



METHODOLOGY

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Directed evolution of a three-finger neurotoxin by using cDNA display yields antagonists as well as agonists of interleukin-6 receptor signaling

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Abstract

Background: Directed evolution of biomolecules such as DNA, RNA and proteins containing high diversity has emerged as an effective method to obtain molecules for various purposes. In the recent past, proteins from non-immunoglobulins have attracted attention as they mimic antibodies with respect to binding potential and provide further potential advantages. In this regard, we have attempted to explore a three-finger neurotoxin protein (3F). 3F proteins are small (~7 kDa), structurally well defined, thermally stable and resistant to proteolysis that presents them as promising candidates for directed evolution.

Results: We have engineered a snake α -neurotoxin that belongs to the 3F family by randomizing the residues in the loops involved in binding with acetylcholine receptors and employing cDNA display to obtain modulators of interleukin-6 receptor (IL-6R). Selected candidates were highly specific for IL-6R with dissociation constants and IC50s in the nanomolar range. Antagonists as well as agonists were identified in an IL-6 dependent cell proliferation assay. Size minimization yielded peptides of about one-third the molecular mass of the original proteins, without significant loss of activities and, additionally, lead to the identification of the loops responsible for function.

Conclusions: This study shows 3F protein is amenable to introduce amino acid changes in the loops that enable preparation of a high diversity library that can be utilized to obtain ligands against macromolecules. We believe this is the first report of protein engineering to convert a neurotoxin to receptor ligands other than the parent receptor, the identification of an agonist from non-immunoglobulin proteins, the construction of peptide mimic of IL-6, and the successful size reduction of a single-chain protein.

Background

In vitro evolution of proteins is an increasingly promising approach for introducing desired, novel changes that can modulate the properties and/or functions of proteins [1]. In this regard, technologies such as phage display, ribosome display, mRNA/cDNA display and others [2-6] that couple the phenotype (expressed proteins) to their genotype (DNA, mRNA or cDNA) have shown

considerable promise, allowing proteins with desired functions to be selected from large totally random and scaffold libraries [7].

Protein scaffolds can be either naturally occurring or *de novo* synthesized, and have defined structures that contain amenable regions such as loops that can be engineered to accommodate completely novel properties, in particular binding and inhibition [8,9]. Disulfide-containing scaffolds, such as α -amylase inhibitor (tendamistat), bovine pancreatic trypsin inhibitor (BPTI; Kunitz domain), EETI-II (knottin) and related proteins are attractive due to their small size, defined structures and remarkable stability. These scaffolds have been successfully engineered for various purposes, such as protease inhibitors, although none have been reported to

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bind or inhibit macromolecules such as receptors [8]. Here, we report the engineering of a three-finger scaffold that provides a benchmark in the development of regulatory proteins that can modulate the function of interleukin 6 receptor (IL-6R).

3F proteins are found in a variety of organisms such as the elapidae snake and mammals, including humans [10-13]. These are small proteins (MW: 7-8 kDa) with 4-5 disulfide bonds, β -structure(s) and three protruding loops that provide the topological basis for the three-finger structure [10,14]. The striking features of the 3F-protein family are strict conservation of cysteine frameworks and high sequence diversity in the loop (corresponding to finger) regions. This may reasonably make 3F toxins in nature with broad spectrum in target molecules, such as ion channels, receptors, proteases, etc [14]. This class of protein exhibits high temperature stability, resistance to proteases and low immunogenicity. The exceptional specificity of 3F proteins for their respective receptors, conferred by the residues on the tips of the loops, has been extensively investigated [15,16]. These properties make 3F proteins excellent candidates for evolutionary engineering aimed at obtaining molecules for research, diagnostics and therapeutics.

We chose IL-6R to obtain modulatory molecules from the 3F snake neurotoxin library. Interleukin-6 (IL-6) is a multifunctional cytokine regulating cell growth, differentiation, and other cellular functions [17]. The activity of IL-6 is exerted through IL-6R [17-19], and IL-6 is known to play a role in the pathogenesis of a variety of diseases such as Rheumatoid arthritis, Castleman's disease and others [18,20,21]. Therefore, molecules regulating IL-6R-mediated signaling of IL-6 are important for research, diagnostics and therapeutics. In this paper, we report the identification of modulators of IL-6R from a 3F library, and demonstrate the potential of the 3F scaffold in generating novel molecules for macromolecular receptors. The approach involved the directed evolution of the 3F protein library using cDNA display [6]. Further, we identified the functional loops by minimizing the size of the scaffold while retaining function comparable to the intact scaffold. We believe this is the first report of the engineering of a three-finger scaffold for a macromolecular receptor, the simultaneous identification of agonist and antagonist molecules of IL-6R, the generation of a peptide mimic of IL-6, and the downstream engineering of the scaffold in order to identify the functional loops.

Results

Overview of cDNA display technology

An overview of the selection process using cDNA display technology is given in Figure 1A. The puromycin linker is central to this technology and facilitates rapid

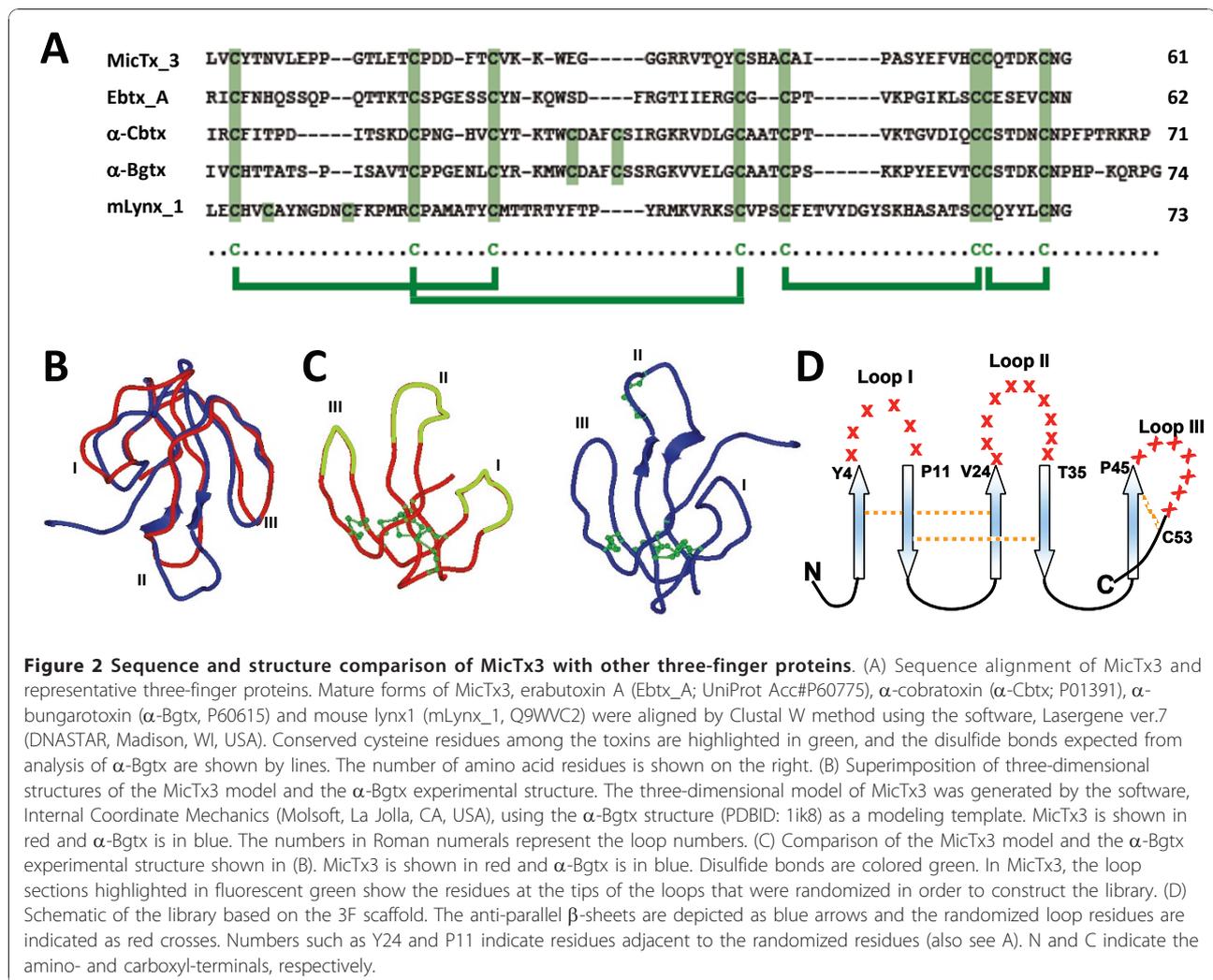
ligation of the mRNA, linking of the expressed protein to its genotype (mRNA), and rapid reverse transcription at relatively low temperature (42°C) (Figure 1B). As a result, unlike related technologies [4,5], the expressed protein is linked to the cDNA (steps 3-5) which provides stability to the complex compared to the unstable mRNA [6]. Furthermore, the stability of cDNA facilitates selection under stringent conditions such as pH, temperature, etc.

Three-finger (3F) library construction and characterization

The 3F scaffold used in this study is a novel snake α -neurotoxin, MicTx3, isolated from the South American coral snake, *Micrurus corallinus*. The cysteine framework of this protein is conserved and is similar to that of other short α -neurotoxins [10] (Figure 2A). Biochemical characterization of MicTx3 provided its affinity (29.5 ± 7.9 nM) for acetylcholine binding protein (AChBP), and electrophysiological studies of its inhibition characteristics were determined by the blockage of nicotinic acetylcholine receptor (nAChR) $\alpha 7$ currents expressed in *Xenopus* oocytes (data not shown). The amino acid residues forming the loops and β -sheets were deduced by secondary structure prediction, together with information from the literature regarding conserved amino acid residues in different toxins involved in binding to the nAChR [15,22]. Computer modeling of MicTx3 by superimposition of its spatial structure (β -sheets and loops) on those of other neurotoxins was found to be in good agreement (Figure 2B). The amino acids predicted to form the loops, including those putatively involved in binding to acetylcholine receptor, were subjected to randomization (Figure 2C). Residues T5-P10 in the Loop I (6 residues), K25-V34 in the loop II (10 residues) and A46-H52 in the loop III (7 residues) were randomized and a library constructed (Figure 2A and 2D). The amino acid residues predicted to form the β -sheets were left unaltered.

The full construct and sequences adopted for cDNA display of the 3F protein library are detailed in Additional file 1, Fig. S1 and Additional file 2, Table S1 online. After transcription, cell-free translation, cDNA display, purification and quantification, an estimated 1.2×10^{11} molecules was obtained and used for the affinity selection of IL-6R (Figure 1A).

The binding characteristics of the 3F initial library (R0) were determined by the affinity of the library for immobilized AChBP (a binder of the parental MicTx3) and IL-6R. It was found that the library binds negligibly to both IL-6R and AChBP (Figure 3A) suggesting that the library does not bind to the receptors via the constant regions (*i.e.*, non-randomized β -sheet residues). These characteristics ensure that the library is suitable for generating novel



binding molecules by randomization of the loops, followed by screening to identify the binding proteins.

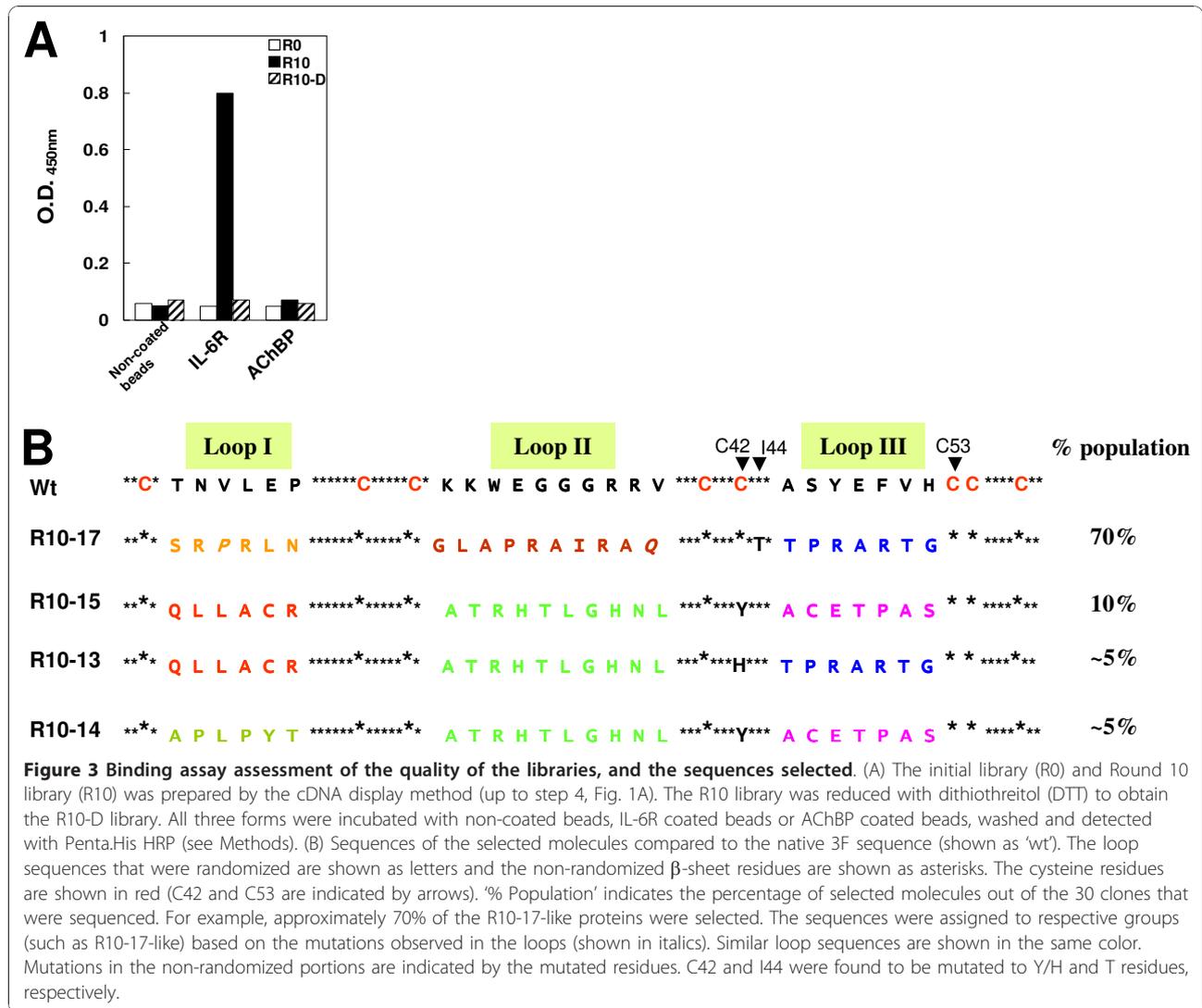
In vitro selection of the 3F library

The potential of the 3F library was assessed by searching the library for novel modulatory molecules of IL-6R. This was performed by affinity selection of binding molecules from the initial library over several rounds during which conditions were progressively made more stringent (see Methods). After 10 rounds of rigorous affinity selection, the DNA pools were cloned and sequenced.

The qualitative binding characteristics of Round 10 library (R10) were analyzed using IL-6R and AChBP (Figure 3A). The R10 displayed proteins showed negligible affinity for non-coated beads but bound significantly to IL-6R, indicating that the R10 library was enriched in comparison with R0, which did not bind to IL-6R. No detectable binding was observed to AChBP. Binding of the 3F-derived proteins is thus specific to

IL-6R. Reduction of the R10 library with DTT resulted in no binding to any of the proteins tested, indicating that binding takes place via the protein part of the displayed protein (*i.e.*, mRNA-protein) and the protein loop(s) protruded by forming disulfide bridges between cysteine residues may contribute to the specific binding.

Analysis of the sequences revealed that the pool could be majorly divided into 4 groups based on the similarity of the loop sequences (Figure 3B). R10-17-like sequences were 70% of the total clones, followed by R10-15, R10-13 and R10-14, at 5-10% each. A few mutations were observed in the constant regions, which could be due to failure of the polymerase proofreading mechanism as the library underwent totally ~650 cycles of PCR. Point mutations were also observed in the loop sequences, indicating that different amino acid residues may be accommodated in these regions. Silent mutations (different coding sequences expressing the same amino acid) were also observed at some positions.

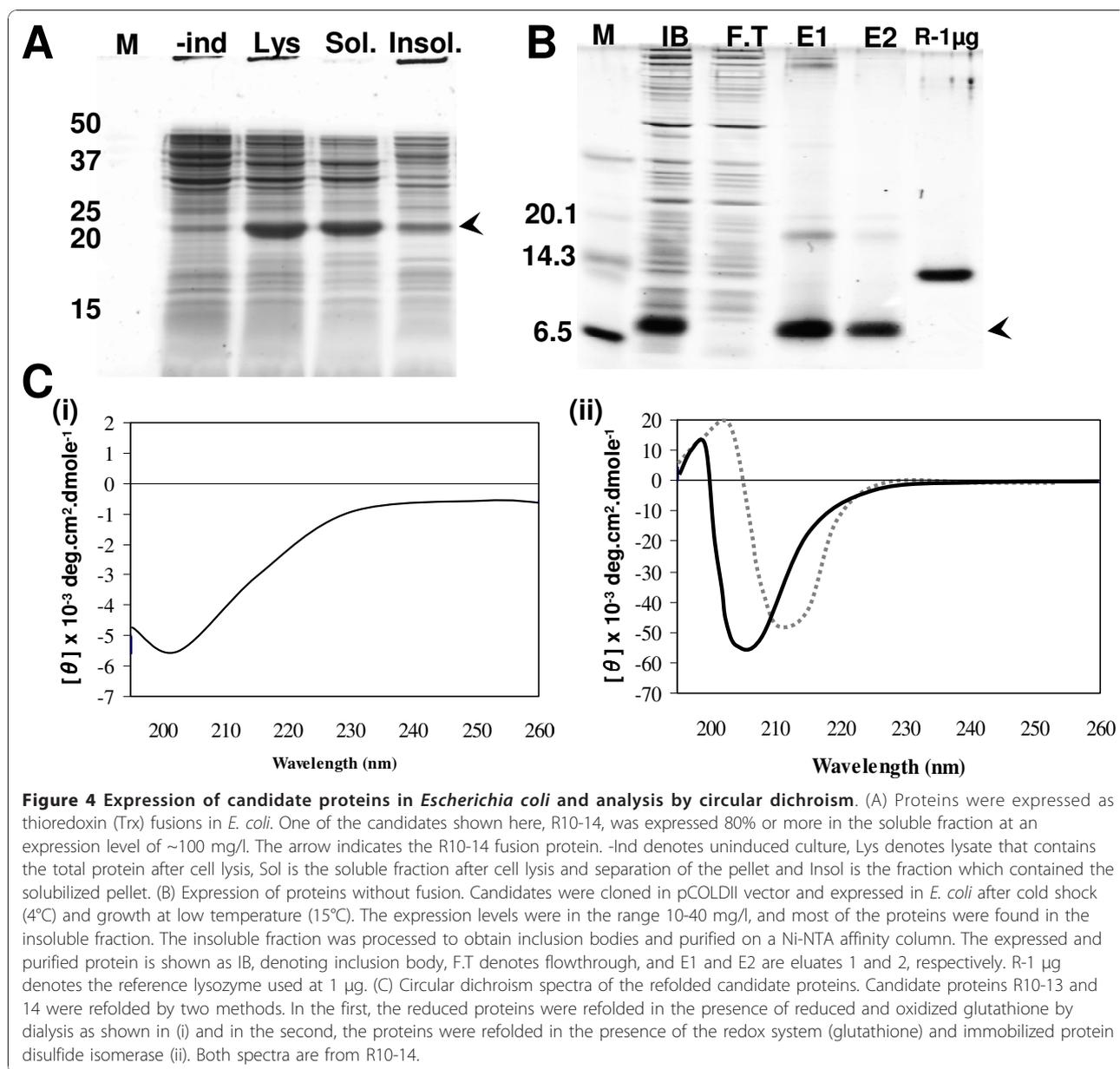


These observations support sequence specific enrichment of sequences from the library. C42, which is supposed to form a disulfide bond with C53 in the parent 3F, was found to be mutated to either Y or H in R10-13, 14 and 15. However, a cysteine residue (C47) was observed in the loop sequences of R10-14 and 15, which were subjected to randomization, so there is the possibility of a disulfide bond between C47 in the third loop and C53 in the β -sheet of the scaffold. In R10-13, no cysteine residue was selected either at the 47th position or any of the other randomized positions in the third loop, which could have implications in the folding of the molecule. The cysteine framework was intact in R10-17 which was in majority.

Expression of candidate proteins

Recombinant proteins were expressed in *Escherichia coli* by two approaches. In the first, the candidates were

fused to the C-terminal of thioredoxin (Trx) and, in general, more than 50% of the expressed protein was soluble fraction. In the case of R10-14, more than 80% soluble protein was observed (Figure 4A). In the second, proteins were expressed without the fusion and were mostly observed in the insoluble fraction. The insoluble proteins were recovered as inclusion bodies, purified and refolded under various conditions (Figure 4B and 4C). We found that proteins refolded in the presence of reduced and oxidized glutathione (a redox pair) had random conformation, as judged from their CD spectra (Figure 4C (i)). However, when immobilized protein disulfide isomerase (PDI), a chaperone known to assist and improve folding/refolding [23], was included in the redox buffer, we observed spectra resembling β -sheets (Figure 4C (ii)). Ellipticities near 200 and 215 nm (spectra in dotted line) indicate a major β -sheet conformation [24]. The fusion and refolded proteins were used



for biochemical assays and only the refolded proteins were used for biological assays.

In vitro biochemical and biological assays

The dissociation constant (K_d) was determined by the method described by Friguet *et al.* [25] with a few modifications. The values were found to be in the 48-115 nM range (Table 1), which are comparable to the dissociation constants of IL-6 (20 ± 3 ; Table 1).

The ability of the selected proteins to inhibit the interaction of IL-6 with IL-6R was analyzed by an *in vitro* biochemical competition assay. We observed that IL-6 inhibited the binding of biotinylated IL-6 to IL-6R with

an IC₅₀ value of 80 ± 3 nM, while the values obtained for R10-13, R10-14 and R10-15 were 113 ± 7 , 164 ± 9 and 102 ± 13 nM, respectively (Table 1). R10-17 was found to be non-competitive.

The selected proteins, R10-14 and R10-15, were found to be highly specific to IL-6R, with no apparent affinity for other proteins such as AChBP and IgG (Figure 5A).

Next, we carried out *in vitro* biological assays using the IL-6-dependent DS-1 human B-lymphoma cell line. As shown in Figure 5B, IL-6R mAb inhibited the IL-6 dependent proliferation of cells in a dose dependent manner (IC₅₀ = 1.2 ± 0.15 nM), and R10-13 was found to inhibit the growth of cells (IC₅₀ = 1.4 ± 0.3 μM).

Table 1 Characteristics of the selected 3F proteins and their derivative peptides

No.	Name	Sequence ¹	aa ²	Disulfide	K _d (nM) ³	Function ⁴	IC50 ⁵ (nM)	IC50/EC50 ⁶ (μM)
1	R10-17	SRPRLN GLAPRAIRAQ TPRARTG	70	-	152 ± 10	NC	NC	-
2	R10-15	QLLACR ATRHTLGHNL ACETPAS	70	-	115 ± 9	C and NI	102 ± 13	NI
3	R10-13	QLLACR ATRHTLGHNL TPRARTG	70	-	48 ± 8	C and I	113 ± 7	1.4 ± 0.3
4	R10-14	APLPYT ATRHTLGHNL ACETPAS	70	-	55 ± 6	C and A	164 ± 9	0.02 ± 0.006
5	13-L1	LVCY <u>QLLAS</u> SRPGTLETGPDFTCV	24	C3 and C23	ND	I	ND	10 ± 1.5
6	13-L2	ETCPDFTGV <u>ATRHTLGHNL</u> TOYCS	25	C3 and C24	ND	NI	ND	NI
7	13-L3	ACA <u>IP</u> TPRARTGC	13	C2 and C13	ND	NI	ND	NI
8	14-L1	LVCY <u>APLPYT</u> PGTLETGPDFTCV	24	C3 and C23	ND	A	ND	0.03 ± 0.005
9	14-L3	IP <u>ACETPAS</u> C	10	C4 and C10	ND	NI	ND	NI

¹Loop sequences of the proteins are for R10-17, 15, 13 and 14. Sequences of individual peptides are shown in full. 13-L1 represents loop 1 of the protein R10-13, bold letters indicate point mutations, and loop sequences are underlined.

²The number of residues in the proteins (e.g., 70) includes 61 residues of the protein, spacer GGS and 6xHis.

³Affinity was measured by Elisa (25). IL-6 was also assayed and the K_d value was found to be 20 ± 5 nM. ND = not determined.

⁴NC = Non-Competitive, C = Competitive, NI = Non-Inhibitory, I = Inhibitory, A = Agonist.

⁵IC50 was determined by ELISA. IL-6 was also assayed and the value was found to be 80 ± 3 nM.

⁶Measured by IL-6 dependent DS-1 proliferation assay. IC50 = concentration for 50% growth inhibition, which for monoclonal antibody was determined to be 1.2 ± 0.15 nM and ED50 = 50% effective concentration, which for IL-6 was found to be 15 ± 2 pM.

The other proteins, R10-15 and 17, showed essentially no effect on the cell growth. However, we observed that R10-14 boosted the proliferation of cells and thus speculated that this molecule could be an agonist of IL-6R. Therefore, we further investigated the potential of R10-14 to induce cell proliferation in the absence of IL-6 and found that R10-14 indeed induces proliferation even in the absence of IL-6 and thus acts as an IL-6 mimetic molecule (Figure 5C, (i) and 5C (ii)). The ED50 (50% effective dose) values were determined to be 15 ± 2 pM for IL-6 and 20 ± 6 nM for R10-14 (Figure 5C and Table 1).

Size optimization of the three-finger scaffold (3F to 1F)

Short peptides retaining the essential features of the 3F scaffold were generated by size minimization. Peptides were designed that included the sequences of the scaffold forming the β-sheets, loops and the disulfide bonds of the selected molecules (see Methods). Thus, the resulting peptides resemble one of the fingers (1F) of the three-finger protein (3F) (Figure 6A and Additional file 3, Fig. S2 online).

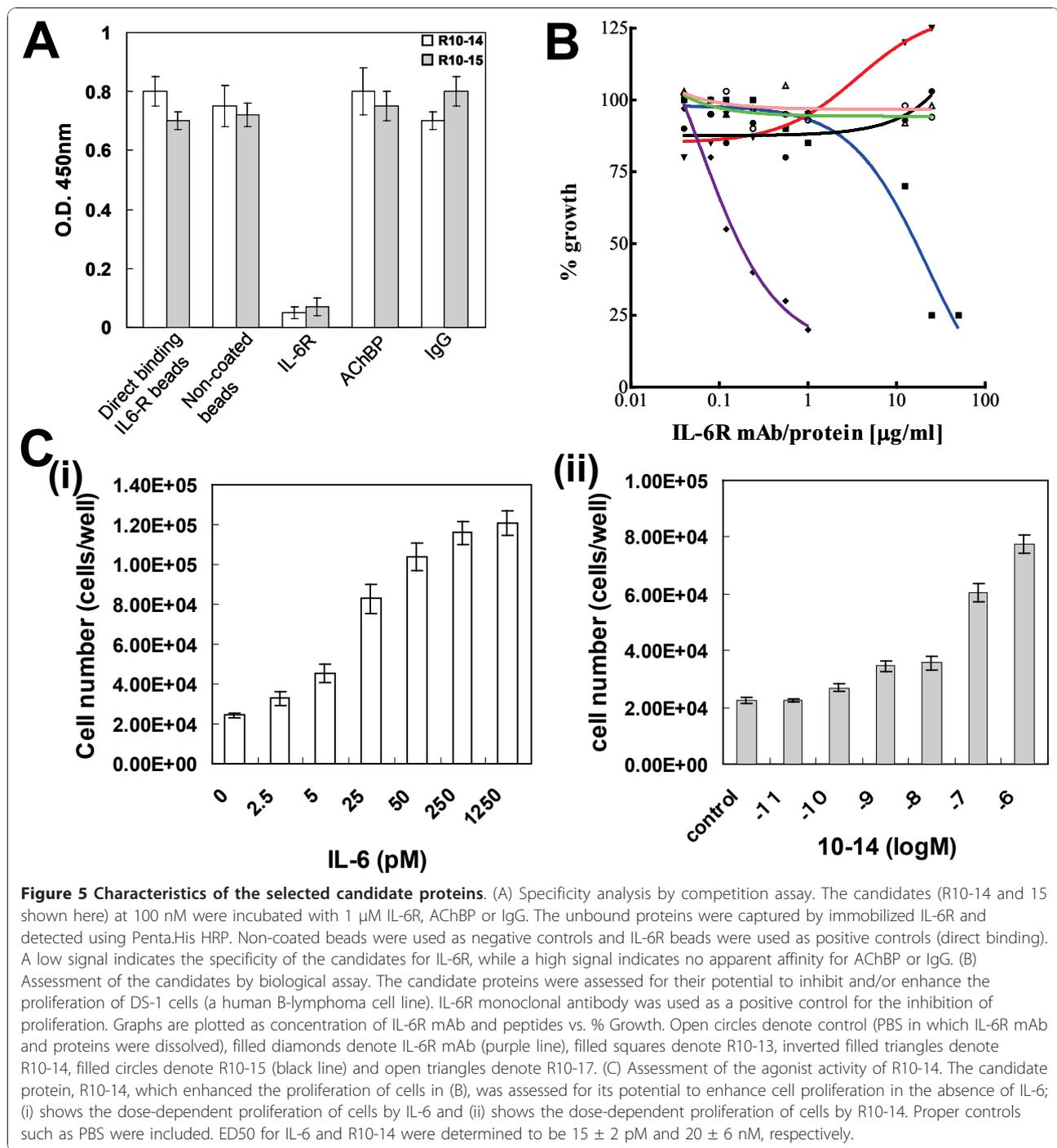
The potency of the designed peptides was assessed using the biological assay described above. In the case of R10-14 derived peptides, 13-L2 (which is identical to the 2nd finger of R10-14; also see Figure 3B) and 14-L3 were ineffective in inducing cell proliferation. However, 14-L1

(ED50 = 30 ± 5 nM) was as effective as the parent molecule, R10-14 (ED50 = 20 ± 6 nM), at inducing cell proliferation. Similarly, in the case of R10-13 derived peptides, 13-L2 and 13-L3 could not inhibit cell proliferation, however 13-L1 inhibited cell proliferation (IC50 = 10 ± 1.5 μM), albeit with a lower potency than the parent R10-13 (IC50 = 1.4 ± 0.3 μM) (Figure 6B, 6C and Table 1).

Discussion

Antibodies and its derivatives such as scFv, minibody, diabody and others have been increasingly used for protein engineering and directed evolution due to their high affinity and specificity towards their targets [26]. Recently, however, alternate protein scaffolds that provide potential advantages over antibodies such as smaller size, easier production and optimization, are showing promise and are attracting attention [8,9]. Such scaffolds are comprised of a single polypeptide chain, have defined structure, and contain amenable regions such as loops that resemble the complementary determining regions (CDR) of antibodies. These CDR-like loops can be utilized to generate large libraries that can be used to obtain molecules with completely novel properties.

In this regard, we identified and explored a new scaffold, three-finger (3F), for directed evolution and protein engineering. 3F proteins are small, compact, highly



specific, stable to thermal and protease exposure, and are found in a variety of organisms ranging from snakes to humans [10-13]. Furthermore, molecular evolution study revealed that the 3F toxin genes show accelerated evolution, by which the gene product could rapidly adapt to the environmental changes including prey species. The evolution rates are significantly higher in the finger regions. These characteristics make them

excellent candidates for protein engineering and directed evolution and, thus, potentially useful for research, diagnostic and therapeutic uses. We have utilized cDNA display for the directed evolution of a 3F scaffold library to obtain molecules that bind to and possibly regulate interleukin-6 receptor (IL-6R).

cDNA display is based on the formation of a covalent fusion between the expressed protein and puromycin

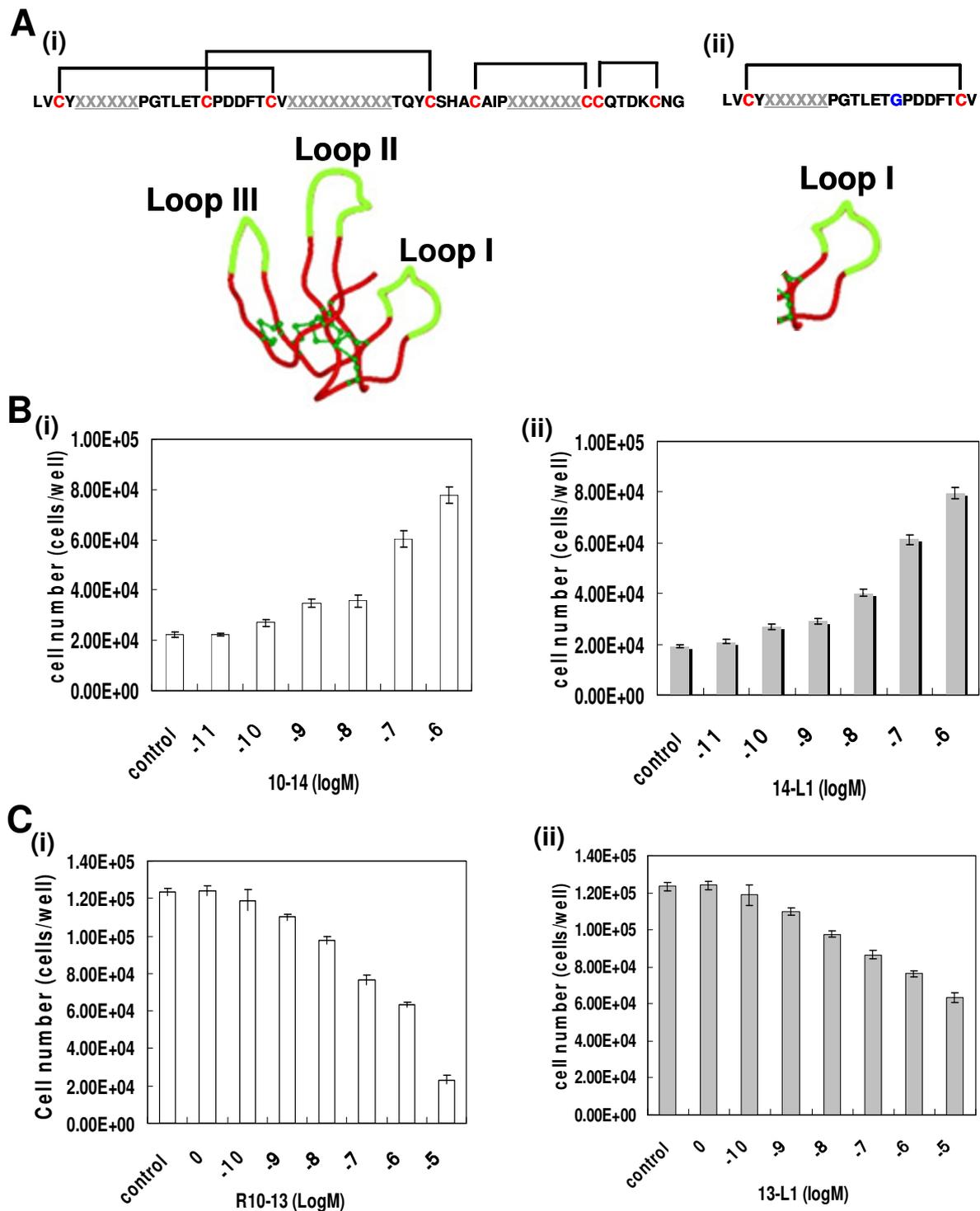


Figure 6 Biological assessment of the size-optimized peptides from the selected candidate proteins. (A) The size optimization strategy was designed to obtain peptides resembling three single fingers between 12-25 residues long (see Additional file 3 Fig. S2) from the parent candidate proteins which are 61 residues long (i). The spatial structures of the parent protein (i) and the derived peptide containing Loop I (ii) are given (disulfide bonds are shown in green and the fluorescent green denotes different loop sequences). (B) Assessment of the peptides as agonists of IL-6R. R10-14 (i) and the designed and chemically synthesized peptides 14-L1 (ii) were assessed for their ability to stimulate the proliferation of DS-1 cells in the absence of IL-6 in a dose-dependent manner. (C) Assessment of the peptides as antagonists. R10-13 (i) and the designed and chemically synthesized peptides 13-L1 (ii) were challenged in the IL-6 dependent proliferation of DS-1 cells in a dose-dependent manner.

linked to the genotype (cDNA). Briefly, a DNA linker containing puromycin is rapidly ligated to the mRNA. Following protein expression, puromycin forms a covalent fusion with the protein on the stalled ribosome [4,5]. This is followed by rapid purification of the complex, facilitated by the biotin-streptavidin pair, and subsequent cDNA synthesis utilizing the 'built-in' primer contained in the linker. Thus, the protein, which is covalently attached to the puromycin-linker, eventually gets linked to the cDNA (Figure 1A) [6]. This process is extremely fast, prevents degradation of the mRNA portion and thus contributes to prepare the library intact in quality and size, which are crucial for the subsequent selection of molecules. The cDNA display technology utilizes a cell-free translation system and, as a result, is capable of generating libraries in the order of 10^{12-14} , compared to 10^8 in the case of cell-dependent methods such as phage display [3].

Affinity selection of the 3F library aimed at identifying novel molecules binding to IL-6R enriched the library with proteins specific for IL-6R; the sequences of these proteins were divided into different major and minor groups (Figure 3). The selected candidates bound to IL-6R with affinity (nanomolar range) and specificity similar to that of antibodies in the biochemical assays. Candidates that competed with the natural ligand, IL-6, were also successfully obtained. Antagonists as well as agonists were identified biologically using an IL-6 dependent cell proliferation assay (Table 1). These results demonstrate the potentials of the 3F scaffold to accommodate residues, different from the parent residues, which are required to generate high-diversity libraries and then to utilize the libraries for obtaining novel ligands for macromolecular targets such as IL-6R by iterative selection. Disulfide-rich proteins such as knottin, tendamistat and others have been shown to be useful as templates to search for protease inhibitors, however, none have been shown to bind and/or regulate the activity of macromolecular receptors [8]. Our results show for the first time that regulatory molecules against receptors can be obtained by the directed evolution of the 3F scaffold.

The 3F scaffold provides potential advantages over related scaffold proteins that have been successfully used for directed evolution. 3F (~6.7 kDa) is smaller than many other proteins such as scFv (~30 kDa), DARPin (~20 kDa), lipocalin (~20 kDa), fibronectin (~10 kDa) and zinc-finger (~9 kDa), and is comparable to Affibody (~6 kDa); these proteins have been used for the purpose of directed evolution [9]. The number of residues in the loops that can be utilized for the construction of libraries is around 38%, which is larger than that of published scaffolds such as ~10% (scFv) [26], ~22% (Affibody) [27], ~22% (fibronectin) [28], ~26% (zinc-finger) [29], ~9%

(lipocalin) [30], ~21% (DARPin) [31], and others. The greater flexibility regarding the residues that can be randomized provides a larger interaction surface between the binding partners during the binding event. The discovery of an agonist in the 3F library also demonstrates the utility of this approach, particularly from the viewpoint that agonists have been identified from immunoglobulin sources [32-34] and random peptide libraries [35,36], but there are no published reports of agonistic molecules from non-immunoglobulin proteins [9]. The high affinity and remarkable specificity of 3F proteins for their targets, along with their stability, make this scaffold suitable for diagnostic, therapeutic and protein chip purposes. Furthermore, the presence of a single lysine (K58) near the C-terminus in the 3F molecule used in this study will facilitate homogeneous labeling of the 3F proteins with chemical moieties such as fluorescein, biotin and other labels and probes that can be exploited for research and diagnostic purposes (e.g., fluorescence imaging of tumors, protein chips, ELISA).

When the candidate proteins were expressed in *E. coli* as recombinant proteins in fusion with thioredoxin, more than 50% of the expressed proteins were found in the soluble fraction; one of the candidates, R10-14, showed more than 80% soluble protein (Figure 4A). Similarly, the candidates also expressed well at 1-2 mg/ml of cell-free lysate when produced as maltose binding protein fusions in a bacterial cell-free translation system such as RTS (Roche, Penzberg, Germany), with nearly all of the protein present in the soluble fraction and checked for their binding activities for IL-6R (data not shown). In contrast, disulfide-rich proteins are known to be poorly expressed in the native form in bacteria, and generally tend to form inclusion bodies [37]. In an attempt to produce non-fusion proteins in bacteria, we observed that the proteins aggregated as inclusion bodies (Figure 4B). However, by using a refolding system that included the redox buffer and immobilized protein disulfide isomerase, the proteins seem to be refolded from the analysis of circular dichroism (Figure 4C). Further, 3F proteins can also be successfully produced in the native state using *Pichia pastoris* as the host system [38].

The 3F proteins are leaf-like, flat molecules with the three fingers extending from a globular head [39,40]. Taking advantage of this structure, we designed a strategy to minimize the size of selected 3F proteins into three, single finger peptides, which lead to three interesting results. First, the size was successfully reduced to 24 residues compared to 61 residues in the parent 3F, and thus can be conveniently prepared by chemically synthesis and facilitate molecular dynamic studies; second, we identified the effector fingers which retained activity; third, function was observed in the 1st finger, i.e., 13-L1 and 14-L1 (Table 1).

Scaffolds based on single-chain repeat proteins such as DARPin and avimers have been successfully moderated in size (reduced or increased) due to the modular nature of parent proteins [31,41], however, there are no reports of other single-chain proteins, such as fibronectin, lipocalins and affibodies, being successfully reduced in size. The possibility of further size reduction of monomers in DARPin (the monomer consists of 33 residues) and avimers (a monomer containing 35 residues) has also not been investigated. Therefore, we believe that this is the first report of the successful reduction of a single-chain 3F scaffold to shorter peptides of only 24 residues which retain activity comparable to that of the parent protein. Recently, ~3 kDa peptides derived by rational design from V_H (complementarity region) and V_L (framework region) domains of Fab have been shown to mimic the parent antibody [42]. Encouraged by these results, the concept of single finger libraries will be interesting to pursue and might be more attractive.

IL-6 is a pleiotropic cytokine involved in the regulation of cell growth, differentiation and various other cellular functions [17], and is known to play a role in the pathogenesis of a variety of diseases [18,20,43-46] and in brain injury [47,48]. Therefore, both antagonist and agonist molecules that can regulate the IL-6R-mediated signaling of IL-6 are important for research, diagnostic and therapeutic purposes. An antibody that acts as an antagonist of IL-6R has been reported [49]; however, there are no reports of agonists of IL-6R. The present study generated R10-13, which has a size $1/20^{\text{th}}$ that of an antibody and an IC_{50} of 9.8 $\mu\text{g/ml}$ ($IC_{50} = 0.18 \mu\text{g/ml}$ of mAb, as determined in this study), and also 13-L1, which has a size $1/57^{\text{th}}$ that of an antibody, and an $IC_{50} = 26 \mu\text{g/ml}$. In different reports, IC_{50} values for peptides selected by phage display and rational design were reported in the range 25-60 $\mu\text{g/ml}$ [50,51]. 3F derived peptides are, therefore, potentially capable of inhibiting the IL-6R-mediated signaling. R10-14 ($ED_{50} = 140 \text{ ng/ml}$) and 14-L1 ($ED_{50} = 79 \text{ ng/ml}$) can potentially be developed and used as agonists of IL-6R ($ED_{50} = 0.5 \text{ ng/ml}$ of IL-6 as determined by this assay) either to supplement IL-6, or used when IL-6 is deficient.

It is noteworthy that the directed evolution of 3F library which was carried out using the soluble extracellular IL-6R generated molecules having comparable affinity and competitive potential to IL-6 (Table 1). However, in cellular assays the peptides were found to be less potent either as antagonist or agonist compared to IL-6R mAb and IL-6 showing approximately 100 times lesser activity. The antagonist and agonist molecules developed in this study, however, have comparable or better efficacy than those previously reported. Martin *et al.* obtained a clone from affibody library with

an affinity in the micromolar range and antagonist function [52], Su *et al.* [50] and Feng *et al.* [51] reported peptides with IC_{50} in the range 25-60 $\mu\text{g/ml}$. Further, phage display based selections of constrained cyclic libraries have been reported to generate agonists in the micromolar range [35,36]. The above peptides and proteins obtained in this study may be regarded as lead molecules from the viewpoint of potency, which can be increased further by exploiting affinity maturation of the parent 3F candidates [53,54] optimization by dimerization or multimerization of the peptides [55-57], and other approaches. These lead molecules were generated by directed evolution of a 3F protein library. Therefore, 3F library used in this study provides a platform to obtain regulatory molecules of IL-6 receptor and, in principle, other macromolecules.

In analogy to 3F scaffold, other peptide scaffolds originating from accelerated evolution such as inhibitor cysteine knot may also be utilized as templates for random peptide libraries.

Conclusions

We have constructed a high diversity library utilizing 3F scaffold as a template and randomizing the loops with which the parent molecule binds to its natural receptor. This library was screened against soluble extracellular IL-6R and yielded ligand molecules that bind with high affinity to the receptor comparable to IL-6. These ligands have both antagonist and agonist function as revealed in a cellular assay. We have also successfully achieved size reduction of this single chain scaffold into single fingers without significant loss of activity. Therefore, 3F proteins are promising candidates for protein engineering, and directed evolution can be used to generate molecules for research, diagnostic and therapeutic uses.

Methods

Preparation of the three-finger library

A full construct for preparing the three-finger (3F) protein library was designed to facilitate the formation and purification of cDNA displayed proteins. The 3F genetic construct was prepared by joining the fragments by overlap PCR (see Additional file 2, Table S1 and Additional file 1, Fig. S1 online). Briefly, to the sequence of the 3F library, SP6 fragment containing the SP6 promoter, cap site, *Xenopus* globin untranslated sequence (UTR) and translation initiation site was added at the 5' end, while the $(G_3-S)_2$ spacer, C-terminal 6xHis, (G_3-S) spacer and Y-tag sequences were added at the 3' end (Additional file 1, Fig. S1). The final library was expressed and purified by Ni-NTA agarose (Qiagen, Valencia, CA, USA) and the library's quality was assessed by sequencing 20 clones

picked at random after cloning them into the TA cloning vector (Invitrogen, Carlsbad, CA, USA).

Transcription of library DNAs

The above library DNAs were subjected to polyacrylamide gel electrophoresis (PAGE), purified from the gel, and then annealed by heating at 94°C and gradient cooling to form the correctly paired double-stranded DNA. The template DNAs were transcribed by SP6 RNA polymerase in a RiboMAX Large Scale Production Systems (Promega, Madison, WI, USA). Reactions were terminated by adding DNase I and products were purified using the phenol/chloroform method. RNA concentration was measured by a spectrophotometer at 260 nm.

Synthesis of the puromycin-linker

The puromycin-linker was synthesized, as described previously [6], by cross-linking two modified oligonucleotides with the hetero-bifunctional reagent, EMCS (N-(6-maleimidocaproyloxy) succinimide) (Figure 1B).

The modified oligonucleotides, Puro-F-S [5'-(S)-TC(F)-(Spec18)-(Spec18)-(Spec18)-(Spec18)-CC-(Puro)-3'] and Biotin-loop [5'-CCCGGTGCAGCTGTTTCATC(T-B)CGGAAACAGCTGCACCCCCCGCCGCCCCCGG(T)CCT-3'] were custom synthesized (BEX Co., Tokyo, Japan). The symbol (S) denotes 5'-thiol-modifier C6, (F), fluorescein-dT; (Puro), puromycin CPG; (Spec18), spacer phosphoramidite 18; (T), amino-modifier C6 dT; (T-B), biotin-dT. The underlined sequence indicates the cutting site of restriction enzyme *PvuII*. All phosphoramidite reagents used for modifications were from Glen Research (Sterling, VA, USA).

Ligation of mRNA to the linker

mRNA was annealed to the biotinylated puromycin-linker DNA (1:1 ratio) via the Y-tag sequence in 1× ligase buffer (Takara, Kyoto, Japan) by heating at 94°C and cooling slowly. mRNA and linker were ligated by the addition of T4 kinase (3 U) and T4 RNA ligase (20 U) (Takara) at 25°C for 1 h, then the conjugated product was purified using an RNeasy Kit (Qiagen). Ligation efficiency and the purity of the products were checked by polyacrylamide gel electrophoresis using FITC and/or VistraGreen (Molecular Probes, USA) staining on a fluorimager (Bio-Rad, Hercules, CA, USA).

cDNA display

mRNA-puromycin linker DNA (3-5 picomoles) was translated in 25 µl using a Retic Lysate IVT Kit (Ambion, Austin, TX, USA) at 30°C for 10 min. After translation, proteins were covalently linked to the puromycin linker in the presence of 65 mM MgCl₂ and

750 mM KCl at 37°C for 2 h. Purification was achieved using the biotin-streptavidin interaction for 10-15 min., followed by M-MLV reverse transcriptase (Takara) reaction to form the cDNA/mRNA hybrid utilizing the 'built-in' primer included in the linker (Figure 1B). Displayed proteins were released from the biotin-streptavidin complex upon digestion with 12 U of *PvuII* restriction enzyme in the supplied buffer at 37°C for 1 h. Full length displayed proteins were purified utilizing the C-terminal 6xHis-tag by Ni-NTA magnetic beads (Qiagen). The purified display proteins were quantified by fluorescence using a Beacon 2000 (Panvera, WI, USA) and a known FITC-labeled DNA as standard.

The whole process was scaled up for library preparation where 200 pmols was used for translation; the subsequent rounds were reduced to 1/5th for R2-R5 and 1/10th for R6-R10.

Library quality assessment

The affinity of the initial 3F library (R0) was checked by a binding assay for immobilized IL-6R and AChBP (see immobilization procedures in the next section). Equal amounts of 3F library prepared by cDNA display (up to step 4, Figure 1A) were incubated with non-coated beads, 250 nM IL-6R or AChBP coated beads for 1 h at room temperature in PBS. Each mixture was washed with PBS-T (0.1% Tween) and subsequently incubated with Penta.His-HRP (Qiagen) in PBS-T for 30 min. at 25°C. The mixtures were washed several times with PBS-T followed by the addition of substrate, 3, 3', 5, 5'-Tetramethylbenzidine (TMB; Sigma, Saint Louis, USA). After color development was complete, the reaction was stopped by the addition of 0.5 M H₂SO₄, each mixture was centrifuged, and absorbance was measured at 450 nm.

Preparation of immobilized proteins

Extracellular IL-6R (PeproTech, London, UK), AChBP (cDNA was cloned from *Aplysia kurodai* [manuscript in preparation] and the recombinant protein was expressed in *E. coli* and purified in our laboratory), IgG (human immunoglobulin; Sigma) and BSA (Ambion) were immobilized on NHS-activated Sepharose 4 Fast Flow (Amersham Biosciences) utilizing the amine coupling chemistries to form a chemically stable amide bond according to manufacturer's instructions. Coupling efficiency was estimated by absorbance measurements of initial and unbound protein at 280 nm. The activity of the immobilized proteins (except for BSA) was measured by ELISA using either biotinylated or 6xHis containing ligands. Non-coated beads were also prepared by the same derivatization procedure, but in the absence of protein.

Selection of IL-6R binders

The above 3F library was screened to identify molecules selectively binding to IL-6R. Each round of selection was performed with the non-coated beads and IL-6R coated beads in a batch-selection mode using Phosphate Buffered Saline (PBS) containing an additional 100 mM NaCl and 0.1% Tween-20. The initial round contained 2 nM cDNA displayed protein and 300 nM IL-6R. Prior to the selection of IL-6R binders, non-specific Sepharose binders were excluded by preincubation of the library with non-coated beads for 1 h. This was followed by incubation of this precleared library with IL-6R coated beads for 1 h. After incubation, the IL-6R beads were washed several times with the PBS/NaCl/Tween-20 buffer and eluted with the same buffer containing 100 mM DTT (dithiothreitol, Sigma). The eluted products were desalted and PCR amplified (conditions described above) with PCR forward and reverse primers (see Additional file 2, Table S1 online), purified and used as templates for PCR amplification with the SP6 fragment. The DNAs were gel purified, transcribed, mRNA ligated to puromycin linker, translated, transformed into display proteins, reverse transcribed, Ni-NTA affinity purified, quantified and used for the next round of selection. The concentration of IL-6R and the incubation time were gradually reduced from 300 nM to 30 nM and from 1 h to 15 min, respectively, while the number of washes increased progressively during further rounds of selection in order to apply selection pressure. In round 9 and 10, 30 nM biotinylated IL-6R was used.

After 10 rounds of stringent selection the eluted pool was PCR amplified, cloned into TA cloning vector (Invitrogen) and sequenced.

Assessment of the selection

Direct binding analysis using displayed proteins: Selected Round 10 library (R10) was prepared by the cDNA display method (up to step 4 of Figure 1A) and purified by spin columns (Bio-Rad). One of the fractions was denatured by treatment with 100 mM dithiothreitol (DTT) for 1 h and purified (designated as R10-D).

Purified display proteins (R10 and R10-D; 5 μ l) were mixed with non-coated beads and 250 nM of IL-6R or AChBP coated beads in PBS-BSA (0.01% BSA) and incubated at 25°C for 1 h. The mixtures were washed with PBS-T (0.1% Tween) and subsequently incubated with Penta.His-HRP (Qiagen) in PBS-T for 30 min. at 25°C. The mixtures were then washed several times with PBS-T followed by addition of the substrate, 3, 3', 5, 5'-tetramethylbenzidine (TMB; Sigma). After color development was complete, the reaction was stopped by the addition of H₂SO₄, the sample was centrifuged, and absorbance measured at 450 nm.

Protein preparation

Proteins were prepared as thioredoxin fusions using the pBAD/TOPO Thiofusion expression Kit (Invitrogen). Thioredoxin (Trx) was also prepared for control experiments. The selected candidates were PCR amplified (encoding C-terminal 6xHis), cloned into pBAD/TOPO Thiofusion expression vector and transformed according to the manufacturer's instructions. Frames were analyzed by sequencing.

The positive clones were cultured in Luria-Bertani (LB) medium containing 50 μ g/ml ampicillin at 37°C for 16 h. A small portion of the culture was transferred to fresh LB-ampicillin medium and grown to an O.D. of 0.5 at 600 nm, then the culture was induced with 0.02% arabinose for 4 h at 37°C. The cells were harvested by centrifugation at 3000 rpm for 20 min and lysed with Bugbuster Protein Extraction Reagent (Novagen, San Diego, USA) in the presence of 1 μ g/ml DNase, 1 μ g/ml RNase and protease inhibitor cocktail (Sigma) at 25°C for 20-30 min. The lysate was centrifuged and separated into supernatant (soluble fraction) and pellet (insoluble fraction) and analyzed on SDS-PAGE. The soluble fraction was purified by Ni-NTA (Qiagen) affinity for the 6xHis tag of the expressed peptide under native conditions to near homogeneity. Proteins were also confirmed by Western blotting for 6xHis by Penta.His HRP (Qiagen). Protein concentration was measured using a Bio-Rad Protein Assay Kit (Bio-Rad) with bovine serum albumin (BSA) as a standard, and by densitometry measurements of the protein bands on SDS-PAGE gels using known protein concentration standards such as Trx.

Non-fusion proteins were prepared by cloning the candidates in pCOLDII vector (Takara). The plasmids were transformed into Rosetta-gami (DE3) pLacI (Novagen). For expression, colonies were grown in LB medium containing 50 μ g/ml ampicillin at 37°C for 16 h. A small portion of the culture was added to fresh LB-ampicillin medium and grown to an O.D. of 0.5 at 600 nm. The culture was incubated at 4°C for 30 min. and induced with 0.1 mM IPTG for 15 h at 15°C. The cells were harvested by centrifugation at 3000 rpm for 20 min and lysed with Bugbuster Protein Extraction Reagent in the presence of 1 μ g/ml DNase, 1 μ g/ml RNase and protease inhibitor cocktail at 25°C for 20-30 min. The lysate was centrifuged and separated into soluble and insoluble fractions. The insoluble fraction was purified to provide inclusion bodies, dissolved in buffer containing 50 mM CAPS, pH 11.0, 0.3% N-lauroylsarcosine and 0.1% β -mercaptoethanol (Sigma), purified on a Ni-NTA affinity column under denaturing conditions to near homogeneity, analyzed on tricine-PAGE and confirmed by Western blotting for 6xHis. Lysozyme was used as a concentration reference.

The denatured proteins were refolded in the presence of 1 mM oxidized glutathione and 10 mM reduced glutathione (Sigma). The proteins were also refolded under identical redox conditions in the presence of immobilized protein disulfide isomerase (PDI; Takara) at a ratio equimolar to the soluble protein. Protein concentration was measured with a Bio-Rad Protein Assay Kit (Bio-Rad) using bovine serum albumin (BSA) as a standard and/or by densitometry of protein bands on tricine-PAGE gels with known protein concentration standards such as lysozyme.

Proteins refolded under both conditions were analyzed by circular dichroism spectroscopy on a Jasco J-805 spectropolarimeter. Proteins were prepared in PBS at 0.3-0.5 mg/ml and measurements taken at 25°C from 195-260 nm.

Binding and inhibition assays

1. Binding assay for dissociation constant (K_d)

Binding affinity of the proteins was assayed as described by Friguier *et al.* [25] with several modifications. A constant amount of protein (10-25 nM) was incubated with varying amounts of IL-6R (1 nM-1 μ M) in PBS-BSA (0.01% BSA) at 25°C for 1 h. Proper controls were included. The mixture was applied to a constant amount of IL-6R coated beads (200 nM) and incubated further for 30 min. After several washings with PBS-T (Tween-20, 0.1%), streptavidin horseradish peroxidase (SA-HRP; Amersham Biosciences) at 1/2000 dilution or Penta.His-HRP (Qiagen) at 1/1000 dilution was added and incubated for 30 min - 1 h. The supernatant was removed and the beads were washed 4-5 times with PBS-T. TMB substrate (200 μ l) was added for color development and the reaction was stopped with 0.5 M H_2SO_4 . Absorbance was monitored at 450 nm. Data were plotted using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA, USA).

2. Inhibition assay for IC₅₀

Inhibition of the IL-6/IL-6R interaction by the proteins was assayed by competitive inhibition experiments. A fixed amount of biotinylated IL-6 (45 nM, *i.e.*, ~3 times the K_d) and varying amounts of protein/peptide (1 nM - 2 μ M) were incubated with a constant amount of IL-6R coated beads (350 nM) in PBS-BSA at 25°C for 1 h. After several washings with PBS-T, SA-HRP at 1/2000 dilution was added and incubated for 30 min. The supernatant was removed and the beads were washed 4-5 times with PBS-T. TMB substrate (200 μ l) was added for color development, the reaction was stopped with 0.5 M H_2SO_4 and absorbance was monitored at 450 nm. Data were plotted using GraphPad Prism 4 (GraphPad Software Inc.).

3. Specificity assay

100 nM of proteins was incubated with 1 μ M of various soluble proteins (IL-6R, AChBP or IgG) at RT for 1 h. The mixtures were further incubated with 300 nM of IL-6R coated beads for 1 h. The subsequent procedures were as described for the binding assay.

Design of minimized peptides

Short cyclic peptides were designed based on the structure and disulfide pattern of 3F proteins. The sequences of the peptides are shown in Table-1. The highly conserved disulfide bonds in 3F proteins are C1-C3, C2-C4, C5-C6 and C7-C8 (numbered according to the position of the cysteine residues going from the N to the C terminus) [10]. Therefore, we designed cyclic peptides containing the individual loops sequences between C1-C3 (24 residues) where C2 was changed to G, C2-C4 (25 residues) where C3 was changed to G and C5-C6 (13 and 10 residues), respectively. If a cysteine was in the loop sequence selected from the library, it was replaced by serine (13-L1). In the case of 14-L3, due to mutation of the C5 residue, a disulfide bond was formed between the cysteine residue in the loop and C6. All the peptides were synthesized and purified by (Invitrogen, Tokyo, Japan).

Cellular assay

Cell culture and reagents: The lymphoblast cell line DS-1 was obtained from the American Type Culture Collection (CRL-11102; Manassas, USA), and were cultured in RPMI 1640 complete culture medium (Gibco, New York, USA) supplemented with 10% fetal bovine serum (FBS; Gibco), 10 mM HEPES buffer (Gibco), 10 U/ml human IL-6 (Sigma, USA), penicillin (16 mg/l) and streptomycin (25 mg/l) at 37°C in a humidified incubator with 5% CO₂ [58]. The cells were routinely passaged every 2 or 3 days in 35 mm flask (Falcon, Akasaka, Japan). Cells were continuously cultured until harvest for analysis.

Analysis of cell proliferation: Logarithmic growing DS-1 cells were dispersed with pipetting, centrifuged at 3000 rpm for 5 min, washed two times in phosphate buffer saline, and were finally resuspended in standard culture medium without IL-6. Prior to the assay, the DS-1 cells (~2 $\times 10^4$ cells/ml) were cultured for 24 h in triplicate in 24 well, flat-bottomed microtiter plates (Falcon) in 1 ml of culture medium. The candidate peptides, IL-6 and anti-human IL-6R antibody (R & D Systems, Minneapolis, USA) were prepared in standard medium at desired concentration. After 24 h pre-cultivation, cells were treated with IL-6 (10 U/ml) and specific antibody (0.03 - 2 μ g/ml), protein or peptide (10^{-10} - 10^{-5} M) for 48 h, and then the cells were counted under an inverted microscope (Olympus, Hatagaya, Japan).

Additional material

Additional file 1: Construction of the three-finger library. (A) Primary structure of the three-finger protein, MicTx3, used in this study. The four disulfide bonds formed between C1-C3, C2-C4, C5-C6 and C7-C8 are conserved among the three-finger proteins across a wide variety of species. The loops are shown in color and were randomized to generate the libraries. (B) Genetic construct adopted for cDNA display. SP6 contains the sp6 promoter, capping site and *Xenopus* globin untranslated sequence (UTR). ATG is the translation initiation codon. The 3F gene with the three randomized loops provides the basis of the library. G3S is spacer, 6XHis facilitates affinity purification by Ni-NTA, and Y-tag facilitates ligation of mRNA to the puromycin linker which contains the complementary Y-tag sequence. (C) Construction of library by overlap PCR. (i) The library was constructed by joining the fragments listed in supplementary Table-1 using overlap PCR in various steps. The portions marked in orange denote the randomized residues. (ii) Analysis of the PCR products by electrophoresis. The bands denote the respective fragments. (D) Direct sequencing of the PCR products of the library. The portions shown by arrows represent loops that were randomized. The peaks are smaller than normal due to the presence of a mixture of residues.

Additional file 2: List of oligonucleotides used for library preparation and amplification. Oligonucleotides that were used in the preparation of 3F library and the primers required for amplification of the selected library are listed.

Additional file 3: Design of shorter peptides containing disulfide bonds from the parent 3F. (A) Scheme for designing peptides. 3F is a leaf-like flat molecule with the three fingers extending from the globular head. The three fingers were designed to split into three individual fingers. (B) Design of the peptides. Primary sequence of the three-finger protein. The four disulfide bonds are formed between C1-C3, C2-C4, C5-C6 and C7-C8. The loops are located between the disulfide bonds. (ii) Short peptides from the 3F sequence. The sequences of each of the peptides correspond to one of the loops. In Loop-1 (L-1), C2 was replaced by G, and in L-2, C3 was changed to G to restore the disulfide bonds as in the parent 3F. In the case of R10-14, C5 was found to be mutated to Y, however, a cysteine residue was selected in the randomized loop. Therefore, a disulfide bond was formed between this cysteine and C6.

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Authors' contributions

MN designed and performed most of the experiments, analyzed the data and wrote the manuscript. SK prepared the proteins, multiple alignment and protein structures. CT and TS performed cellular assays and wrote a part of the manuscript. MM and NN analyzed the data. TK conceived the study, analyzed the data and wrote the manuscript. All authors approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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