was prepared by mixing IL-13 and DARPin at a molar ratio of 1.1:1 (excess IL-13) and incubated for 2 h at 4°C. The unbound species were separated by size-exclusion chromatography using a Superdex 200 column. For

crystallization, the complex was concentrated to 15 mg/mL in 20 mM HEPES pH 7.5, 100 mM NaCl.

Crystallization was carried out by the vapor-diffusion method at 20°C using an Oryx4 robot (Douglas Instruments). The experiments were composed of equal volumes of protein and reservoir solution in a sitting drop format in 96-well Corning 3550 plates. The screening was performed with the Hampton HT Screen and in-house screens. Large multifaceted crystals of DARPin 6G9 suitable for X-ray analysis were obtained from 0.4 M $\text{NH}_4\text{H}_2\text{PO}_4$ (Hampton conditions A3). The plate-shaped crystals of the complex were obtained after optimization of the initial hits from 11% PEG 3350, 0.2 M Li_2SO_4 in 0.1 M Na acetate buffer, pH 4.5. The crystal data are given in Table I.

X-ray data collection and structure determination

For X-ray data collection, the crystals were soaked for a few seconds in the respective mother liquor supplemented with 20% glycerol and were frozen in liquid nitrogen. Diffraction data were collected at the Swiss Light Source synchrotron over a 180° crystal rotation with 0.25-s exposures per 0.25°-image and were processed with the program XDS (Kabsch, 2010). X-ray data statistics are given in Table I.

The DARPin 6G9 structure was solved by molecular replacement using PDB entry 1J8S (Sennhauser *et al.*, 2007) as a search model. All crystallographic calculations were performed with the CCP4 suite of programs (Winn *et al.*, 2011). Model adjustments were carried out using the program Coot (Emsley *et al.*, 2010). The refined DARPin structure was used as a search model to solve the structure of the complex. The refinement statistics are given in Table I.

Accession numbers

The atomic coordinates and structure factors were deposited in the Protein Data Bank under accession codes 5KNG (DARPin 6G9) and 5KNH (DARPin:IL-13 complex).

Results

Unbound DARPin 6G9

The crystal structure of DARPin 6G9 was determined at 1.35 Å resolution. Apart from the N-terminal His tag, the entire polypeptide chain can be traced in the electron density. There are four DARPin molecules in the asymmetric unit. Molecules A and C as well as Molecules B and D superimpose very well with the RMSD of only 0.26 Å for all C α atoms. However, the two pairs deviate substantially in the relative position of the N-cap which includes the first two helices. The second of the helices (Residues 26–36) is off by up to 2 Å when the structures are superimposed on the three ankyrin repeats. This difference indicates some flexibility within the molecule and perhaps is a sign of the imperfect packing of the N-cap to the following ankyrin repeat. For comparison, no significant deviation is detected in the C-cap. The observed differences in the N-cap are most likely caused by the different crystal environment of the DARPin molecules.

The atomic B factors averaged over the main-chain atoms in Molecule A are plotted in Fig. 1. The other three molecules have similar distributions, all of which indicate an elevated mobility of the β -turns of the ankyrin repeats as compared to α -helices. The N- and C-termini are somewhat disordered as also reflected in the weaker electron density.

Table I. Crystal data, X-ray data and refinement statistics

	DARPin® 6G9		IL-13 + DARPin® 6G9	
PDB code	5KNG		5KNH	
<i>Crystal data</i>				
Space group	R3		C222 ₁	
Unit cell axes (Å)	96.08, 96.08, 204.43		52.41, 78.49, 119.44	
Molecules per asymmetric unit	4		1	
V _m (Å ³ /Da)	2.52		2.0	
Solvent content (%)	51		39	
<i>X-ray data</i>				
Resolution (Å)	30–1.35	(1.39–1.35)	30–1.60	(1.64–1.60)
Number of measured reflections	799 852	(58 459)	210 223	(14 049)
Number of unique reflections	156 050	(11 473)	32 998	(2329)
Completeness (%)	100	(100)	99.6	(96.4)
Redundancy	5.1	(5.1)	6.4	(6.0)
R-merge (I)	0.055	(0.469)	0.065	(0.542)
< I/σ >	15.5	(3.3)	17.1	(3.7)
B-factor (Wilson plot) (Å ²)	21.3		25.7	
<i>Refinement</i>				
Resolution (Å)	15–1.35		15–1.60	
Number of all atoms	5348		2129	
Number of water molecules	699		218	
R-factor (%)	0.174		0.169	
R-free (%)	0.197		0.196	
RMSD bond lengths (Å)	0.007		0.008	
RMSD bond angles (°)	1.1		1.1	
Mean B-factor (Å ²)	22.4		23.2	

Numbers in parentheses refer to the highest-resolution shell.

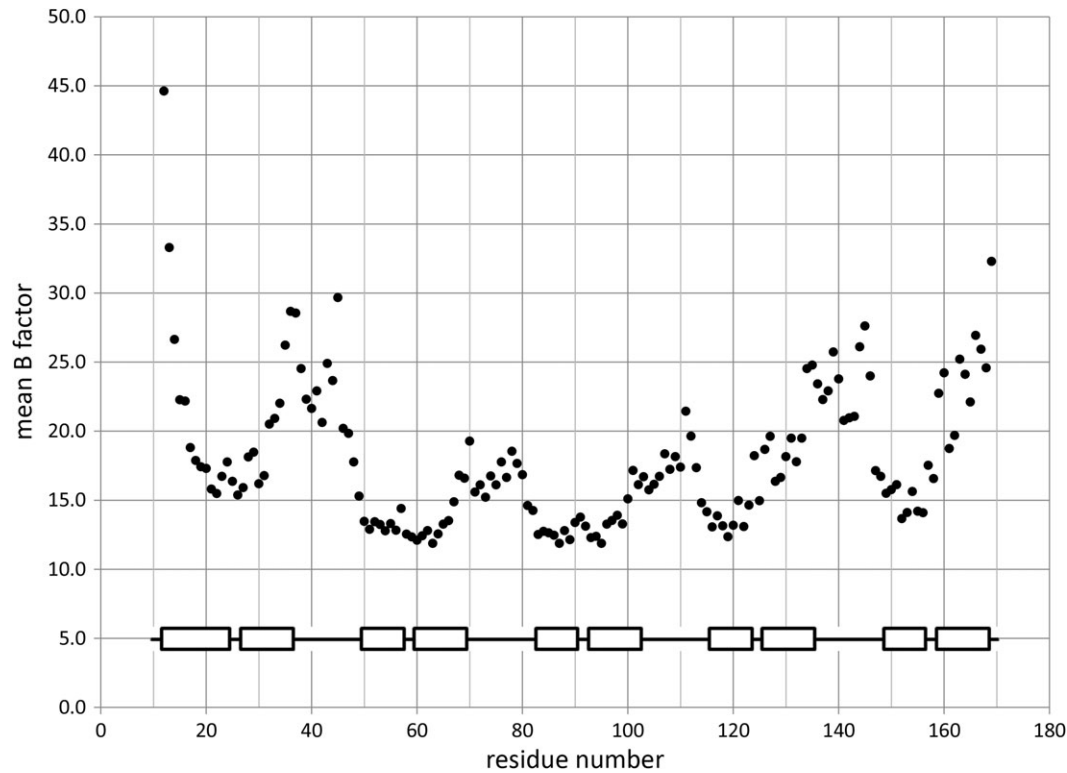


Fig. 1 Distribution of mean atomic B factors along the protein chain in Molecule A. Boxes indicate positions of α-helices in the sequence.

DARPin 6G9 in complex with IL-13

The structure of the IL-13:DARPin complex was determined at 1.6 Å resolution. The polypeptide chain of IL-13 folds into a 4-helical bundle where Helices A and B run in one direction and Helices C and D run in the opposite direction. Consequently, the loop connecting Helices B and C is short, whereas the crossover loops AB and CD are long. The IL-13 structure is available for comparisons from the receptor complexes and antibody complexes determined previously (LaPorte *et al.*, 2008; Teplyakov *et al.*, 2009; Fransson *et al.*, 2010; Lupardus *et al.*, 2010; Ultsch *et al.*, 2013).

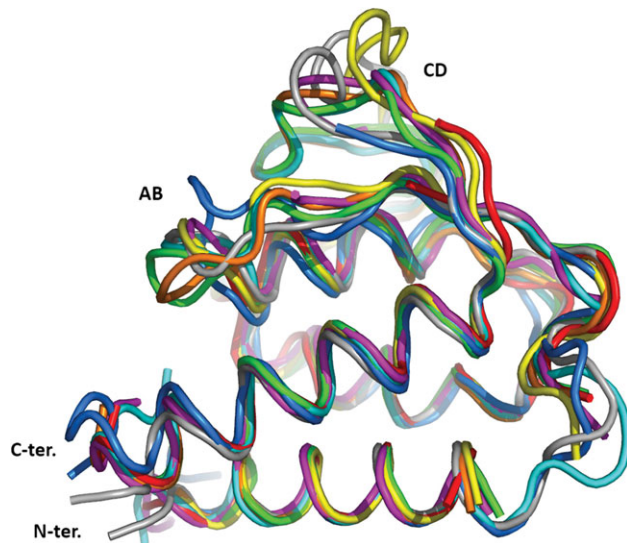


Fig. 2 Superposition of IL-13 structures from the complexes with DARPin (this work, blue), IL-4R α /IL-13R α 1 (3BPO, magenta), IL-13R α 2 (3LB6, cyan), CNTO607 Fab (3G6D, gray), C836 Fab (3L5W, yellow), H2L6 Fab (3L5X, orange), M1295 Fab (4PS4, red) and lebrikizumab (4I77, green). Long loops AB and CD are disordered and have missing residues in some structures.

The orientation of helices is essentially the same in all structures (Fig. 2). Some differences at the N- and C-termini may be due to interactions with the corresponding binders. Most deviations occur in the loop regions, and in many structures those loops are disordered.

The receptor chains, IL-4R α and IL-13R α 1, bind IL-13 at the surfaces formed by Helices A, C and D (LaPorte *et al.*, 2008). All structurally characterized mAbs compete for the same epitopes (Teplyakov *et al.*, 2009; Fransson *et al.*, 2010; Ultsch *et al.*, 2013). DARPin 6G9 binds IL-13 at the surface formed by Helices A and D. The major groove of the DARPin molecule accommodates Helix D, while the ridge formed by four β -turns of the ankyrin repeats fits into the space between Helices A and D (Fig. 3B). The neutralization effect of DARPin 6G9 is due to blocking the IL-13 interaction with the receptor chain IL-13R α 1 (Fig. 3A). DARPin 6G9 would not interfere with the other receptor chain, IL-4R α .

Comparison of the DARPin structures in the complex and in the unbound form indicates that the DARPin molecule opens by $\sim 8^\circ$ upon binding IL-13 (Fig. 4A). The structures were superimposed using the C α atoms of the N-cap (Residues 12–43). The following three ankyrin repeats and the C-cap show significant deviation between the structures with the magnitude gradually increasing towards the C-terminus. The RMSDs for repeats one, two and three and the C-cap are 1.1 Å, 1.5 Å, 2.6 Å and 3.5 Å, respectively, when superimposed on Molecule A of the free DARPin. When superimposed on Molecule B, the deviations are also consistent with the opening of the DARPin molecule, although they are twice smaller in magnitude and range from 0.4 Å to 1.9 Å. This is because the N-cap in the bound DARPin matches its relative position in Molecule B. Consequently, the RMSD for the first internal repeat is also very small (0.4 Å).

The large-scale rearrangement in the DARPin molecule upon binding IL-13 is achieved by rigid-body movements of the repeats and the terminal caps, all of which maintain their individual conformation. Crystal contacts are unlikely to be the source of this conformational rearrangement. On the one hand, the repeats of the four unbound DARPin molecules superimpose very well despite different

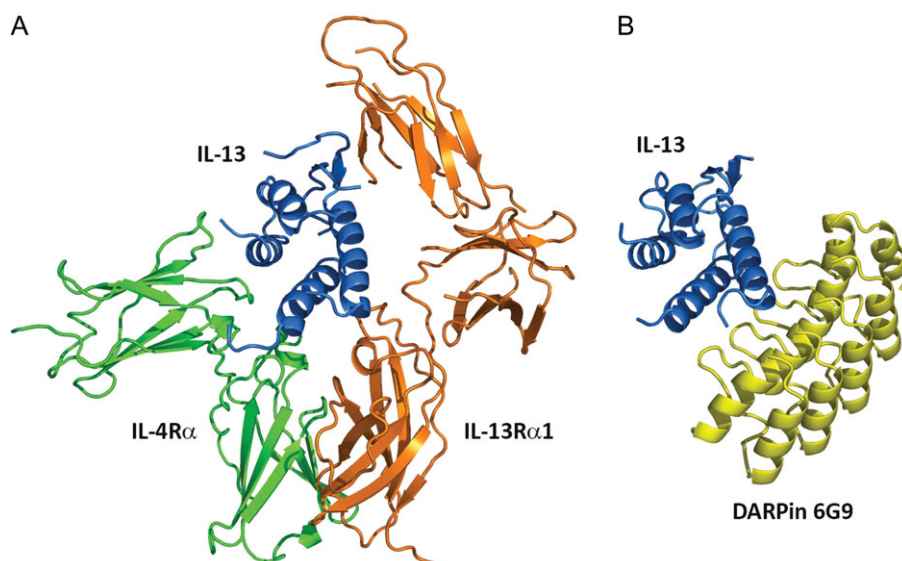


Fig. 3 IL-13 interaction with the receptor and with DARPin 6G9. (A) IL-13 complex with IL-4R α /IL-13R α 1 receptor (LaPorte *et al.*, 2008). (B) IL-13:DARPin 6G9 complex. IL-13 is in the same orientation as in (A).

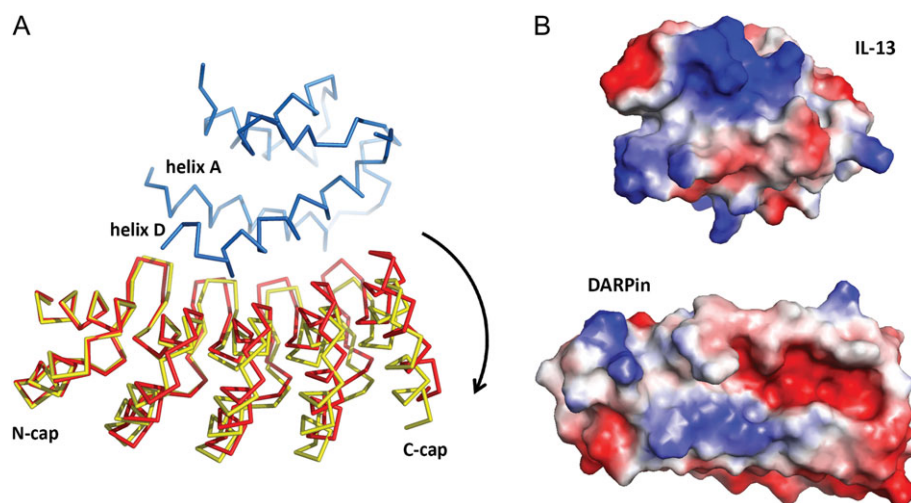


Fig. 4 (A) C-alpha trace of the complex between IL-13 (blue) and DARPin 6G9 (yellow) superimposed on the free DARPin structure (red) using the C α atoms of the N-cap. The arrow indicates 'opening' of the DARPin molecule upon binding IL-13. (B) Electrostatic potential of the interacting surfaces of IL-13 and DARPin (positive blue, negative red). Open-shell view with respect to (A).

crystallographic environment suggesting that the scaffold is rigid enough to withstand the crystal lattice forces. On the other hand, that conformation is inconsistent with the complex. When superimposed on the first repeat, the clashes with IL-13 would occur at the second repeat and the C-cap. Reciprocally, when superimposed on the third repeat, the clashes occur at the first repeat. This indicates the induced-fit rearrangement of the DARPin molecule in the complex.

IL-13-DARPin interface

Target recognition by DARPin 6G9 involves all four β -turns and four out of five helices forming the groove. The interface is extensive and covers nearly 1000 Å² on each molecule. In total, 20 DARPin residues are involved in the interactions, based on the 4-Å cut-off distance. The binding epitope on IL-13 includes 17 residues, 10 from Helix D and 7 from Helix A.

DARPin 6G9 binds IL-13 through both the groove and the ridge. Intermolecular interactions at the ridge are mostly hydrophobic. Interactions at the groove involve both hydrophobic and charged residues. Although charged residues play a major role in the interactions, their distribution is quite unexpected. The binding surface of IL-13 formed by Helices A and D is positively charged due to a number of basic residues (Fig. 4B). The groove of DARPin, however, also bears a positive charge in the N-terminal half, i.e. exactly where it binds IL-13. This cluster includes Arg23, Arg56 and Arg90 and apparently is balanced by the IL-13 C-terminal charge and the dipole of Helix D. The acidic patch in the C-terminal half of the groove that includes Asp77, Asp81, Asp110, Glu114, Asp143, Asp151 and Asp155 does not contribute much to the short-range interactions, however may provide a long-range electrostatic attraction.

The C-terminal residue of IL-13, Asn113, is a key element of the epitope (Fig. 5). Besides the van-der-Waals contact to Tyr46, it forms six hydrogen bonds to the DARPin residues Asp44 and Ser48 (through the side chain) and Arg23 and Arg56 (through the carboxyl group). The dead-end mode of binding suggests that DARPin 6G9 must be sensitive to the presence of Asn113 as the C-terminal

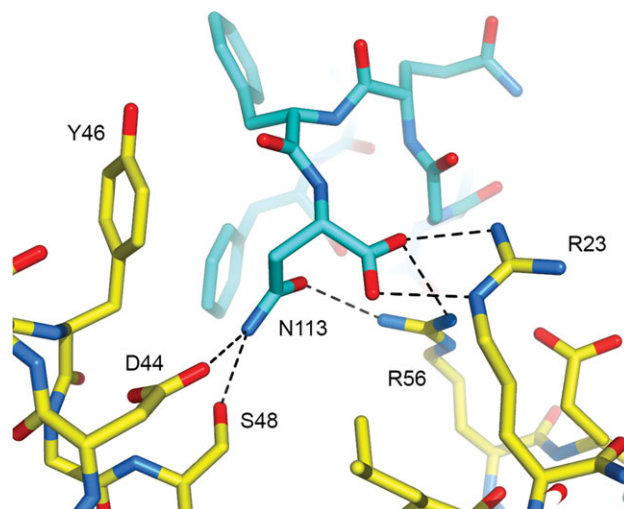


Fig. 5 Interactions at the C-terminus of IL-13. DARPin residues are yellow, IL-13 residues are blue. Hydrogen bonds are shown as dashed lines.

residue of IL-13. This is one of the reasons DARPin 6G9 would not bind mouse IL-13, which is one residue shorter at the C-terminus.

Discussion

Neutralization mechanism

IL-13 signals through cell surface heterodimeric receptor composed of two chains, IL-13R α 1 and IL-4R α . IL-13 binds IL-13R α 1 first with low affinity ($K_D = 40$ nM) and then recruits IL-4R α to the complex, generating a high-affinity receptor ($K_D = 0.5$ nM) (Andrews *et al.*, 2002). Recent study (Tiware *et al.*, 2016) has shown that the clearance of IL-13 in patients treated with anti-IL-13 mAbs with similar pharmacokinetic profiles may be epitope dependent.

The crystal structure of the IL-13:DARPin complex reveals the DARPin binding epitope, which is located at Helices A and D of IL-13. The structure suggests that DARPin 6G9 blocks the interaction of IL-13 with IL-13R α 1, but not with IL-4R α . Interfering

with the formation of the initial IL-13:IL-13R α 1 complex may have certain benefits since DARPin would compete for IL-13 with the low-affinity receptor chain (IL-13R α 1) rather than with the high-affinity chain (IL-4R α).

Cynomolgus monkey IL-13 used in this work differs from human IL-13 in seven positions. All of them are outside the DARPin 6G9 epitope, which explains its species cross-reactivity. One of the differences between human and monkey IL-13 is at Position 111 (Arg in human, Gln in monkey). Interestingly, the human IL-13 natural variant R111Q (aka R130Q) is known to be associated with bronchial asthma in several ethnically diverse populations (Heinzmann *et al.*, 2000). This variant has a lower affinity for IL-13R α 2 than the wild type, whereas both types show the same affinity for IL-13R α 1, which would cause up-regulation of the IL-13 concentration in the body (Arima *et al.*, 2002). Mutational studies have led to a conclusion that the substitution R111Q may change the conformation of IL-13 (Arima *et al.*, 2005). Our results do not support this hypothesis. The structure of monkey IL-13, which has the same substitution R111Q with respect to human IL-13, is essentially identical to the human IL-13 structures determined to date. The similarity is even more convincing since all IL-13 structures were in the form of complexes with other molecules. Recent structures of human IL-13 with IL-13R α 1 (LaPorte *et al.*, 2008) and with IL-13R α 2 (Lupardus *et al.*, 2010) have shown that Residue 111 is not involved in the ligand-receptor interactions. Therefore, it is difficult to explain the decrease in affinity for IL-13R α 2 of the R111Q variant in structural terms.

Randomization design

The target recognition by DARPin is achieved by randomization at five positions of potential interaction residues within each repeat (red and blue in Fig. 6). The residues may be any natural amino acids except Cys, Gly and Pro. The binding surface of DARPin 6G9 is quite extended and includes both the groove and the ridge. Not

surprisingly, a number of residues in the β -turns are hydrophobic since the ‘ridge’ intercalates into the hydrophobic interior between Helices A and D. Residues in the groove are mostly polar since they interact with a hydrophilic surface of Helix D.

Interestingly, almost half of the residues forming the binding paratope were not randomized. Three of them are not within the variable ankyrin repeats but rather in the terminal caps, Arg23 in the N-cap, Lys144 and Phe145 in the C-cap. Arg23 was frequently found to interact with various target proteins (Seeger *et al.*, 2013). In DARPin 6G9 it is the key anchor that binds the C-terminal carboxyl group of IL-13. In addition to these, invariant Asp residues in the first position of each β -turn that were preserved to stabilize the β -turn, appeared to form numerous hydrogen bonds to IL-13 residues. Taken together, this demonstrates a remarkable adaptability of the DARPin scaffold to recognize the target through both the ridge and the groove as well as through both randomized and non-randomized residues.

The importance of providing a continuous interaction surface was reiterated in a recent analysis of DARPin complexes that showed that DARPins tend to exclude their unrandomized caps from binding the target (Schilling *et al.*, 2014). All randomized N-cap positions were involved in forming the interface suggesting that cap randomization can be employed to further increase the continuous interaction surface of DARPins. Our results support this view as both caps of DARPin 6G9 are involved in binding even though they were not randomized.

Flexibility of DARPins

A series of DARPin variants were designed to increase stability of the molecule by improving the packing of the N- and C-caps to the adjacent internal repeat. The new design made the C-cap more similar to the consensus sequence of the internal repeats (Kramer *et al.*, 2010; Interlandi *et al.*, 2008). Notably, the Ala to Pro substitution restored the conserved TPLH motif while the C-terminal extension by three residues elongated the helix. Both changes together with some other mutations were implemented in the C-cap utilized in the present study. Based on the comparison of the bound and unbound forms of DARPin 6G9 and the distribution of temperature factors in the structures, one may conclude that the C-cap was indeed stabilized.

DARPin 6G9 utilizes the original design of the N-cap (Kohl *et al.*, 2003). The crystal structure of this DARPin with four independent molecules shows multiple conformations of the N-cap indicative of its loose packing to the first internal repeat which may lead to the instability of the entire molecule. Indeed, the N-cap was considered a possible reason for the two-state unfolding of DARPin in GdnHCl where a pre-transition occurred at 3.7 M GdnHCl, before the main transition at 5.6 M GdnHCl (Interlandi *et al.*, 2008). Subsequently the N-cap has been stabilized by mutation of residues Met34-Ala35-Asn36 in the loop following the second helix. Substitution of Leu for Met34 was the most beneficial (Binz, 2012).

While the DARPin molecule becomes more rigid through improved intramolecular interactions, it gains stability but loses flexibility. In the case of DARPin 6G9, the imperfect N-cap appeared to be an advantage as it allowed some ‘breathing’ of the molecule that promoted binding of the target. The relative position of the N-cap in the complex exactly corresponds to that observed in Molecules B and D in the unbound state indicating that this ‘open’ conformation fits the target better than the ‘closed’ one.

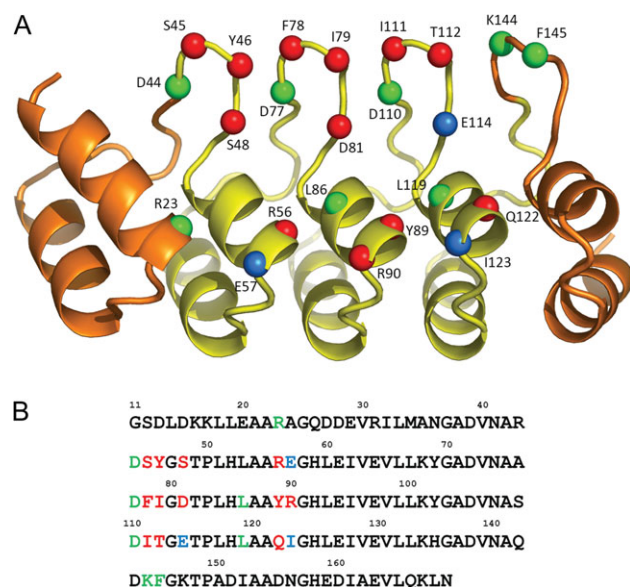


Fig. 6 (A) Ribbon presentation of DARPin 6G9 with variable and binding positions shown as spheres. Internal repeats are yellow, N- and C-caps are orange. Residues involved in IL-13 binding are red and green, residues varied in the selection process are red and blue. (B) Amino acid sequence of DARPin 6G9 aligned by ankyrin repeats. Residue colors as in (A).

Moreover, not only the N-cap but the entire DARPIn molecule displays a remarkable conformational flexibility upon binding IL-13. The concerted rearrangement of each repeat with respect to the adjacent repeats results in the more open binding site. The induced-fit mode of operation of DARPIn 6G9 is unusual among DARPins that were designed to be rigid. It was even postulated that target binding by DARPins is governed by rigid-body interactions, and conformational changes of the DARPIn backbone do not occur on binding (Binz *et al.*, 2004). Although this mode of binding is entropically favored, some epitopes on target proteins might be accommodated more easily through a higher degree of flexibility. The effect is probably more pronounced for the extended interfaces where mutual adjustment of the DARPIn and target protein may be of prime importance.

One example of the extreme induced fit was reported by Bandeiras *et al.* (2008) who studied the DARPIn complex with Plk-1 kinase. Interaction with the target triggered the complete unfolding of the C-cap that was apparently destabilized by a frame-shift mutation of the five C-terminal residues. The intact C-cap would have clashed with Plk-1. The removal of the C-cap in a DARPIn variant dramatically improved binding to Plk-1.

The fact that induced-fit binding by DARPins is rarely observed may simply reflect the lack of structural studies of DARPins in both bound and unbound states that would reveal accompanying conformational changes. The present study provides such an example that will advance our understanding of the molecular mechanisms of target recognition and facilitate the design of novel binding proteins.

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