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(54) Title: DESIGNED ANKYRIN REPEAT PROTEINS BINDING TO PLATELET-DERIVED GROWTH FACTOR

(57) Abstract: New designed ankyrin repeat proteins with binding specificity for PDGF-BB are described, as well as nucleic acids encoding such PDGF binding proteins, pharmaceutical compositions comprising such proteins and the use of such proteins in the treatment of diseases.

Designed ankyrin repeat proteins binding to platelet-derived growth factor

Field of the invention

The present invention relates to designed ankyrin repeat proteins with binding specificity for platelet-derived growth factor (PDGF), as well as nucleic acids encoding such PDGF binding proteins, pharmaceutical compositions comprising such proteins and the use of such proteins in the treatment of diseases.

Background of the invention

Platelet-derived growth factor (PDGF) was identified more than three decades ago as a serum growth factor for fibroblasts, smooth muscle cells and glia cells. Its role in physiology and medicine is extensively described in a recent review (Andrae, J., Gallini, R. and Betsholtz, C., Genes Dev., *22*, 1276-1312, 2008). Human PDGF was originally identified as a disulfide-linked dimer of two different polypeptide chains, A (PDGF-A; human PDGF-A has the UniProtKB/Swiss-Prot number P04085) and B (PDGF-B; human PDGF-B has the UniProtKB/Swiss-Prot number P01127). Thereby, three protein dimers can be formed: PDGF-AA, PDGF-AB and PDGF-BB. Recently, two additional PDGF polypeptide chains, PDGF-C and PDGF-D, were identified. The currently known PDGF genes and polypeptides belong to a family of structurally and functionally related growth factors including also the vascular endothelial growth factors (VEGFs). PDGF/VEGF growth factors are conserved throughout the animal kingdom.

PDGFs act via two receptor tyrosine kinases (RTKs), PDGF receptor (PDGFR) alpha (PDGFRalpha) and beta (PDGFRbeta), with common domain structures, including five extracellular immunoglobulin (Ig) loops and a split intracellular tyrosine kinase domain. The VEGFs act through a distinct but structurally related subfamily of RTKs. Ligand binding promotes receptor dimerization, which initiates signaling. Depending on ligand configuration and the pattern of receptor expression, different receptor dimers may form. However, only a few interactions seem to be relevant in vivo; i.e., those of PDGF-AA and PDGF-CC via PDGFRalpha, and PDGF-BB via PDGFRbeta.

The PDGFs have crucial roles during development, but there is limited evidence for normal physiological functions in the adult. Studies of PDGFs and PDGFRs in animal development have revealed roles for PDGFRalpha signaling in gastrulation and in the

development of the cranial and cardiac neural crest, gonads, lung, intestine, skin, CNS, and skeleton. Similarly, roles for PDGFRbeta signaling have been established in blood vessel formation and early hematopoiesis. PDGF signaling is implicated in a range of diseases. Autocrine activation of PDGF signaling pathways is involved in certain gliomas, sarcomas, and leukemias. Paracrine PDGF signaling is commonly observed in epithelial cancers, where it triggers stromal recruitment and may be involved in epithelial–mesenchymal transition, thereby affecting tumor growth, angiogenesis, invasion, and metastasis. PDGFs drive pathological mesenchymal responses in vascular disorders such as atherosclerosis, restenosis, pulmonary hypertension, and retinal diseases, as well as in fibrotic diseases, including pulmonary fibrosis, liver cirrhosis, scleroderma, glomerulosclerosis, and cardiac fibrosis.

Thus, increased PDGF activity has been linked with several diseases and pathological conditions. Causal pathogenic roles of the PDGFs have been established for some diseases, providing prospects for therapy using PDGF antagonists, such as PDGF specific antibodies. In addition, it has been suggested that the combination of anti-VEGF and anti-PDGF agents affords synergistic therapeutic benefits for treating certain ocular neovascular diseases (WO 2005/020972; Jo, N., Mailhos, C., Ju, M., Cheung, E., Bradley, J., Nishijima, K., Robinson, G.S., Adamis, A.P. and Shima, D.T., Am. J. Pathol., *168(6)*, 2036-2053, 2006).

There are, beside antibodies, novel binding proteins or binding domains that can be used to specifically bind a target molecule (e.g. Binz, H.K., Amstutz, P. and Plückthun, A., Nat. Biotechnol. *23*, 1257-1268, 2005) and thereby act as an antagonist. One such novel class of binding proteins or binding domains not possessing an Fc are based on designed repeat proteins or designed repeat domains (WO 2002/020565; Binz, H.K., Amstutz, P., Kohl, A., Stumpp, M.T., Briand, C., Forrer, P., Grütter, M.G., and Plückthun, A., Nat. Biotechnol. *22*, 575-582, 2004; Stumpp, M.T., Binz, H.K and Amstutz, P., Drug Discov. Today *13*, 695-701, 2008). WO 2002/020565 describes how large libraries of repeat proteins can be constructed and their general application. Nevertheless, WO 2002/020565 does neither disclose the selection of repeat domains with binding specificity for PDGF-BB nor concrete repeat modules or repeat sequence motifs of repeat domains that specifically bind to PDGF-BB. Furthermore, WO 2002/020565 does not suggest that repeat domains with binding specificity for PDGF-BB could be used to regulate the PDGF-BB mediated signaling pathways to successfully treat diseases. These designed repeat domains harness the modular nature of repeat proteins and may possess N-terminal and C-

terminal capping modules to prevent the designed repeat domains from aggregation by shielding the hydrophobic core of the domain (Forrer, P., Stumpp, M.T., Binz, H.K. and Plückthun, A., FEBS letters *539*, 2-6, 2003).

The technical problem underlying the present invention is identifying novel binding proteins, such as ankyrin repeat proteins or domains, with binding specificity to PDGF-BB to regulate PDGF-BB mediated signaling pathways for an improved treatment of certain cancers, vascular disorders such as retinal diseases, fibrotic diseases and other pathological conditions. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Summary of the invention

The present invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain, wherein said ankyrin repeat domain binds PDGF-BB in PBS with a Kd below 10^{-7}M.

More particularly, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain, wherein said ankyrin repeat domain competes for binding to PDGF-BB with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60, or wherein said ankyrin repeat domain is selected from the group consisting of SEQ ID NOs:23 to 60 wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

In a further embodiment, the invention relates to a recombinant PDGF-BB binding protein comprising at least one ankyrin repeat domain, which comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO: 12, 14, 15, 17, 18 and 19 and sequences, wherein up to 9 amino acids in SEQ ID NO: 12, 14, 15, 17, 18 and 19 are exchanged by any amino acid.

In particular the invention relates to a recombinant PDGF-BB binding protein comprising a peptide of any one of the sequences SEQ ID NO: 12 to 19 and 23 to 61.

The invention further relates to nucleic acid molecules encoding the binding proteins of the present invention, and to a pharmaceutical composition comprising one or more of the above mentioned binding proteins or nucleic acid molecules.

The invention further relates to a method of treatment of a pathological condition using the binding proteins of the invention.

Brief Description of the Figures

Figure 1. *Inhibition of NHI-3T3 fibroblast proliferation*
Inhibition of proliferation of NIH-3T3 fibroblasts by various concentrations of a DARPin with specificity for PDGF-BB (exemplified by DARPin #49) and a corresponding fitted inhibition curve are shown. The IC_{50} value was then calculated from the fitted inhibition curve to be 1.9 nM for DARPin #49.
OD, optical density at 450 nm; C, concentration of DARPin #49 in nM; D1, DARPin #49. The X axis is shown in logarithmic scale. See below for the definition of DARPin #49.

Figure 2. *PDGFRbeta competition assay.*
The inhibition of the binding of PDGF-BB to PDGFRbeta by various concentrations of DARPins with specificity for PDGF-BB and the corresponding fitted inhibition curves are shown for a distinct single experiment. The IC_{50} values were then calculated to be about 20 and 18 pM for the DARPins #50 (D1) and #28 (D2), respectively. OD, optical density at 450 nm; C, concentration of DARPins in pM. The X axis is shown in logarithmic scale. See below for the definitions of DARPin #50 and 28.

Figure 3. *Effects of an anti-PDGF-BB DARPin vs vehicle on the development of choroidal neovascularization.*
Mice were daily injected intraperitoneal with vehicle or DARPin #61-PEG20 (i.e. DARPin #61 conjugated to PEG20 over its C-terminal Cys residues by standards means (e.g. as described in WO 2011/135067)) from day 0 until day 14. At day 2 the laser burns were applied to the eye to induce choroidal neovascularization (CNV) and at day 14 the extent of CNV was measured. Symbols represent individual eyes and represent mean values of three induced CNV spots each. Bars represent median values of the individual groups. A, Area of CNV in mm^2; V, vehicle (i.e. PBS); D1, DARPin #61-PEG20 in PBS at a 10 mg/kg per dose injected; D2, DARPin #61-PEG20 in PBS at a 1 mg/kg per dose injected.

Detailed description of the invention

The recombinant binding protein or domain according to the invention is specific for a mammalian PDGF-BB. Preferably, the recombinant binding domain according to the invention is specific for a PDGF-BB of mice, rat, dog, rabbit, monkey or human origin. More preferably, the recombinant binding domain according to the invention is specific for a PDGF-BB of human origin.

The term "protein" refers to a polypeptide, wherein at least part of the polypeptide has, or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its polypeptide chain(s). If a protein comprises two or more polypeptides, the individual polypeptide chains may be linked non-covalently or covalently, e.g. by a disulfide bond between two polypeptides. A part of a protein, which individually has, or is able to acquire, a defined three-dimensional arrangement by forming secondary or tertiary structures, is termed "protein domain". Such protein domains are well known to the practitioner skilled in the art.

The term “recombinant” as used in recombinant protein, recombinant protein domain, recombinant binding protein and the like, means that said polypeptides are produced by the use of recombinant DNA technologies well known by the practitioner skilled in the relevant art. For example, a recombinant DNA molecule (e.g. produced by gene synthesis) encoding a polypeptide can be cloned into a bacterial expression plasmid (e.g. pQE30, Qiagen), yeast expression plasmid or mammalian expression plasmid. When, for example, such a constructed recombinant bacterial expression plasmid is inserted into an appropriate bacteria (e.g. *Escherichia coli*), this bacteria can produce the polypeptide encoded by this recombinant DNA. The correspondingly produced polypeptide is called a recombinant polypeptide.

In the context of the present invention, the term "polypeptide" relates to a molecule consisting of one or more chains of multiple, i.e. two or more, amino acids linked via peptide bonds. Preferably, a polypeptide consists of more than eight amino acids linked via peptide bonds.

The term "polypeptide tag" refers to an amino acid sequence attached to a polypeptide/protein, wherein said amino acid sequence is useful for the purification, detection, or targeting of said polypeptide/protein, or wherein said amino acid sequence

improves the physicochemical behavior of the polypeptide/protein, or wherein said amino acid sequence possesses an effector function. The individual polypeptide tags, moieties and/or domains of a binding protein may be connected to each other directly or via polypeptide linkers. These polypeptide tags are all well known in the art and are fully available to the person skilled in the art. Examples of polypeptide tags are small polypeptide sequences, for example, His (e.g. the His-tag of SEQ ID NO:9), myc, FLAG, or Strep-tags or moieties such as enzymes (for example enzymes like alkaline phosphatase), which allow the detection of said polypeptide/protein, or moieties which can be used for targeting (such as immunoglobulins or fragments thereof) and/or as effector molecules.

The term "polypeptide linker" refers to an amino acid sequence, which is able to link, for example, two protein domains, a polypeptide tag and a protein domain, a protein domain and a non-polypeptide moiety such as polyethylene glycol or two sequence tags. Such additional domains, tags, non-polypeptide moieties and linkers are known to the person skilled in the relevant art. A list of example is provided in the description of the patent application WO 2002/020565. Particular examples of such linkers are glycine-serine-linkers and proline-threonine-linkers of variable lengths; preferably, said linkers have a length between 2 and 24 amino acids; more preferably, said linkers have a length between 2 and 16 amino acids. An example of a glycine-serine-linker is provided in SEQ ID NO:10 and an example of a proline-threonine-linker is provided in SEQ ID NO:11. Preferably, the proline-threonine-linker of SEQ ID NO:11 is preceded by GS and/or followed by GS.

The term “polymer moiety” refers to either a proteinaceous polymer moiety or a non-proteinaceous polymer moiety. A “proteinaceous polymer moiety” preferably is a polypeptide that does not form a stable tertiary structure. Examples of proteinaceous polymer moieties are XTEN® (a registered trademark of Amunix; WO 2007/103515) polypeptides, or polypeptides comprising proline, alanine and serine residues as described in WO 2008/155134. Such proteinaceous polymer moieties can be covalently attached to, for example, a binding domain of the invention by the generation of genetic fusion polypeptides using standard DNA cloning technologies, followed by their standard expression and purification. A “non-proteinaceous polymer moiety” is a polymer moiety not built from polypeptides. Examples of non-proteinaceous polymer moieties are hydroxyethyl starch (HES), polyethylene glycol (PEG), polypropylene glycol, or polyoxyalkylene. The term “PEGylated” means that a PEG moiety is covalently attached

to, for example, a polypeptide of the invention. A polymer moiety of the invention may vary widely in molecular weight. Preferably, said polymer moiety is connected by a polypeptide linker to a binding domain.

In a specific embodiment, a PEG moiety or any other non-proteinaceous polymer can, e.g., be coupled to a cysteine thiol via a maleimide linker with the cysteine being coupled via a peptide linker to the N- or C-terminus of a binding domain as described herein.

The term "binding protein" refers to a protein comprising one or more binding domains, one or more bioactive compounds and one or more polymer moieties as further explained below. Preferably, said binding protein comprises up to four binding domains. More preferably, said binding protein comprises up to two binding domains. Most preferably, said binding protein comprises only one binding domain. Furthermore, any such binding protein may comprise additional protein domains that are not binding domains, multimerization moieties, polypeptide tags, polypeptide linkers and/or a single Cys residue. Examples of multimerization moieties are immunoglobulin heavy chain constant regions which pair to provide functional immunoglobulin Fc domains, and leucine zippers or polypeptides comprising a free thiol which forms an intermolecular disulfide bond between two such polypeptides. The single Cys residue may be used for conjugating other moieties to the polypeptide, for example, by using the maleimide chemistry well known to the person skilled in the art. Preferably, said binding protein is a recombinant binding protein. Also preferably, the binding domains of binding protein possess different target specificities.

The term “bioactive compound” refers to a compound that is disease modifying when applied to a mammal having said disease. A bioactive compound may have antagonistic or agonistic properties and can be a proteinaceous bioactive compound or a non-proteinaceous bioactive compound. Such proteinaceous bioactive compounds can be covalently attached to, for example, a binding domain of the invention by the generation of genetic fusion polypeptides using standard DNA cloning technologies, followed by their standard expression and purification. Such non-proteinaceous bioactive compounds can be covalently attached to, for example, a binding domain of the invention by chemical means, e.g., by coupling to a cysteine thiol via a maleimide linker with a cysteine being coupled via a peptide linker to the N- or C-terminus of a binding domain as described herein. Examples of proteinaceous bioactive compounds are binding domains having a distinct target specificity (e.g. neutralizing a growth factor by binding to it), cytokines (e.g.

interleukins), growth factors (e.g. human growth hormone), antibodies and fragments thereof, hormones (e.g. GLP-1) and any possible proteinaceous drug. Examples of non-proteinaceous bioactive compounds are, toxins (e.g. DM1 from ImmunoGen), small molecules targeting GPCRs, antibiotics and any possible non-proteinaceous drug.

The term “binding domain” means a protein domain exhibiting the same "fold" (three-dimensional arrangement) as a protein scaffold and having a predetermined property, as defined below. Such a binding domain may be obtained by rational, or most commonly, combinatorial protein engineering techniques, skills which are known in the art (Binz et al., 2005, loc. cit.). For example, a binding domain having a predetermined property can be obtained by a method comprising the steps of (a) providing a diverse collection of protein domains exhibiting the same fold as a protein scaffold as defined further below; and (b) screening said diverse collection and/or selecting from said diverse collection to obtain at least one protein domain having said predetermined property. The diverse collection of protein domains may be provided by several methods in accordance with the screening and/or selection system being used, and may comprise the use of methods well known to the person skilled in the art, such as phage display or ribosome display. Preferably, said binding domain is a recombinant binding domain.

The term "protein scaffold" means a protein with exposed surface areas in which amino acid insertions, substitutions or deletions are highly tolerable. Examples of protein scaffolds that can be used to generate binding domains of the present invention are antibodies or fragments thereof such as single-chain Fv or Fab fragments, protein A from *Staphylococcus aureus*, the bilin binding protein from *Pieris brassicae* or other lipocalins, ankyrin repeat proteins or other repeat proteins, and human fibronectin. Protein scaffolds are known to the person skilled in the art (Binz et al., 2005, loc. cit.; Binz et al., 2004, loc. cit.).

The term "target" refers to an individual molecule such as a nucleic acid molecule, a polypeptide or protein, a carbohydrate, or any other naturally occurring molecule, including any part of such individual molecule, or complexes of two or more of such molecules. The target may be a whole cell or a tissue sample, or it may be any non-natural molecule or moiety. Preferably, the target is a naturally occurring or non-natural polypeptide or a polypeptide containing chemical modifications, for example modified by natural or non-natural phosphorylation, acetylation, or methylation. In the particular application of the present invention, the target is PDGF-BB.

The term "predetermined property" refers to a property such as binding to a target, blocking of a target, activation of a target-mediated reaction, enzymatic activity, and related further properties. Depending on the type of desired property, one of ordinary skill will be able to identify format and necessary steps for performing screening and/or selection of a binding domain with the desired property. Preferably, said predetermined property is binding to a target.

The definitions hereinafter for repeat proteins are based on those in patent application WO 2002/020565. Patent application WO 2002/020565 further contains a general description of repeat protein features, techniques and applications.

The term "repeat proteins" refers to a protein comprising one or more repeat domains. Preferably, each of said repeat proteins comprises up to four repeat domains. More preferably, each of said repeat proteins comprises up to two repeat domains. Most preferably, each of the repeat proteins comprises only one repeat domain. Furthermore, said repeat protein may comprise additional non-repeat protein domains, polypeptide tags and/or polypeptide linkers.

The term "repeat domain" refers to a protein domain comprising two or more consecutive repeat units (modules) as structural units, wherein said structural units have the same fold, and stack tightly to create a superhelical structure having a joint hydrophobic core. Preferably, a repeat domain further comprises an N-terminal and/or a C-terminal capping unit (or module). Even more preferably, said N-terminal and/or C-terminal capping units (or modules) are capping repeats.

The term "designed repeat protein" and “designed repeat domain” refer to a repeat protein or repeat domain, respectively, obtained as the result of the inventive procedure explained in patent application WO 2002/020565. Designed repeat proteins and designed repeat domains are synthetic and not from nature. They are man-made proteins or domains, respectively, obtained by expression of correspondingly designed nucleic acids. Preferably, the expression is done in eukaryotic or prokaryotic cells, such as bacterial cells, or by using a cell-free *in vitro* expression system. Accordingly, a designed ankyrin repeat protein (i.e. a DARPin) corresponds to a recombinant binding protein of the invention comprising at least one ankyrin repeat domain.

The term "structural unit" refers to a locally ordered part of a polypeptide, formed by three-dimensional interactions between two or more segments of secondary structure that are near one another along the polypeptide chain. Such a structural unit exhibits a structural motif. The term "structural motif" refers to a three-dimensional arrangement of secondary structure elements present in at least one structural unit. Structural motifs are well known to the person skilled in the art. Structural units alone are not able to acquire a defined three-dimensional arrangement; however, their consecutive arrangement, for example as repeat modules in a repeat domain, leads to a mutual stabilization of neighboring units resulting in a superhelical structure.

The term "repeat unit" refers to amino acid sequences comprising repeat sequence motifs of one or more naturally occurring repeat proteins, wherein said "repeat units" are found in multiple copies, and which exhibit a defined folding topology common to all said motifs determining the fold of the protein. Such repeat units correspond to the “repeating structural units (repeats)” of repeat proteins as described by Forrer et al., 2003, loc. cit. or the “consecutive homologous structural units (repeats)” of repeat proteins as described by Binz et al, 2004, loc. cit.. Such repeat units comprise framework residues and interaction residues. Examples of such repeat units are armadillo repeat units, leucine-rich repeat units, ankyrin repeat units, tetratricopeptide repeat units, HEAT repeat units, and leucine-rich variant repeat units. Naturally occurring proteins containing two or more such repeat units are referred to as "naturally occurring repeat proteins". The amino acid sequences of the individual repeat units of a repeat protein may have a significant number of mutations, substitutions, additions and/or deletions when compared to each other, while still substantially retaining the general pattern, or motif, of the repeat units.

Accordingly, the term “ankyrin repeat unit” shall mean a repeat unit, which is an ankyrin repeat as described, for example, by Forrer et al., 2003, loc. cit.. Ankyrin repeats are well known to the person skilled in the art. The term "ankyrin repeat domain" refers to a repeat domain comprising two or more consecutive ankyrin repeat units (modules) as structural units, and, preferably, an N-terminal and/or a C-terminal capping unit (or module).

The term "framework residues" relates to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which contribute to the folding topology, i.e. which contribute to the fold of said repeat unit (or module) or which contribute to the interaction with a neighboring unit (or module). Such contribution might be the interaction with other residues in the repeat unit (or module), or the influence on the

polypeptide backbone conformation as found in α-helices or β-sheets, or amino acid stretches forming linear polypeptides or loops.

The term "target interaction residues" refers to amino acid residues of the repeat units, or the corresponding amino acid residues of the repeat modules, which contribute to the interaction with target substances. Such contribution might be the direct interaction with the target substances, or the influence on other directly interacting residues, e.g. by stabilizing the conformation of the polypeptide of a repeat unit (or module) to allow or enhance the interaction of directly interacting residues with said target. Such framework and target interaction residues may be identified by analysis of the structural data obtained by physicochemical methods, such as X-ray crystallography, NMR and/or CD spectroscopy, or by comparison with known and related structural information well known to practitioners in structural biology and/or bioinformatics.

Preferably, the repeat units used for the deduction of a repeat sequence motif are homologous repeat units, wherein the repeat units comprise the same structural motif and wherein more than 70% of the framework residues of said repeat units are homologous to each other. Preferably, more than 80% of the framework residues of said repeat units are homologous. Most preferably, more than 90% of the framework residues of said repeat units are homologous. Computer programs to determine the percentage of homology between polypeptides, such as Fasta, Blast or Gap, are known to the person skilled in the art. Further preferably, the repeat units used for the deduction of a repeat sequence motif are homologous repeat units obtained from repeat domains selected on a defined target.

The term "repeat sequence motif" refers to an amino acid sequence, which is deduced from one or more repeat units or repeat modules. Preferably, said repeat units or repeat modules are from repeat domains having binding specificity for the same target. Such repeat sequence motifs comprise framework residue positions and target interaction residue positions. Said framework residue positions correspond to the positions of framework residues of the repeat units (or modules). Likewise, said target interaction residue positions correspond to the positions of target interaction residues of the repeat units (or modules). Repeat sequence motifs comprise fixed positions and randomized positions. The term "fixed position" refers to an amino acid position in a repeat sequence motif, wherein said position is set to a particular amino acid. Most often, such fixed positions correspond to the positions of framework residues and/or the positions of target interaction residues that are specific for a certain target. The term "randomized position"

refers to an amino acid position in a repeat sequence motif, wherein two or more amino acids are allowed at said amino acid position, for example, wherein any of the usual twenty naturally occurring amino acids are allowed, or wherein most of the twenty naturally occurring amino acids are allowed, such as amino acids other than cysteine, or amino acids other than glycine, cysteine and proline. Most often, such randomized positions correspond to the positions of target interaction residues. However, some positions of framework residues may also be randomized.

The term "folding topology" refers to the tertiary structure of said repeat units or repeat modules. The folding topology will be determined by stretches of amino acids forming at least parts of α-helices or β-sheets, or amino acid stretches forming linear polypeptides or loops, or any combination of α-helices, β-sheets and/or linear polypeptides/loops. For example, an ankyrin repeat unit/module consists of a β-turn, followed by two antiparallel α-helices and a loop that reaches the turn of the next repeat unit/module.

The term "consecutive" refers to an arrangement, wherein the repeat units or repeat modules are arranged in tandem. In designed repeat proteins, there are at least 2, usually about 2 to 6, in particular at least about 6, frequently 20 or more repeat units (or modules). In most cases, repeat units (or modules) of a repeat domain will exhibit a high degree of sequence identity (same amino acid residues at corresponding positions) or sequence similarity (amino acid residues being different, but having similar physicochemical properties), and some of the amino acid residues might be key residues being strongly conserved. However, a high degree of sequence variability by amino acid insertions and/or deletions, and/or substitutions between the different repeat units (or modules) of a repeat domain may be possible as long as the common folding topology of the repeat units (or modules) is maintained.

Methods for directly determining the folding topology of repeat proteins by physico-chemical means such as X-ray crystallography, NMR or CD spectroscopy, are well known to the practitioner skilled in the art. Methods for identifying and determining repeat units or repeat sequence motifs or for identifying families of related proteins comprising such repeat units or motifs, such as homology searches (BLAST etc.), are well established in the field of bioinformatics, and are well known to the practitioner in the art. The step of refining an initial repeat sequence motif may comprise an iterative process.

The term "repeat modules" refers to the repeated amino acid sequences of the designed repeat domains, which are originally derived from the repeat units of naturally occurring repeat proteins. Each repeat module comprised in a repeat domain is derived from one or more repeat units of the family or subfamily of naturally occurring repeat proteins, e.g. the family of armadillo repeat proteins or ankyrin repeat proteins. Further preferably, each repeat module comprised in a repeat domain comprises a repeat sequence motif deduced from homologous repeat units obtained from repeat domains selected on a target, for example as described in Example 1 and having the same target specificity.

Accordingly, the term “ankyrin repeat module” shall mean a repeat module, which is originally derived from the repeat units of naturally occurring ankyrin repeat proteins. Ankyrin repeat proteins are well known to the person skilled in the art.

"Repeat modules" may comprise positions with amino acid residues present in all copies of corresponding repeat modules ("fixed positions") and positions with differing or "randomized" amino acid residues ("randomized positions").

The term “capping module” refers to a polypeptide fused to the N- or C-terminal repeat module of a repeat domain, wherein said capping module forms tight tertiary interactions (i.e. tertiary structure interactions) with said repeat module thereby providing a cap that shields the hydrophobic core of said repeat module at the side not in contact with the consecutive repeat module from the solvent. Said N- and/or C-terminal capping module may be, or may be derived from, a capping unit or other structural unit found in a naturally occurring repeat protein adjacent to a repeat unit. The term “capping unit” refers to a naturally occurring folded polypeptide, wherein said polypeptide defines a particular structural unit which is N- or C-terminally fused to a repeat unit, wherein said polypeptide forms tight tertiary structure interactions with said repeat unit thereby providing a cap that shields the hydrophobic core of said repeat unit at one side from the solvent. Preferably, capping modules or capping units are capping repeats. The term “capping repeat” refers to capping module or capping unit having a similar or the same fold as said adjacent repeat unit (or module) and/or sequence similarities to said adjacent repeat unit (or module). Capping modules and capping repeats are described in WO 2002/020565 and by Interlandi et al., 2008 (loc. cit.). Examples of N-terminal ankyrin capping modules (i.e. N-terminal capping repeats) are SEQ ID NO:1 to 3 and examples of ankyrin C-terminal capping modules (i.e. C-terminal capping repeats) are SEQ ID NO:4 to 8, 13 and 16.

For example, the N-terminal ankyrin capping module of SEQ ID NO:49 is encoded by the amino acids from position 1 to 32 and the C-terminal capping module of SEQ ID NO:49 is encoded by the amino acids form position 132 to 159.

A recombinant binding protein according to the invention comprises at least one ankyrin repeat domain, wherein said ankyrin repeat domain has binding specificity for mammalian PDGF-BB.

The term “has binding specificity for a target”, “specifically binding to a target” or “target specificity” and the like means that a binding protein or binding domain binds in PBS to a target with a lower dissociation constant than to an unrelated protein such as the *E. coli* maltose binding protein (MBP). Preferably, the dissociation constant in PBS for the target is at least 10, more preferably at least 10^2, even more preferably at least 10^3, or most preferably at least 10^4 times lower than the corresponding dissociation constant for MBP.

Recombinant binding proteins comprising an ankyrin repeat domain with binding specificity for PDGF-BB are shown in the Examples.

In particular, the invention relates to a recombinant binding protein as defined herein comprising an ankyrin repeat domain with binding specificity for PDGF-BB, which binds PDGF-BB in PBS with a dissociation constant (Kd) below 10^{-6}M. Preferably, said ankyrin repeat domain binds PDGF-BB with a Kd in PBS below 10^{-7}M, more preferably below 10^{-8}M, 10^{-9}M, 10^{-10}M, or most preferably below 10^{-11}M.

Methods to determine dissociation constants of protein-protein interactions, such as surface plasmon resonance (SPR) based technologies (e.g. SPR equilibrium analysis) or isothermal titration calorimetry (ITC) are well known to the person skilled in the art. The measured Kd values of a particular protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of Kd values are preferably made with standardized solutions of protein and a standardized buffer, such as PBS.

Recombinant binding proteins comprising an ankyrin repeat domain binding PDGF-BB with a Kd in PBS below 10^{-6}M are shown in Example 2.

Preferred is a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for human PDGF-BB.

Further preferred is a recombinant binding protein comprising an ankyrin repeat domain comprising between 70 and 300 amino acids, in particular between 90 and 200 amino acids.

A binding domain of the invention is an ankyrin repeat domain or a designed ankyrin repeat domain, preferably as described in WO 2002/020565. Examples of designed ankyrin repeat domains with binding specificity for PDGF-BB are shown in the Examples.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein the ankyrin repeat domain inhibits the binding of PDGF-BB to PDGFRbeta in PBS with an IC_{50} value below 10^{-7}M. Preferably, said ankyrin repeat domain inhibits the binding of PDGF-BB to PDGFRbeta in PBS with an IC_{50} value below 10^{-7}M, more preferably below 10^{-8}M, 10^{-9}M, 10^{-10}M, or most preferably below 10^{-11}M.

The half maximal inhibitory concentration (IC_{50}) is a measure of the effectiveness of a compound, such as a binding domain of the invention, in inhibiting a biological, biochemical or biophysical function. Methods to determine IC_{50} values of inhibition of protein-protein interactions, such as competition ELISAs are well known to the person skilled in the art. The measured IC_{50} values of a particular inhibitor of a protein-protein interaction can vary if measured under different conditions (e.g., salt concentration, pH). Thus, measurements of IC_{50} values are preferably made with standardized solutions of protein and a standardized buffer, such as PBS.

Recombinant binding proteins comprising an ankyrin repeat domain inhibiting the binding of PDGF-BB to PDGFRbeta in PBS with an IC_{50} value below 10^{-7}M are shown in Example 4.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for PDGF-BB, which inhibits the PDGF-BB stimulated proliferation of NIH-3T3 fibroblasts (ATCC, cat number: CRL-1658)

with an IC_{50} value below 10^{-6}M. Preferably, said repeat domain inhibits the PDGF-BB stimulated proliferation of NIH-3T3 fibroblasts with an IC_{50} value below 10^{-7}M, more preferably below 10^{-8}M, 10^{-9}M, 10^{-10}M, or most preferably 10^{-11}M.

NIH-3T3 cells are responsive to PDGF-BB for growth and as such can be used to measure the functional inhibitory capability of the compounds of the invention. NIH-3T3 cells are grown in culture medium and then starved of nutrients for 7 hours prior to addition of PDGF-BB and a titration of the anti-PDGF-BB DARPin. Assessment of the ability of the compounds of the invention to inhibit PDGF-BB is determined by the proliferative capacity of the NIH-3T3 cells as measured by standard measurements well known to the person skilled in the art. Recombinant binding proteins comprising an ankyrin repeat domain inhibiting the PDGF-BB stimulated proliferation of NIH-3T3 fibroblasts with an IC_{50} value below 10^{-6}M are shown in Example 3.

The invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for PDGF-BB, wherein said binding protein and/or ankyrin repeat domain has a midpoint denaturation temperature (Tm) above 40°C upon thermal unfolding in PBS and forms less than 5% (w/w) insoluble aggregates at concentrations up to 10 g/L when incubated at 37°C for 1 day in PBS.

The term “PBS” means a phosphate buffered water solution containing 137 mM NaCl, 10 mM phosphate and 2.7 mM KCl and having a pH of 7.4.

Preferably, the recombinant binding protein and/or binding domain has a midpoint denaturation temperature (Tm) above 45°C, more preferably above 50°C, more preferably above 55°C, and most preferably above 60°C upon thermal unfolding in PBS at pH 7.4. A binding protein or a binding domain of the invention possesses a defined secondary and tertiary structure under physiological conditions. Thermal unfolding of such a polypeptide results in a loss of its tertiary and secondary structure, which can be followed, for example, by circular dichroism (CD) measurements. The midpoint denaturation temperature of a binding protein or binding domain upon thermal unfolding corresponds to the temperature at the midpoint of the cooperative transition in physiological buffer upon heat denaturation of said protein or domain by slowly increasing the temperature from 10°C to about 100°C. The determination of a midpoint denaturation temperature upon thermal unfolding is well known to the person skilled in the art. This midpoint denaturation

temperature of a binding protein or binding domain upon thermal unfolding is indicative of the thermal stability of said polypeptide.

Also preferred is a recombinant binding protein and/or ankyrin repeat domain forming less than 5% (w/w) insoluble aggregates at concentrations up to 20 g/L, preferably up 40 g/L, more preferably up to 60 g/L, even more preferably up to 80 g/L, and most preferably up to 100 g/L when incubated for over 5 days, preferably over 10 days, more preferably over 20 days, more preferably over 40 days, and most preferably over 100 days at 37°C in PBS. The formation of insoluble aggregates can be detected by the appearance of visual precipitations, gel filtration or dynamic light scattering, which strongly increases upon formation of insoluble aggregates. Insoluble aggregates can be removed from a protein sample by centrifugation at 10'000 x g for 10 minutes. Preferably, a recombinant binding protein and/or ankyrin repeat domain forms less than 2%, more preferably less than 1%, 0.5%, 0.2%, 0.1%, or most preferably less than 0.05% (w/w) insoluble aggregates under the mentioned incubation conditions at 37°C in PBS. Percentages of insoluble aggregates can be determined by separation of the insoluble aggregates from soluble protein, followed by determination of the protein amounts in the soluble and insoluble fraction by standard quantification methods.

Also preferred is a recombinant binding protein and/or ankyrin repeat domain that does not lose its native three-dimensional structure upon incubation in PBS containing 100 mM dithiothreitol (DTT) for 1 or 10 hours at 37°C.

In one particular embodiment the invention relates to a recombinant binding protein comprising an ankyrin repeat domain, specifically binding to PDGF-BB and having the indicated or preferred midpoint denaturation temperature and non-aggregating properties as defined above.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein the ankyrin repeat domain competes for binding to a mammalian PDGF-BB with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60; preferably SEQ ID NOs: 24, 45 and 50, in particular SEQ ID NO:24 and 50.

Also preferably said ankyrin repeat domain competes for binding to a mammalian PDGF-BB with a binding protein selected from the group of DARPins #23 to 60. Preferably, said

repeat domain competes for binding to a mammalian PDGF-BB with a binding protein from the group of DARPins #24, 45 and 50. More preferably, said ankyrin repeat domain competes for binding to a mammalian PDGF-BB with binding protein DARPin #24 or 50.

The term “compete for binding” means the inability of two different binding domains of the invention to bind simultaneously to the same target, while both are able to bind the same target individually. Thus, such two binding domains compete for binding to said target. Preferably, said two competing binding domains bind to an overlapping or the same binding epitope on said target. Methods, such as competition Enzyme-Linked Immuno Sorbent Assay (ELISA) or competition SPR measurements (e.g. by using the Proteon instrument from BioRad), to determine if two binding domains compete for binding to a target, are well known to the practitioner in the art. For example, the ankyrin repeat domain of SEQ ID No: #49 or SEQ ID No: #58 competes for binding to human PDGF with the ankyrin repeat domain of SEQ ID No: #50.

The term “epitope” means the specific site on the surface of a target protein, such as PDGF-BB, to which a binding domain of the invention, such as an ankyrin repeat domain, attaches itself. This term is defined in analogy to epitopes of antibodies, which are well known to the person skilled in the art. If two binding domains of the invention bind to the same epitope, they will compete for binding for PDGF-BB. The exact molecular arrangement of an epitope can be elucidated, for example, by protein X-ray crystallography (a method well known to the person skilled in the art) of the binding domain of the invention in complex with PDGF-BB.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein said ankyrin repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60,
wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

Preferably, such an ankyrin repeat domain in a recombinant binding protein of the invention comprises an amino acid sequence that has at least 70% amino acid sequence

identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NO: 24, 45 and 50; more preferably, 24 and 50.

Preferably, such an ankyrin repeat domain in a recombinant binding protein of the invention comprises an amino acid sequence with at least 70% amino acid sequence identity, for example 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% amino acid sequence identity with one, two or three ankyrin repeat modules present between the N-terminal and C-terminal capping modules of an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60.

Preferably, instead of 70% amino acid sequence identity, such an ankyrin repeat domain or such one, two or three repeat modules present between the N-terminal and C-terminal capping modules in an ankyrin repeat domain in a recombinant binding protein of the invention comprises an amino acid sequence with at least 75%, more preferably at least 76%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, or most preferred at least 95% amino acid sequence identity. Preferably, the mentioned percentages of amino acid sequence identity is in the framework positions.

Preferably, up to 30 amino acids, for example 30, 29, 28, 27, 26, 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or no amino acid(s) in the repeat domains SEQ ID NO:23 to 60 are exchanged by another amino acid. In particular, up to 25 amino acids, more preferably up to 20 amino acids, more preferably up to 15 amino acids, even more preferably up to 11 amino acids, more preferably up to 8 amino acids, more preferably up to 5 amino acids, more preferably up to 2 amino acid, and most preferably no amino acid in SEQ ID NO: 23 to 60 is exchanged.

Preferably, when amino acids are exchanged in the capping modules of SEQ ID NO:13 or 16, the repeat modules of SEQ ID NO:12, 14, 15, 17, 18 and 19 or the repeat domains of SEQ ID NO:23 to 60, these amino acids are selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y; more preferably from the group consisting of A, D, E, H, I, K, L, Q, R, S, T, V, and Y. Also preferably, an amino acid is exchanged by a homologous amino acid; i.e. an amino acid is exchanged by an amino acid having a side chain with similar biophysical properties. For example, the negative charged amino acid D may be replaced by the negative charged amino acid E, or a hydrophobic amino acid such

as L may be replaced by A, I or V. The techniques of exchanging an amino acid by another amino acid in a polypeptide are well known to the person skilled in the art.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein said ankyrin repeat domain is selected from the group consisting of SEQ ID NOs: 23 to 60,
wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

Preferably, such an ankyrin repeat domain is selected from the group consisting of SEQ ID NO: 24, 45 and 50; more preferably, 24 and 50.

In a further embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain attaches to the same epitope as an ankyrin repeat domain selected from the group consisting of SEQ ID NOs:23 to 60. Preferably, such an ankyrin repeat domain is selected from the group consisting of SEQ ID NO: 24, 45 and 50; more preferably, 24 and 50.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO:12, 14, 15, 17, 18 and 19 and sequences, wherein up to 9 amino acids in SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by any amino acid.

Preferably, such an ankyrin repeat module of said ankyrin repeat domain is selected from the group consisting of SEQ ID NO: 12, 14 and 17; more preferably, 12 and 17.

Preferably, up to 8 amino acids in the repeat modules of SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by another amino acid, more preferably up to 7 amino acids, more preferably up to 6 amino acids, more preferably up to 5 amino acids, even more preferably up to 4 amino acids, more preferably up to 3 amino acids, more preferably up to 2 amino acids, and most preferably 1 amino acid. Preferably, the mentioned exchanges of amino

acid are in the framework positions. Accordingly, up to 8 amino acids in framework positions of SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by any amino acid, preferably up to 7, 6, 5, 4, 3 or 2 amino acids, and most preferably 1 amino acid.

In a further embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a repeat module with the ankyrin repeat sequence KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA (SEQ ID NO:12) and sequences, wherein up to 9 amino acids in SEQ ID NO:11 are exchanged by any amino acid and wherein

E at position 3 is optionally exchanged by an amino acid selected from the group consisting of D, W, Q, I and Y, preferably of D and W;

E at position 4 is optionally exchanged by an amino acid selected from the group consisting of T, D, Y, and S, preferably of T and D;

T at position 6 is optionally exchanged by an amino acid selected from the group consisting of S and F, preferably by S;

Y at position 11 is optionally exchanged by F;

V at position 14 is optionally exchanged by an amino acid selected from the group consisting of A, Y and T, preferably by A; and

W at position 15 is optionally exchanged by an amino acid selected from the group consisting of F, K, V, and Y, preferably of F and Y.

In a further embodiment, the invention relates to a recombinant binding protein comprising at least one ankyrin repeat domain with binding specificity for a mammalian PDGF-BB, wherein said ankyrin repeat domain comprises a capping module having an amino acid sequence selected from the group consisting of SEQ ID NO:13 and 16 and sequences, wherein up to 9 amino acids in SEQ ID NO:13 and 16 are exchanged by any amino acid.

Preferably, up to 8 amino acids in the capping modules of SEQ ID NO:13 and 16 comprised in said ankyrin repeat domain are exchanged by an other amino acid, more preferably up to 7 amino acids, more preferably up to 6 amino acids, more preferably up to 5 amino acids, even more preferably up to 4 amino acids, more preferably up to 3 amino acids, more preferably up to 2 amino acids, more preferably up to 1 amino acid, and most preferably no amino acid in SEQ ID NO:13 and 16 is exchanged.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a C-terminal capping module with the sequence QDIYGATPADLAALVGHEDIAEVLQKLN (SEQ ID NO:13) and sequences, wherein up to 9 amino acids in SEQ ID NO:13 are exchanged by any amino acid
wherein
I at position 3 is optionally exchanged by an amino acid selected from the group consisting of K, L, A and V, preferably L, A and V;
Y at position 4 is optionally exchanged by an amino acid selected from the group consisting of W. F and S, preferably, of W and F;
A at position 6 is optionally exchanged by K;
L at position 14 is optionally exchanged by an amino acid selected from the group consisting of F, Y and D, preferably of F and Y;
V at position 15 is optionally exchanged by an amino acid selected from the group consisting of L, I , A and N, preferably, L and I; and
V at position 23 is exchanged by an amino acid selected from the group consisting of I and L.

Preferred is a recombinant binding protein, wherein the ankyrin repeat domain comprises the ankyrin repeat module of SEQ ID NO:12 and the C-terminal capping module SEQ ID NO:13. Preferably, said C-terminal capping module directly follows said ankyrin repeat module in said ankyrin repeat domain.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a repeat module with the ankyrin repeat sequence KDQEGTTPLHFAASVGHLEIVEVLLKAGADVNA (SEQ ID NO:15) and sequences, wherein up to 9 amino acids in SEQ ID NO:15 are exchanged by any amino acid and wherein
Q at position 3 is optionally exchanged by A;
E at position 4 is optionally exchanged by D;
T at position 6 is optionally exchanged by E;
F at position 11 is optionally exchanged by Y;
S at position 14 is optionally exchanged by V; and
V at position 15 is optionally exchanged by W.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a C-terminal capping module with the sequence QDHYGATPADLAALIGHEDIAEVLQKLN (SEQ ID NO:16) and sequences, wherein up to 9 amino acids in SEQ ID NO:15 are exchanged by any amino acid and wherein
H at position 3 is optionally exchanged by I; and
Y at position 4 is optionally exchanged by W.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a repeat module with the ankyrin repeat sequence KDLNGQTPLHLAADIGHLEIVEVLLKAGADVNA (SEQ ID NO:17) and sequences, wherein up to 9 amino acids in SEQ ID NO:17 are exchanged by any amino acid and wherein
K at position 1 is optionally exchanged by Q or I;
L at position 3 is optionally exchanged by N; and
A at position 27 is optionally exchanged by H.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a repeat module with the ankyrin repeat sequence KDYAGSTPLRLAAWAGHLEIVEVLLKAGADVNA (SEQ ID NO:18) and sequences, wherein up to 9 amino acids in SEQ ID NO:18 are exchanged by any amino acid and wherein
K at position 1 is optionally exchanged by Q;
W at position 14 is optionally exchanged by H;
A at position 15 is optionally exchanged by V; and
A at position 27 is optionally exchanged by N or Y.

In yet another embodiment, the invention relates to a recombinant binding protein, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a repeat module with the ankyrin repeat sequence KDYFGYTPLHLAAYFGHLEIVEVLLKAGADVNA (SEQ ID NO:19) and sequences, wherein up to 9 amino acids in SEQ ID NO:19 are exchanged by any amino acid and wherein

K at position 1 is optionally exchanged by N;
A at position 12 is optionally exchanged by T;
A at position 13 is optionally exchanged by T;
E at position 22 is optionally exchanged by D;
A at position 27 is optionally exchanged by H or Y.

Further preferred is a N-terminal or C-terminal ankyrin capping module comprising an N-terminal or C-terminal ankyrin capping repeat, respectively, wherein one or more of the amino acids residues in said capping repeat are replaced by an amino acid residue found at the corresponding position on alignment of a corresponding ankyrin capping unit or ankyrin repeat unit.

The replacement of amino acids can be by any of the 20 most often naturally occurring amino acids, preferably by amino acids selected from the group consisting of A, D, E, F, H, I, K, L, M, N, Q, R, S, T, V, W and Y; and more preferably from the group consisting of A, D, E, H, I, K, L, Q, R, S, T, V, and Y. Also preferably, the replacement of amino acids is by a homologous amino acid; i.e. an amino acid is replaced by an amino acid having a side chain with similar biophysical properties. For example, the negative charged amino acid D may be replaced by the negative charged amino acid E, or a hydrophobic amino acid such as L may be replaced by A, I or V. The replacement of an amino acid by a homologous amino acid is well known to the person skilled in the art.

Also preferred is a C-terminal ankyrin capping module comprising the amino acid A at position 27 and 28 of any of the above C-terminal capping modules based on SEQ ID NO:4 to 8, 13 and 16.

Also preferred is a C-terminal capping module comprising the amino acids from position 1 to 26 or from position 1 to 27 of any of the above C-terminal capping modules based on SEQ ID NO:4 to 8, 13 and 16.

Amino acids G at position 1 and/or S at position 2 of SEQ ID NO:1 to 3 can be removed from N-terminal ankyrin capping modules without any apparent influence on the properties. These two amino acids serve as linkers to connect the ankyrin repeat domain to further amino acids and proteins. The invention also comprises such ankyrin repeat domains comprising N-terminal ankyrin capping modules wherein G at position 1 and/or S at position 2 are removed. It is understood that the amino acid positions (e.g. “position

33") in an ankyrin repeat domain as defined herein are adapted accordingly, resulting in a number shift, e.g. “position 33” will become “position 32”, if one amino acid is missing, or “position 33” will become “position 31”, if two amino acid are missing.

An ankyrin capping module of an ankyrin repeat domain of the invention can be exchanged by an ankyrin capping module by combining techniques, such as alignment of amino acid sequences, mutagenesis and gene synthesis, known to the person skilled in the art. For example, the C-terminal capping repeat of SEQ ID NO:49 can be replaced by the C-terminal capping repeat of SEQ ID NO:8 by (i) determination of the C-terminal capping repeat of SEQ ID NO:49 (i.e. sequence position 132 to 159) by sequence alignment with SEQ ID NO:8, (ii) replacing the sequence of the determined C-terminal capping repeat of SEQ ID NO:49 with the sequence of SEQ ID NO:8, (iii) generation of a gene encoding the repeat domain encoding the exchanged C-terminal capping module, (iv) expressing of the modified repeat domain in the cytoplasm of *E. coli* and (v) purification of the modified repeat domain by standard means. As a further example, the N-terminal capping repeat of SEQ ID NO:49 can be replaced by the N-terminal capping repeat of SEQ ID NO:2 by (i) determination of the N-terminal capping repeat of SEQ ID NO:49 (i.e. sequence position 1 to 32) by sequence alignment with SEQ ID NO:2, (ii) replacing the sequence of the determined N-terminal capping repeat of SEQ ID NO:49 with the sequence of SEQ ID NO:2, (iii) generation of a gene encoding the repeat domain encoding the exchanged N-terminal capping module, (iv) expressing of the modified repeat domain in the cytoplasm of *E. coli* and (v) purification of the modified repeat domain by standard means.

Furthermore, an ankyrin repeat domain of the invention can be constructed genetically by assembling a N-terminal ankyrin capping module (e.g. the N-terminal capping repeat of SEQ ID NO:2) followed by one or more repeat modules (e.g. the three ankyrin repeat modules comprising the amino acid residues from position 33 to 131 of SEQ ID NO:49) and a C-terminal capping module (e.g. the C-terminal capping repeat of SEQ ID NO:8) by means of gene synthesis. The genetically assembled repeat domain gene can then be expressed in *E. coli* as described above.

Further preferred is a recombinant binding protein, repeat domain, repeat module, N-terminal capping module or C-terminal capping module having an amino acid sequence devoid of amino acids C, M or N.

Further preferred is a recombinant binding protein, repeat domain, repeat module, N-terminal capping module or C-terminal capping module having an amino acid sequence devoid of amino acid N followed by G.

Further preferred is a recombinant binding protein or repeat domain comprising any such N-terminal or C-terminal capping module.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the N-terminal capping module of said repeat domain is exchanged by an amino acid residue found at the corresponding position on alignment of an N-terminal capping unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such an N-terminal capping unit is a naturally occurring N-terminal capping unit.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the C-terminal capping module of said repeat domain is exchanged by an amino acid residue found at the corresponding position on alignment of a C-terminal capping unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such a C-terminal capping unit is a naturally occurring C-terminal capping unit.

In still another particular embodiment, up to 30% of the amino acid residues, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units, N-terminal capping units or C-terminal capping units.

The term "consensus sequence" refers to an amino acid sequence, wherein said consensus sequence is obtained by structural and/or sequence aligning of multiple repeat units. Using two or more structural and/or sequence aligned repeat units, and allowing for gaps in the alignment, it is possible to determine the most frequent amino acid residue at each position. The consensus sequence is that sequence which comprises the amino acids which are most frequently represented at each position. In the event that two or

more amino acids are represented above-average at a single position, the consensus sequence may include a subset of those amino acids. Said two or more repeat units may be taken from the repeat units comprised in a single repeat protein, or from two or more different repeat proteins.

Consensus sequences and methods to determine them are well known to the person skilled in the art.

A “consensus amino acid residue” is the amino acid found at a certain position in a consensus sequence. If two or more, e.g. three, four or five, amino acid residues are found with a similar probability in said two or more repeat units, the consensus amino acid may be one of the most frequently found amino acids or a combination of said two or more amino acid residues.

Further preferred are non-naturally occurring capping modules, repeat modules, binding proteins or binding domains.

The term “non-naturally occurring” means synthetic or not from nature, more specifically, the term means made from the hand of man. The term “non-naturally occurring binding protein" or "non-naturally occurring binding domain” means that said binding protein or said binding domain is synthetic (i.e. produced by chemical synthesis from amino acids) or recombinant and not from nature. “Non-naturally occurring binding protein" or "non-naturally occurring binding domain” is a man-made protein or domain, respectively, obtained by expression of correspondingly designed nucleic acids. Preferably, the expression is done in eukaryotic or bacterial cells, or by using a cell-free *in vitro* expression system. Further, the term means that the sequence of said binding protein or said binding domain is not present as a non-artificial sequence entry in a sequence database, for example in GenBank, EMBL-Bank or Swiss-Prot. These databases and other similar sequence databases are well known to the person skilled in the art.

In one particular embodiment the invention relates to a recombinant binding protein comprising an ankyrin repeat domain specifically binding to PDGF-BB and further comprising an ankyrin repeat domain specifically binding to vascular endothelial growth factors A (VEGF-A). Examples of ankyrin repeat domains with specificity for PDGF-BB are given herein and examples of ankyrin repeat domains with specificity to VEGF-A are described in WO 2010/060748 (US 2011/0207668) and WO 2011/135067 (US

2013/0116197), the entire disclosures of which are incorporated by reference herein. Such two repeat domains can be linked by a polypeptide linker by genetic means by methods known to the person skilled in the art. In one embodiment of the invention, a recombinant binding protein comprising an ankyrin repeat domain specifically binding PDGF-BB and an ankyrin repeat domain specifically binding VEGF-A may be used to treat diseases of the retina and choroidal neovascular diseases, such as exudative age-related macular degeneration, polypoidal choroidal neovascularization, and pathological myopia.

Another preferred embodiment is a recombinant binding protein comprising an ankyrin repeat domain with binding specificity for PDGF-BB comprising one, two, three or more internal repeat modules that will participate in binding to PDGF-BB. Preferably, such an ankyrin repeat domain comprises an N-terminal capping module, two to four internal repeat modules, and a C-terminal capping module. Preferably, said capping modules are capping repeats. Also preferably, said capping modules will participate in binding to PDGF-BB.

Further preferred is a recombinant binding protein comprising two or more of said ankyrin repeat domains with binding specificity for PDGF-BB. Preferably, said binding protein comprises 2 or 3 of said repeat domains. Said two or more repeat domains have the same or different amino acid sequence.

In a further preferred embodiment of a recombinant binding protein comprising an ankyrin repeat domain according to the present invention, one or more of the amino acid residues of the repeat modules of said ankyrin repeat domain are exchanged by an amino acid residue found at the corresponding position on alignment of a repeat unit. Preferably, up to 30% of the amino acid residues are exchanged, more preferably, up to 20%, and even more preferably, up to 10% of the amino acid residues are exchanged. Most preferably, such a repeat unit is a naturally occurring repeat unit.

In still another particular embodiment, up to 30% of the amino acid residues, for example 29%, 28%, 27%, 26%, 25%, 24%, 23%, 22%, 21%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or 0% of the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units. More preferably, up to 20%, and even more preferably, up to

10% of the amino acid residues are exchanged with amino acids which are not found in the corresponding positions of repeat units.

In further embodiments, any of the recombinant PDGF-BB binding proteins or domains described herein may be covalently bound to one or more additional moieties, including, for example, a moiety that binds to a different target to create a dual-specificity binding agent, a bioactive compound, a labeling moiety (e.g. a fluorescent label such as fluorescein, or a radioactive tracer), a moiety that facilitates protein purification (e.g. a small peptide tag, such as a His- or strep-tag), a moiety that provides effector functions for improved therapeutic efficacy (e.g. the Fc part of an antibody to provide antibody-dependent cell-mediated cytotoxicity, a toxic protein moiety such as *Pseudomonas aeruginosa* exotoxin A (ETA) or a small molecular toxic agent such as maytansinoids or DNA alkylating agents) or a moiety that provides improved pharmacokinetics. Improved pharmacokinetics may be assessed according to the perceived therapeutic need. Often it is desirable to increase bioavailability and/or increase the time between doses, possibly by increasing the time that a protein remains available in the serum after dosing. In some instances, it is desirable to improve the continuity of the serum concentration of the protein over time (e.g., decrease the difference in serum concentration of the protein between the concentration shortly after administration and the concentration shortly before the next administration). Moieties that tend to slow clearance of a protein from the blood include hydroxyethyl starch (HES), polyethylene glycol (PEG), sugars (e.g. sialic acid), well-tolerated protein moieties (e.g. Fc fragments or serum albumin), and binding domains or peptides with specificity and affinity for abundant serum proteins, such as antibody Fc fragments or serum albumin. Examples of such binding domains with affinity for serum albumin are provided in WO 2012/069654. The recombinant binding protein of the invention may be attached to a moiety that reduces the clearance rate of polypeptides in a mammal (e.g. in mouse, rat, or human) by greater than three-fold relative to the unmodified polypeptides.

In a further embodiment, the invention relates to nucleic acid molecules encoding the particular recombinant binding proteins, the particular ankyrin repeat domains, the particular ankyrin repeat modules and the particular capping modules. Further, a vector comprising said nucleic acid molecule is considered.

Further, a pharmaceutical composition comprising one or more of the above mentioned recombinant binding proteins, in particular binding proteins comprising repeat domains, or

nucleic acid molecules encoding the particular binding proteins, and optionally a pharmaceutical acceptable carrier and/or diluent is considered. Pharmaceutical acceptable carriers and/or diluents are known to the person skilled in the art and are explained in more detail below. Even further, a diagnostic composition comprising one or more of the above mentioned recombinant binding proteins, in particular binding proteins comprising repeat domains, is considered.

A pharmaceutical composition comprises recombinant binding proteins as described above and a pharmaceutically acceptable carrier, excipient or stabilizer, for example as described in Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. [1980]. Suitable carriers, excipients or stabilizers known to the skilled man are saline, Ringer's solution, dextrose solution, Hank's solution, fixed oils, ethyl oleate, 5% dextrose in saline, substances that enhance isotonicity and chemical stability, buffers and preservatives. Other suitable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids and amino acid copolymers. A pharmaceutical composition may also be a combination formulation, comprising an additional active agent, such as an anti-cancer agent or an anti-angiogenic agent.

The formulations to be used for *in vivo* administration must be aseptic or sterile. This is readily accomplished by filtration through sterile filtration membranes.

The pharmaceutical composition may be administered by any suitable method within the knowledge of the person skilled in the art.

Further, any of the above mentioned pharmaceutical composition is considered for the treatment of a disorder.

The invention further provides methods of treatment. The method comprises administering, to a patient in need thereof, a therapeutically effective amount of a recombinant binding protein of the invention, that is, an amount that is sufficient to produce a desired effect on a patient.

Further, a method of treating a pathological condition in a mammal including man, comprising administering to a patient in need thereof an effective amount of the above mentioned pharmaceutical composition is considered.

Examples of such pathological conditions are atherosclerosis, restenosis, pulmonary hypertension, ocular and retinal diseases and fibrotic diseases, including pulmonary fibrosis, liver cirrhosis, scleroderma, glomerulosclerosis and cardiac fibrosis. In addition, anti-PDGF-BB therapy is useful for oncology pathological conditions, such as gliomas, sarcomas, leukemias, lymphomas and epithelial cancers.

The recombinant binding protein or ankyrin repeat domain according to the invention may be obtained and/or further evolved by several methods such as display on the surface of bacteriophages (WO 1990/002809, WO 2007/006665) or bacterial cells (WO 1993/010214), ribosomal display (WO 1998/048008), display on plasmids (WO 1993/008278) or by using covalent RNA-repeat protein hybrid constructs (WO 2000/032823), or intracellular expression and selection / screening such as by protein complementation assay (WO 1998/341120). Such methods are known to the person skilled in the art.

A library of ankyrin repeat proteins used for the selection/screening of a recombinant binding protein or ankyrin repeat domain according to the invention may be obtained according to protocols known to the person skilled in the art (WO 2002/020565, Binz, H.K., et al., J. Mol. Biol., *332*, 489-503, 2003, and Binz et al., 2004, loc. cit). The use of such libraries for the selection of ankyrin repeat domains with specificity for PDGF-BB is exemplified in Example 1. Furthermore, ankyrin repeat domains of the present invention may be modularly assembled from ankyrin repeat modules according to the current invention and appropriate capping modules or capping repeats (Forrer, P., et al., FEBS letters *539*, 2-6, 2003) using standard recombinant DNA technologies (e.g. WO 2002/020565, Binz et al., 2003, loc. cit. and Binz et al., 2004, loc. cit).

The invention is not restricted to the particular embodiments described in the Examples. Other sources may be used and processed following the general outline described below.

Examples

All of the starting materials and reagents disclosed below are known to those skilled in the art, and are available commercially or can be prepared using well-known techniques.

Materials

Chemicals were purchased from Fluka (Switzerland). Oligonucleotides were from Microsynth (Switzerland). Unless stated otherwise, DNA polymerases, restriction enzymes and buffers were from New England Biolabs (USA) or Fermentas (Lithuania). The cloning and protein production strain was *E. coli* XL1-blue (Stratagene, USA) or BL21 (Novagen, USA). Recombinant human and murine PDGF-BB was purchased from Reliatech (Germany; product numbers 200-055 and M10-125, respectively). Biotinylated PDGF-BB was obtained chemically via coupling of the biotin moiety to primary amines of the protein using standard biotinylation reagents and methods (Pierce, USA).

Molecular Biology

Unless stated otherwise, methods are performed according to described protocols (Sambrook J., Fritsch E.F. and Maniatis T., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory 1989, New York).

Designed ankyrin repeat protein libraries

Methods to generate designed ankyrin repeat protein libraries are described (WO 2002/020565; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). By such methods designed ankyrin repeat protein libraries having randomized ankyrin repeat modules and/or randomized capping modules can be constructed. For example, such libraries could accordingly be assembled based on a fixed N-terminal capping module (e.g. the N-terminal capping module of SEQ ID NO: 2) or a randomized N-terminal capping module according to SEQ ID NO: 64, one or more randomized repeat modules according to the sequence motif of SEQ ID NO: 20, 62 or 63, and a fixed C-terminal capping module (e.g. the C-terminal capping module of SEQ ID NO: 8) or a randomized C-terminal capping module according to SEQ ID NO: 65. Preferably, such libraries are assembled to not have the amino acids C, G, M, N (in front of a G residue) or P at randomized positions of repeat or capping modules. In addition, randomized repeat modules according to the sequence motif of SEQ ID NO: 20, 62 or 63 could be further randomized at position 10 and/or position 17; the randomized N-terminal capping module according to the sequence motif of SEQ ID NO: 64 could be further randomized at position 7 and/or position 9; and the randomized C-terminal capping modules according to the sequence motif of SEQ ID NO: 65 could be further randomized at positions 10, 11 and/or 17.

Furthermore, such randomized modules in such libraries may comprise additional polypeptide loop insertions with randomized amino acid positions. Examples of such polypeptide loop insertions are complement determining region (CDR) loop libraries of antibodies or de novo generated peptide libraries. For example, such a loop insertion could be designed using the structure of the N-terminal ankyrin repeat domain of human ribonuclease L (Tanaka, N., Nakanishi, M, Kusakabe, Y, Goto, Y., Kitade, Y, Nakamura, K.T., EMBO J. *23(30)*, 3929-3938, 2004) as guidance. In analogy to this ankyrin repeat domain where ten amino acids are inserted in the beta-turn present close to the boarder of two ankyrin repeats, ankyrin repeat proteins libraries may contain randomized loops (with fixed and randomized positions) of variable length (e.g. 1 to 20 amino acids) inserted in one or more beta-turns of an ankyrin repeat domain.

Any such N-terminal capping module of an ankyrin repeat protein library preferably possesses the RELLKA or RILKAA motif instead of the RILLAA motif (e.g. present from position 21 to 26 in SEQ ID NO:64) and any such C-terminal capping module of an ankyrin repeat protein library preferably possesses the KAA or KLA motif instead of the KLN motif (e.g. the last three amino acids in SEQ ID NO:65).

The design of such an ankyrin repeat protein library may be guided by known structures of an ankyrin repeat domain interacting with a target. Examples of such structures, identified by their Protein Data Bank (PDB) unique accession or identification codes (PDB-IDs), are 1WDY, 3V31, 3V30, 3V2X, 3V2O, 3UXG, 3TWQ-3TWX, 1N11, 1S70 and 2ZGD.

Examples of designed ankyrin repeat protein libraries, such as the N2C and N3C designed ankyrin repeat protein libraries, are described (WO 2002/020565; Binz et al. 2003, loc. cit.; Binz et al. 2004, loc. cit.). The digit in N2C and N3C describes the number of randomized repeat modules present between the N-terminal and C-terminal capping modules.

The nomenclature used to define the positions inside the repeat units and modules is based on Binz et al. 2004, loc. cit. with the modification that borders of the ankyrin repeat modules and ankyrin repeat units are shifted by one amino acid position. For example, position 1 of an ankyrin repeat module of Binz et al. 2004 (loc. cit.) corresponds to position 2 of a ankyrin repeat module of the current disclosure and consequently position 33 of a ankyrin repeat module of Binz et al. 2004, loc. cit. corresponds to position 1 of a following ankyrin repeat module of the current disclosure.

All the DNA sequences were confirmed by sequencing, and the calculated molecular weight of all described proteins was confirmed by mass spectrometry.

Example 1: *Selection of binding proteins comprising an ankyrin repeat domain with binding specificity for PDGF-BB*

Using ribosome display (Hanes, J. and Plückthun, A., PNAS *94*, 4937-42, 1997) many designed ankyrin repeat proteins (DARPins) with binding specificity for PDGF-BB were selected from DARPin libraries as described by Binz et al. 2004 (loc. cit.). The binding of the selected clones toward specific (PDGF-BB) and unspecific (MBP, *E. coli* maltose binding protein) targets was assessed by crude extract ELISA indicating that hundreds PDGF-BB binding proteins were successfully selected. For example, the ankyrin repeat domains of SEQ ID NO: 23 to 61 constitute amino acid sequences of selected binding proteins comprising an ankyrin repeat domain with binding specificity for PDGF-BB. Individual ankyrin repeat modules from such ankyrin repeat domains with binding specificity to PDGF-BB are provided in SEQ ID NO: 12, 14, 15, 17, 18 and 19. Individual capping modules of such ankyrin repeat domains with binding specificity to PDGF-BB are provided in SEQ ID NO: 13 and 16.

Selection of PDGF-BB specific ankyrin repeat proteins by ribosome display

The selection of PDGF-BB specific ankyrin repeat proteins was performed by ribosome display (Hanes and Plückthun, loc. cit.) using human and mouse PDGF-BB as target proteins, libraries of designed ankyrin repeat proteins as described above and established protocols (Zahnd, C., Amstutz, P. and Plückthun, A., Nat. Methods *4*, 69-79, 2007). The number of reverse transcription (RT)-PCR cycles after each selection round was constantly reduced from 40 to 30, adjusting to the yield due to enrichment of binders. The first four rounds of selection employed standard ribosome display selection, using decreasing target concentration and increasing washing stringency to increase selection pressure from round 1 to round 4 (Binz et al. 2004, loc. cit.). To enrich high affinity anti-PDGF-BB DARPins, the output from the fourth round of standard ribosome display selection (above) was subjected to an off-rate selection round with increased selection stringency (Zahnd, 2007, loc. cit.). A final standard selection round was performed to amplify and recover the off-rate selected binding proteins.

Selected clones bind specifically to PDGF-BB as shown by crude extract ELISA

Individual selected DARPins specifically binding PDGF-BB were identified by an enzyme-linked immunosorbent assay (ELISA) using crude *Escherichia coli* extracts of DARPin expression cells using standard protocols. DARPins selected by ribosome display were cloned into the pQE30 (Qiagen) expression vector, transformed into *E. coli* XL1-Blue (Stratagene) and then grown overnight at 37°C in a 96-deep-well plate (each clone in a single well) containing 1 ml growth medium (2YT containing 1% glucose and 100 µg/ml ampicillin). 1 ml of fresh 2YT containing 50 µg/ml ampicillin was inoculated with 100 µl of the overnight culture in a fresh 96-deep-well plate. After incubation for 2 h at 37°C, expression was induced with IPTG (1 mM final concentration) and continued for 3 h. Cells were harvested, resuspended in 100 µl B-PERII (Pierce) and incubated for 15 min at room temperature with shaking. Then, 900 µl PBS-TC (PBS supplemented with 0.25% Casein hydrolysate, 0.1% Tween 20®, pH 7.4) were added and cell debris were removed by centrifugation. 100 µl of each lysed clone were applied to a well of a Neutravidin coated MaxiSorp plate containing either PDGF-BB or the unrelated MBP immobilized via their biotin moiety and incubated for 1 h at RT. After extensive washing with PBS-T (PBS supplemented with 0.1% Tween 20®, pH 7.4) the plate was developed using standard ELISA procedures using the monoclonal horse-radish-labeled anti-RGS$(His)_4$ antibody (34650, Qiagen) Binding was then detected by POD substrate (Roche). The color development was measured at 405 nm. Screening of several hundred clones by such a crude cell extract ELISA revealed more than hundred different DARPins with specificity for PDGF-BB. These binding proteins were chosen for further analysis. Examples of amino acid sequences of selected ankyrin repeat domains that specifically bind to PDGF-BB are provided in SEQ ID NO:23 to 61.

These ankyrin repeat domains with binding specificity for PDGF-BB and negative control DARPins with no binding specificity for PDGF-BB (i.e. DARPin #21 and #22) were cloned into a pQE (QIAgen, Germany) based expression vector providing an N-terminal His-tag to facilitate simple protein purification as described below. Thus, expression vectors encoding the following DARPins were constructed:

DARPin #21 (SEQ ID NO:21 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #22 (SEQ ID NO:22 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #23 (SEQ ID NO:23 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #24 (SEQ ID NO:24 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #25 (SEQ ID NO:25 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #26 (SEQ ID NO:26 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #27 (SEQ ID NO:27 with a His-tag (SEQ ID NO:9) fused to its N-terminus);

DARPin #28 (SEQ ID NO:28 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #29 (SEQ ID NO:29 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #30 (SEQ ID NO:30 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #31 (SEQ ID NO:31 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #32 (SEQ ID NO:32 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #33 (SEQ ID NO:33 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #34 (SEQ ID NO:34 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #35 (SEQ ID NO:35 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #36 (SEQ ID NO:36 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #37 (SEQ ID NO:37 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #38 (SEQ ID NO:38 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #39 (SEQ ID NO:39 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #40 (SEQ ID NO:40 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #41 (SEQ ID NO:41 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #42 (SEQ ID NO:42 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #43 (SEQ ID NO:43 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #44 (SEQ ID NO:44 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #45 (SEQ ID NO:45 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #46 (SEQ ID NO:46 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #47 (SEQ ID NO:47 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #48 (SEQ ID NO:48 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #49 (SEQ ID NO:49 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #50 (SEQ ID NO:50 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #51 (SEQ ID NO:51 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #52 (SEQ ID NO:52 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #53 (SEQ ID NO:53 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #54 (SEQ ID NO:54 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #55 (SEQ ID NO:55 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #56 (SEQ ID NO:56 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #57 (SEQ ID NO:57 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #58 (SEQ ID NO:58 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #59 (SEQ ID NO:59 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #60 (SEQ ID NO:60 with a His-tag (SEQ ID NO:9) fused to its N-terminus);
DARPin #61 (SEQ ID NO:61 with a His-tag (SEQ ID NO:9) fused to its N-terminus);

High level and soluble expression of DARPins

For further analysis, the selected clones showing specific PDGF-BB binding in the crude cell extract ELISA as described above were expressed in *E. coli* BL21 or XL1-Blue cells and purified using their His-tag using standard protocols. 50 ml of stationary overnight cultures (TB, 1% glucose, 100 mg/l of ampicillin; 37°C) were used to inoculate 1 l cultures (same medium without glucose). At an absorbance of 0.7 (1 for BL21) at 600 nm, the cultures were induced with 0.5 mM IPTG and incubated at 37°C for 4-5 h. The cultures were centrifuged and the resulting pellets were resuspended in 40 ml of TBS500 (50 mM Tris–HCl, 500 mM NaCl, pH 8) and sonicated. The lysate was recentrifuged, and glycerol (10% (v/v) final concentration) and imidazole (20 mM final concentration) were added to the resulting supernatant. Proteins were purified over a Ni-nitrilotriacetic acid column (2.5 ml column volume) according to the manufacturer's instructions (QIAgen, Germany). Alternatively, DARPins or selected repeat domains devoid of a 6xHis-tag were purified by anion exchange chromatography followed by size exclusion chromatography according to standard resins and protocols known to the person skilled in the art. Up to 200 mg of highly soluble DARPins with binding specificity to PDGF-BB can be purified from one liter of *E. coli* culture with a purity > 95% as estimated from SDS-15% PAGE. Such purified DARPins are used for further characterizations.

Example 2: *Characterization of the DARPins with binding for specificity for PDGF-BB by Surface Plasmon Resonance Analysis*

Biotinylated PDGF-BB molecules from human and mouse were immobilized in a flow cell through binding to coated Streptavidin and the interaction with various selected DARPins was analyzed.

Surface Plasmon Resonance (SPR) analysis

SPR was measured using a ProteOn instrument (BioRad) and measurement was performed according standard procedures known to the person skilled in the art. The running buffer was PBS, pH 7.4, containing 0.005% Tween 20®. Neutravidin was covalently immobilized on a GLC chip (BioRad) to a level of about 8000 resonance units (RU). Immobilization of PDGF-BB on the neutravidin coated chip was then performed. The interaction of DARPin PDGF-BB was then measured by injecting 100 µl running buffer (PBS containing 0.005% Tween®) containing serial dilutions of DARPins of concentration of 12.5, 6.26, 3.13 and 1.67 nM (on-rate measurement), followed by a running buffer flow for between 10 minutes and up to to 3 hours at a constant flow rate of 30 µl/min (off-rate measurement). The signals (i.e. resonance unit (RU) values) of an uncoated reference

cell and a reference injection (i.e. injection of running buffer only) were subtracted from the RU traces obtained after injection of PDGF-BB (double-referencing). From the SRP traces obtained from the on-rate and off-rate measurements the on- and off-rate of the corresponding DARPin PDGF-BB interaction can be determined.

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The results are summarized in Table 1. Dissociation constants (Kd) were calculated from the estimated on- and off-rates using standard procedures known to the person skilled in the art.

Table 1: Dissociation constants of DARPin PDGF-BB interactions (human and mouse) determined by SPR

DARPin#	Kd [M] (human)	Kd [M] (mouse)
23	2.14E-11	1.72E-11
24	3.01E-11	n.d.
25	1.47E-11	1.28E-11
26	1.77E-11	1.74E-11
28	1.71E-11	n.d.
29	1.05E-10	n.d.
30	1.10E-10	n.d.
31	1.09E-10	n.d.
32	6.38E-11	8.34E-11
33	8.06E-11	9.04E-11
34	7.75E-11	5.92E-11
35	9.56E-11	9.81E-11
36	2.42E-11	5.30E-11
37	1.52E-10	8.28E-11
38	9.41E-11	5.83E-11
39	1.72E-10	3.82E-10
40	3.44E-11	6.08E-11
42	8.05E-11	9.74E-11
43	1.29E-06	1.51E-06
44	7.68E-11	9.02E-11
45	1.08E-10	n.d.
46	1.12E-10	n.d.
47	9.37E-11	n.d.
48	1.13E-10	1.21E-10
49	7.69E-11	1.02E-10
50	1.15E-10	n.d.
51	1.21E-10	n.d.
53	1.28E-10	n.d.
54	2.45E-10	n.d.
55	5.55E-11	n.d.
56	1.50E-10	n.d.
57	1.23E-10	n.d.
58	2.57E-10	n.d.
59	1.71E-10	n.d.

n.d.: not determined.

Example 3*: Inhibition of fibroblast proliferation by DARPins with binding specificity for PDGF-BB*

NIH-3T3 fibroblast cells are a standard cell line used for assays involving PDGF-BB. On day 1 at 70%-80% confluence, the cells were harvested and seeded in a 96-well culture plate with density of 5000 cells/well in a growth medium, followed by starving of cells around 7-8 hours later by changing of medium to assay medium and incubated for 24 hours. All incubation condition was 37°C with 5% CO_2 flow. Following the starving of cells, on day 2 the media was changed into a fresh assay media containing the dilution series of growth factor human PDGF-BB (for the proliferation assay) or a inhibition mixture of 20 ng/mL human PDGF-BB with a 2.5-fold dilution series of DARPins (200 nM to 0.05 nM) for the inhibition assay. The cells were incubated for another 48h in this condition upon the addition of 20 µL of WST-1 reagent (Roche product no. 11644807001). This reagent enables a colorimetric assay that analyzes the number of viable cells. Readout of the signals was done at A_{450} nm with a correction background at A_{600} nm at several time points of 2, 4 and 6 hours after the addition of WST-1.

Example results are summarized in Table 2. IC_{50} values were calculated from the titration curves obtained as described above using GraphPad Prism software and standard procedures known to the person skilled in the art. An example titration curve is given for DARPin #49 in Figure 1.

Table 2: Inhibition potency by various DARPins of NIH-3T3 cell proliferation induced by PDGF-BB

DARPin#	IC_{50} [nM]
24	1.4
28	1.6
30	3.2
49	1.9
59	2.0

Example 4: *Characterization of DARPins with binding specificity for PDGF-BB by receptor competition assay*

The potency of PEGylated anti-PDGF-BB DARPins to inhibit the binding of human PDGF-BB to its receptor PDGFRbeta was determined in a in a receptor competition ELISA (based on PDGF-BB Quantikine, R&D Systems). PDGFRbeta/Fc chimera has been pre-coated on a microplate. The DARPins were preincubated in assay diluent from the PDGF-BB Quantikine kit (R&D Systems) with a defined amount of PDGF-BB and incubated at room temperature for 2 hours with shaking at 750 rpm. These preincubation mixtures were then transferred into the precoated wells and any PDGF-BB that is not blocked by the DARPins was bound by the immobilized receptor. After washing away any unbound substances, a horse radish peroxidase-linked polyclonal antibody specific for PDGF-BB was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and color was developing in proportion to the amount of PDGF-BB bound. The color development was stopped and the intensity of the color was measured at 405 nm. In this assay, the tested DARPins showed high PDGF-BB inhibition potency as summarized in Table 3. Example titration curves are given for a set of DARPins in Figure 2. IC_{50} values were calculated from such titration curves obtained as described above using Graph Pad Prism software and standard procedures known to the person skilled in the art.

Table 3: Inhibition of the PDGF-BB interaction with its receptor PDGFRbeta by DARPins (mean IC_{50} values are given)

DARPin#	IC_{50} [pM]
23	22
24	15
28	16
29	15
30	490
31	480
34	210
37	> 400
38	85
44	130
45	160
46	150
47	140
49	66
50	32
51	8
52	68
53	36
54	210
55	14
56	170
57	204
58	470

Example 5: *Inhibition of laser-induced choroidal neovascularization in mouse by DARPins with binding specificity for PDGF-BB*

The effect on the growth of neovessels was tested *in vivo*. A mouse laser choroidal neovascularization model was chosen and performed as published described (Takahashi, K., Saishin, Y., Saishin, Y., King, A.G., Levin, R. and Campochiaro, P.A., Arch. Ophthalmol. *127(4)*, 494 - 499, 2009).

Choroidal neovascularization (CNV) was induced by laser photocoagulation-induced rupture of Bruch's membrane as previously described. On day 2, adult C57BL/6 mice were anesthetized with ketamine hydrochloride (100 mg/kg body weight), and pupils were dilated with 1% tropicamide. Three burns of 532 nm diode laser photocoagulation (75 µm spot size, 0.1 seconds duration, 120 mW) were delivered to each retina with the slit lamp delivery system of an OcuLight GL diode laser using a handheld cover slip as a contact lens to view the retina. Burns were performed in the 9, 12, and 3 o'clock positions of the posterior pole of the retina. Production of a bubble at the time of laser, which indicates rupture of Bruch's membrane, is an important factor in obtaining choroidal neovascularization, and therefore, only burns in which a bubble was produced were included in the study. DARPin #61-PEG20 was administered daily with a concentration of 10 or 1 mg/kg, respectively, as indicated in Figure 3. On day 14, mice were heart perfused with fluorescein-labeled dextran, retinal flat-mounts were prepared as described (Takahashi et al., loc cit) and the area of neovascularization was quantified by image analysis. Statistical analysis was performed using 1-way ANOVA and Dunnett post-test to compare all DARPin groups to the vehicle group. These techniques are all known to persons skilled in the art. The results are presented in Figure 3.

Claims

1. A recombinant binding protein comprising at least one ankyrin repeat domain, wherein said ankyrin repeat domain binds PDGF-BB in PBS with a Kd below 10^{-7}M.

2. The binding protein of claim 1, wherein said ankyrin repeat domain inhibits the binding of PDGF-BB to PDGFRbeta in PBS with an IC_{50} value below 10^{-7}M.

3. The binding protein of claim 1 or 2, wherein said ankyrin repeat domain inhibits the PDGF-BB stimulated proliferation of 3T3 fibroblasts with an IC_{50} value below 10^{-7}M.

4. The binding protein of any one of claims 1 to 3, wherein said ankyrin repeat domain competes for binding to PDGF-BB with an ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60.

5. The binding protein of any one of claims 1 to 4, wherein said ankyrin repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60,
wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

6. The binding protein of claim 5 , wherein said ankyrin repeat domain comprises an amino acid sequence that has at least 76% amino acid sequence identity with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60,
wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

7. The binding protein of claim 5 , wherein said ankyrin repeat domain comprises an amino acid sequence that has at least 70% amino acid sequence identity in its framework positions with one ankyrin repeat domain selected from the group consisting of SEQ ID NOs: 23 to 60,

wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

8. The binding protein of claim 5 , wherein said ankyrin repeat domain is selected from the group consisting of SEQ ID NOs:23 to 60
wherein G at position 1 and/or S at position 2 of said ankyrin repeat domain are optionally missing; and
L at the second last position and/or N at the last position of said ankyrin repeat domain are optionally exchanged by A.

9. The binding protein of any one of claims 1 to 8, wherein said ankyrin repeat domain attaches to the same epitope as an ankyrin repeat domain selected from the group consisting of SEQ ID NOs:23 to 60.

10. The binding protein of any one of claims 1 to 7 or 9, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO:12, 14, 15, 17, 18 and 19 and sequences, wherein up to 9 amino acids in SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by any amino acid.

11. The binding protein of claim 10, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO:12, 14, 15, 17, 18 and 19 and sequences, wherein up to 2 amino acids in SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by any amino acid.

12. The binding protein of claim 10, wherein said ankyrin repeat domain comprises an ankyrin repeat module having an amino acid sequence selected from the group consisting of SEQ ID NO:12, 14, 15, 17, 18 and 19 and sequences, wherein up to 8 amino acids in the framework positions of SEQ ID NO:12, 14, 15, 17, 18 and 19 are exchanged by any amino acid.

13. The binding protein of claim 10, wherein said ankyrin repeat module has the amino acid sequence
KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA (SEQ ID NO:12)

and sequences, wherein up to 9 amino acids in SEQ ID NO:12 are exchanged by any amino acid and wherein

E at position 3 is optionally exchanged by an amino acid selected from the group consisting of D, W, Q, I and Y;

E at position 4 is optionally exchanged by an amino acid selected from the group consisting of T, D, Y, and S;

T at position 6 is optionally exchanged by an amino acid selected from the group consisting of S and F;

Y at position 11 is optionally exchanged by F;

V at position 14 is optionally exchanged by an amino acid selected from the group consisting of A, Y and T;

W at position 15 is optionally exchanged by an amino acid selected from the group consisting of F, K, V, and Y.

14. The binding protein of claim 13, wherein said ankyrin repeat module has the amino acid sequence

KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA (SEQ ID NO:12)

wherein up to 8 amino acids in framework positions of SEQ ID NO:12 are exchanged by any amino acid and wherein

E at position 3 is optionally exchanged by an amino acid selected from the group consisting of D, W, Q, I and Y;

E at position 4 is optionally exchanged by an amino acid selected from the group consisting of T, D, Y, and S;

T at position 6 is optionally exchanged by an amino acid selected from the group consisting of S and F;

Y at position 11 is optionally exchanged by F;

V at position 14 is optionally exchanged by an amino acid selected from the group consisting of A, Y and T;

W at position 15 is optionally exchanged by an amino acid selected from the group consisting of F, K, V, and Y.

15. The binding protein of any one of claims 1 to 14, wherein said ankyrin repeat domain comprises a capping module having an amino acid sequence selected from the group consisting of SEQ ID NO:13 and 16 and sequences, wherein up to 8-amino acids in SEQ ID NO:13 and 16 are exchanged by any amino acid.

16. The binding protein of claim 15, wherein said ankyrin repeat domain comprises a capping module having an amino acid sequence selected from the group consisting of SEQ ID NO:13 and 16 and sequences, wherein up to 7 amino acids in framework positions of SEQ ID NO:13 and 16 are exchanged by any amino acid.

17. The binding protein of claim 15, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a C-terminal capping module with the amino acid sequence

QDIYGATPADLAALVGHEDIAEVLQKLN (SEQ ID NO:13)

and sequences, wherein up to 8 amino acids in SEQ ID NO:13 are exchanged by any amino acid and wherein

I at position 3 is optionally exchanged by an amino acid selected from the group consisting of K, L, A and V;

Y at position 4 is optionally exchanged by an amino acid selected from the group consisting of W. F and S;

A at position 6 is optionally exchanged by K;

L at position 14 is optionally exchanged by an amino acid selected from the group consisting of F, Y and D;

V at position 15 is optionally exchanged by an amino acid selected from the group consisting of L, I , A and N; and

V at position 23 is exchanged by an amino acid selected from the group consisting of I and L.

18. The binding protein of claim 15, wherein the ankyrin repeat domain with binding specificity to PDGF-BB comprises a C-terminal capping module with the amino acid sequence

QDIYGATPADLAALVGHEDIAEVLQKLN (SEQ ID NO:13)

wherein up to 7 amino acids in framework positions of SEQ ID NO:13 are exchanged by any amino acid and wherein

I at position 3 is optionally exchanged by an amino acid selected from the group consisting of K, L, A and V;

Y at position 4 is optionally exchanged by an amino acid selected from the group consisting of W. F and S;

A at position 6 is optionally exchanged by K;

L at position 14 is optionally exchanged by an amino acid selected from the group consisting of F, Y and D;

V at position 15 is optionally exchanged by an amino acid selected from the group consisting of L, I , A and N; and
V at position 23 is exchanged by an amino acid selected from the group consisting of I and L.

19. The binding protein of any one of claims 1 to 4, wherein said ankyrin repeat domain comprises the ankyrin repeat module of claim 13 and the C-terminal capping module of claim 17.

20. The binding protein of claim 19 wherein said ankyrin repeat domain comprises the ankyrin repeat module of SEQ ID NO:12 directly followed by the C-terminal capping module of SEQ ID NO:13.

21. The binding protein of any one of claims 1 to 7 and 9 to 19, wherein one or more of the amino acid residues of the ankyrin repeat modules of said ankyrin repeat domain are exchanged by an amino acid residue found at the corresponding position on alignment of an ankyrin repeat unit.

22. The binding protein of any one of claims 1 to 4, comprising a peptide of any one of the sequences SEQ ID NO:12 to 19 and 23 to 61.

23. A nucleic acid encoding a binding protein of any one of claims 1 to 22 .

24. A pharmaceutical composition comprising the binding protein of any one of claims 1 to 22 or the nucleic acid of claim 23 , and optionally a pharmaceutical acceptable carrier and/or diluent.

25. A method of treating a condition selected from exudative age-related macular degeneration, polypoidal choroidal neovascularization, and pathological myopia, the method comprising the step of administering, to a patient in need of such treatment, a therapeutically effect amount of a binding protein of any one of claims 1 to 22 .

26. A method of treating a condition selected from exudative age-related macular degeneration, polypoidal choroidal neovascularization, and pathological myopia, the method comprising the step of administering, to a patient in need of such treatment, a

therapeutically effect amount of a binding protein comprising an ankyrin repeat domain specifically binding PDGF-BB and an ankyrin repeat domain specifically binding VEGF-A.

Fig. 1

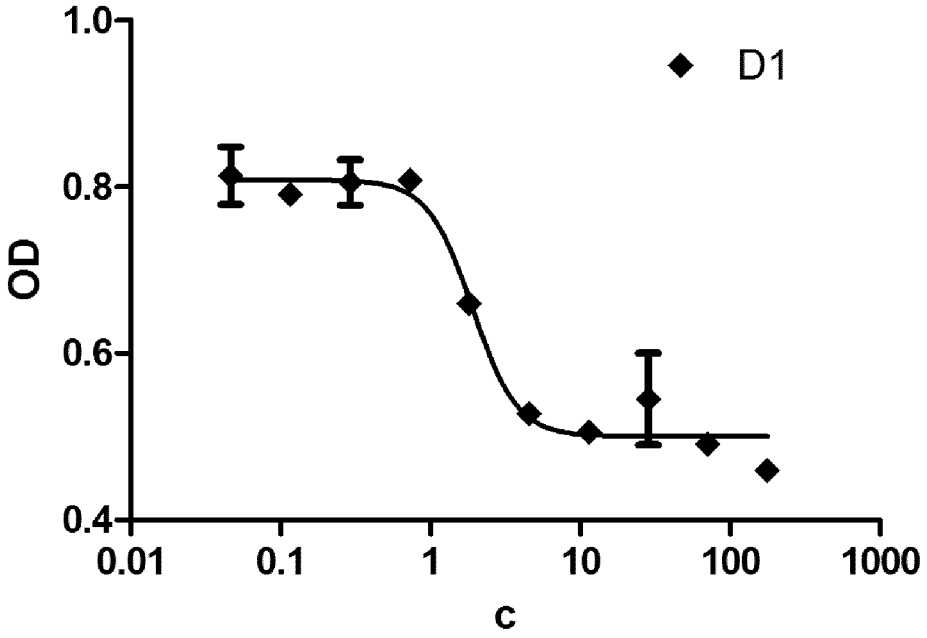


Fig. 2

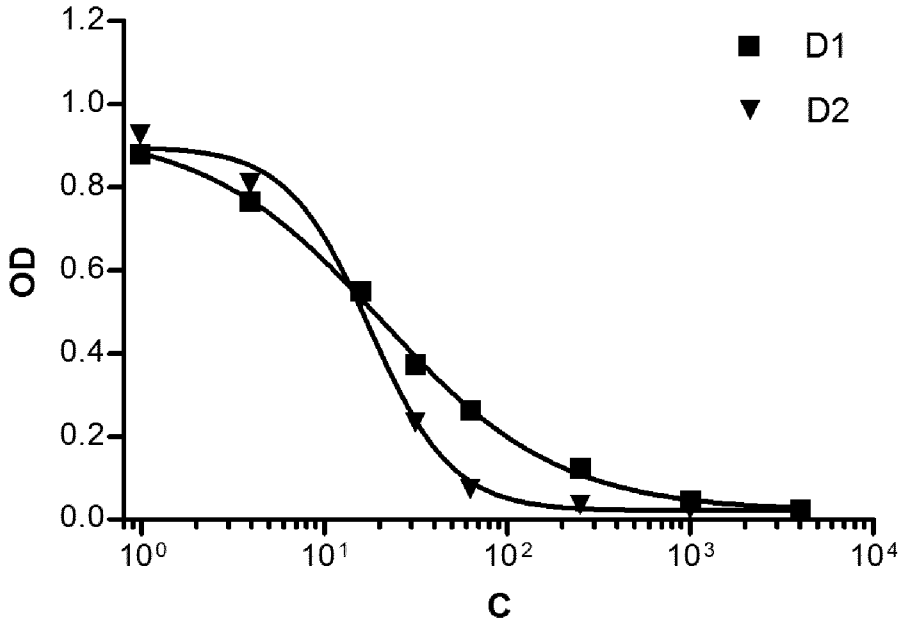
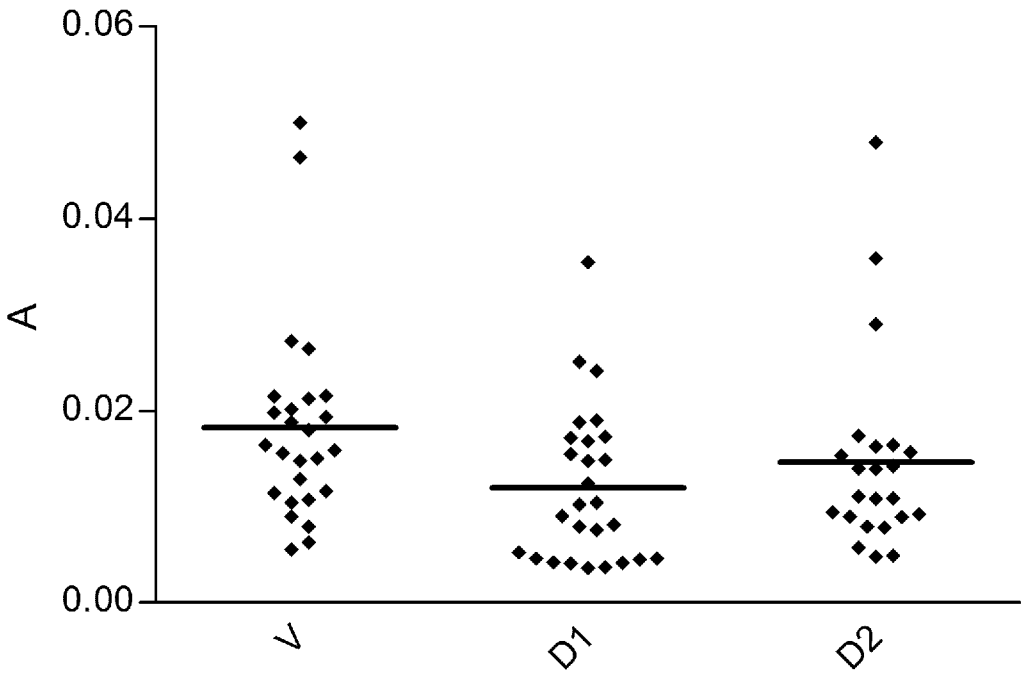


Fig. 3



INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/063488

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/063488

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/10 C07K14/49 C07K14/71 G01N33/68
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2013	19/09/2013

Name and mailing address of the ISA/	Authorized officer
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3

Form PCT/ISA/210 (second sheet) (April 2005)

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Information on patent family members

International application No
PCT/EP2013/063488

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代理人 陈文平 徐志明
(51)Int.Cl.
C12N 15/10(2006.01)
C07K 14/49(2006.01)
C07K 14/71(2006.01)
G01N 33/68(2006.01)

权利要求书3页 说明书26页
序列表52页 附图2页

(54)发明名称

设计的与血小板衍生生长因子结合的锚蛋白重复序列蛋白

(57)摘要

描述了具有与PDGF-BB的结合特异性的新设计的锚蛋白重复蛋白以及编码这类PDGF结合蛋白的核酸，包含这类蛋白的药物组合物以及这类蛋白质在治疗疾病中的用途。

CN 104508129 A

1.包含至少一个锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域在PBS中与PDGF-BB结合的Kd小于10^{-7}M。

2.根据权利要求1所述的结合蛋白，其中所述锚蛋白重复结构域在PBS中抑制PDGF-BB与PDGFRβ结合的IC_{50}值小于10^{-7}M。

3.根据权利要求1或2所述的结合蛋白，其中所述锚蛋白重复结构域抑制PDGF-BB刺激的3T3成纤维细胞增殖的IC_{50}值小于10^{-7}M。

4.根据权利要求1-3任一项所述的结合蛋白，其中所述锚蛋白重复结构域与选自SEQ ID NO:23-60的锚蛋白重复结构域竞争结合PDGF-BB。

5.根据权利要求1-4任一项所述的结合蛋白，其中所述锚蛋白重复结构域包含与选自SEQ ID NO:23-60的一个锚蛋白重复结构域有至少70％氨基酸序列同一性的氨基酸序列，

其中所述锚蛋白重复结构域中位置1的G和/或位置2的S任选地缺失，且

所述锚蛋白重复结构域中倒数第二位置的L和/或最后位置的N任选地被A置换。

6.根据权利要求5所述的结合蛋白，其中所述锚蛋白重复结构域包含与选自SEQ ID NO:23-60的一个锚蛋白重复结构域有至少76％氨基酸序列同一性的氨基酸序列，

其中所述锚蛋白重复结构域中位置1的G和/或位置2的S任选地缺失，且

所述锚蛋白重复结构域中倒数第二位置的L和/或最后位置的N任选地被A置换。

7.根据权利要求5所述的结合蛋白，其中所述锚蛋白重复结构域包含与选自SEQ ID NO:23-60的一个锚蛋白重复结构域在其框架位置中有至少70％氨基酸序列同一性的氨基酸序列，

其中所述锚蛋白重复结构域中位置1的G和/或位置2的S任选地缺失，且

所述锚蛋白重复结构域中倒数第二位置的L和/或最后位置的N任选地被A置换。

8.根据权利要求5所述的结合蛋白，其中所述锚蛋白重复结构域选自SEQ ID NO:23-60，

其中所述锚蛋白重复结构域中位置1的G和/或位置2的S任选地缺失，且

所述锚蛋白重复结构域中倒数第二位置的L和/或最后位置的N任选地被A置换。

9.根据权利要求1-8任一项所述的结合蛋白，其中所述锚蛋白重复结构域与选自SEQ ID NOs:23-60的锚蛋白重复结构域结合相同的表位。

10.根据权利要求1-7或9任一项所述的结合蛋白，其中所述锚蛋白重复结构域包含有选自如下组的氨基酸序列的锚蛋白重复模块:SEQ ID NO:12、14、15、17、18和19及SEQ ID NO:12、14、15、17、18和19中最多9个氨基酸被任意氨基酸置换的序列。

11.根据权利要求10所述的结合蛋白，其中所述锚蛋白重复结构域包含有选自下组的氨基酸序列的锚蛋白重复模块:SEQ ID NO:12、14、15、17、18和19及SEQ ID NO:12、14、15、17、18和19中最多2个氨基酸被任意氨基酸置换的序列。

12.根据权利要求10所述的结合蛋白，其中所述锚蛋白重复结构域包含有选自下组的氨基酸序列的锚蛋白重复模块:SEQ ID NO:12、14、15、17、18和19及SEQ ID NO:12、14、15、17、18和19的框架位置中最多8个氨基酸被任意氨基酸置换的序列。

13.根据权利要求10所述的结合蛋白，其中所述锚蛋白重复模块具有氨基酸序列KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA(SEQ ID NO:12)和SEQ ID NO:12中最多9个氨基酸被任意氨基酸所置换的序列，其中

位置 3 的 E 任选地被选自 D、W、Q、I 和 Y 的氨基酸置换；

位置 4 的 E 任选地被选自 T、D、Y 和 S 的氨基酸置换；

位置 6 的 T 任选地被选自 S 和 F 的氨基酸置换；

位置 11 的 Y 任选地被 F 置换；

位置 14 的 V 任选地被选自 A、Y 和 T 的氨基酸置换；

位置 15 的 W 任选地被选自 F、K、V 和 Y 的氨基酸置换。

14. 根据权利要求 13 所述的结合蛋白，其中所述锚蛋白重复模块具有氨基酸序列 KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA(SEQ ID NO:12)，其中 SEQ ID NO:12 的框架位置中最多 8 个氨基酸被任意氨基酸所置换，且其中

位置 3 的 E 任选地被选自 D、W、Q、I 和 Y 的氨基酸置换；

位置 4 的 E 任选地被选自 T、D、Y 和 S 的氨基酸置换；

位置 6 的 T 任选地被选自 S 和 F 的氨基酸置换；

位置 11 的 Y 任选地被 F 置换；

位置 14 的 V 任选地被选自 A、Y 和 T 的氨基酸置换；

位置 15 的 W 任选地被选自 F、K、V 和 Y 的氨基酸置换。

15. 根据权利要求 1-14 任一项所述的结合蛋白，其中所述锚蛋白重复结构域包含具有选自下组的氨基酸序列的加帽模块：SEQ ID NO:13 和 16 以及 SEQ ID NO:13 和 16 中最多 8 个氨基酸被任意氨基酸所置换的序列。

16. 根据权利要求 15 所述的结合蛋白，其中所述锚蛋白重复结构域包含具有选自下组的氨基酸序列的加帽模块：SEQ ID NO:13 和 16 以及 SEQ ID NO:13 和 16 的框架位置中最多 7 个氨基酸被任意氨基酸所置换的序列。

17. 根据权利要求 15 所述的结合蛋白，其中所述具有与 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有氨基酸序列 QDIYGATPADLAALVGHEDIAEVLQKLN(SEQ ID NO:13) 或 SEQ ID NO:13 中最多 8 个氨基酸被任意氨基酸所置换的序列的 C- 末端加帽模块，且其中

位置 3 的 I 任选地被选自 K、L、A 和 V 的氨基酸置换；

位置 4 的 Y 任选地被选自 W、F 和 S 的氨基酸置换；

位置 6 的 A 任选地被 K 置换；

位置 14 的 L 任选地被选自 F、Y 和 D 的氨基酸置换；

位置 15 的 V 任选地被选自 L、I、A 和 N 的氨基酸置换；以及

位置 23 的 V 任选地被选自 I 和 L 的氨基酸置换。

18. 根据权利要求 15 所述的结合蛋白，其中所述具有与 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有氨基酸序列 QDIYGATPADLAALVGHEDIAEVLQKLN(SEQ ID NO:13) 的 C- 末端加帽模块，其中 SEQ ID NO:13 的框架位置中最多 7 个氨基酸被任意氨基酸置换，且其中

位置 3 的 I 任选地被选自 K、L、A 和 V 的氨基酸置换；

位置 4 的 Y 任选地被选自 W、F 和 S 的氨基酸置换；

位置 6 的 A 任选地被 K 置换；

位置 14 的 L 任选地被选自 F、Y 和 D 的氨基酸置换；

位置 15 的 V 任选地被选自 L、I、A 和 N 的氨基酸置换；以及

位置 23 的 V 任选地被选自 I 和 L 的氨基酸置换。

19. 根据权利要求 1-4 任一项所述的结合蛋白，其中所述锚蛋白重复结构域包含权利要求 13 的锚蛋白重复模块和权利要求 17 的 C- 末端加帽模块。

20. 根据权利要求 19 所述的结合蛋白，其中所述锚蛋白重复结构域包含 SEQ ID NO:12 的锚蛋白重复模块，紧接着是 SEQ ID NO:13 的 C- 末端加帽模块。

21. 根据权利要求 1-7 和 9-19 任一项所述的结合蛋白，其中所述锚蛋白重复结构域的锚蛋白重复模块中的一个或多个氨基酸残基被锚蛋白重复单元比对时对应位置处存在的氨基酸残基置换。

22. 根据权利要求 1-4 任一项所述的结合蛋白，包含序列 SEQ ID NO :12-19 和 23-61 中任一序列的肽。

23. 编码根据权利要求 1-22 任一项所述的结合蛋白的核酸。

24. 包含根据权利要求 1-22 任一项所述的结合蛋白或根据权利要求 23 所述的核酸及任选的药学上可接受的载体和 / 或稀释剂的药物组合物。

25. 治疗选自渗出型年龄相关性黄斑变性、息肉状脉络膜血管新生和病理性近视的状况的方法，所述方法包括向需要所述治疗的患者施用治疗有效量的根据权利要求 1-22 任一项所述的结合蛋白。

26. 治疗选自渗出型年龄相关性黄斑变性、息肉状脉络膜血管新生、和病理性近视的状况的方法，所述方法包括向需要所述治疗的患者施用治疗有效量的包含与 PDGF-BB 特异性结合的锚蛋白重复结构域和与 VEGF-A 特异性结合的锚蛋白重复结构域的结合蛋白。

设计的与血小板衍生生长因子结合的锚蛋白重复序列蛋白

技术领域

[0001] 本发明涉及设计的具有与血小板衍生生长因子 (PDGF) 的结合特异性的锚蛋白重复蛋白，以及编码这类 PDGF 结合蛋白的核酸序列、包含这类蛋白质的药物组合物和这类蛋白质治疗疾病的用途。

背景技术

[0002] 血小板衍生生长因子 (PDGF) 在三十多年前被确认为成纤维细胞、平滑肌细胞和神经胶质细胞的血清生长因子。最近的综述中 (Andrae, J., Gallini, R. 和 Betsholtz, C., Genes Dev., 22, 1276-1312, 2008) 广泛描述了它在生理学和医学上的作用。人 PDGF 最初被确定为二硫键连接的两条不同肽链 (A(PDGF-A ;人 PDGF-A 具有 UniProtKB/Swiss-Prot 编 号 P04085) 和 B(PDGF-B ; 人 PDGF-B 具 有 UniProtKB/Swiss-Prot 编 号 P01127)) 的二聚体。因此，可以形成三种蛋白质二聚体 :PDGF-AA、PDGF-AB 和 PDGF-BB。最近，鉴定了两种额外的 PDGF 多肽链，PDGF-C 和 PDGF-D。目前已知的 PDGF 基因和多肽属于结构和功能相关的生长因子家族，其也包括血管内皮生长因子 (VEGF)。PDGF/VEGF 生长因子在整个动物界是保守的。

[0003] PDGF 通过两种具有共同域结构的受体酪氨酸激酶 (RTK)，PDGF 受体 (PDGFR) α (PDGFRα) 和 β (PDGFRβ)，起作用，其包括 5 个细胞外免疫球蛋白 (Ig) 环和分裂的细胞内酪氨酸激酶结构域。VEGF 通过不同的但结构相关的 RTK 亚家族起作用。配体结合促进受体二聚化，这启动信号传导。取决于配体的构造和受体表达的模式，可以形成不同的受体二聚体。然而，在体内似乎只有少数的相互作用似乎是相关的 ;即，经由 PDGFRα 的 PDGF-AA 和 PDGF-CC，及经由 PDGFRβ 的 PDGF-BB 的那些相互作用。

[0004] PDGF 在发育过程中有关键作用，但在成人中对于正常生理功能证据有限。动物发育中 PDGF 和 PDGFR 的研究揭示了 PDGFRα 信号传导在原肠胚形成以及在脑和心神经嵴、生殖腺、肺、肠、皮肤、中枢神经系统和骨骼发育中的作用。相似地，确定了 PDGFRβ 信号传导在血管形成和早期造血中的作用。PDGF 信号传导牵涉广泛疾病。PDGF 信号传导途径的自分泌激活与某些胶质瘤、肉瘤和白血病相关。通常在上皮细胞癌中观察到旁分泌的 PDGF 信号传导，其中它触发了基质募集 (stromal recruitment) 且可能参与上皮 - 间质转变，从而影响肿瘤生长、血管形成、侵袭和转移。PDGF 驱动血管障碍（例如动脉粥样硬化、再狭窄、肺动脉高血压和视网膜疾病）以及纤维化疾病（包括肺纤维化、肝硬化、硬皮病、肾小球硬化和心脏纤维化）中的病理间质反应。

[0005] 因此，提高的 PDGF 活性已经与几种疾病和病理状况关联。对于某些疾病已经确认了 PDGF 的致病性作用，为使用 PDGF 拮抗剂例如 PDGF 特异性抗体的治疗提供了前景。此外，已表明抗 VEGF 和抗 PDGF 剂的组合对于治疗某些眼部新生血管疾病有协同治疗效益 (WO 2005/020972 ;Jo, N., Mailhos, C., Ju, M., Cheung, E., Bradley, J., Nishijima, K., Robinson, G. S., Adamis, A. P. 和 Shima, D. T., Am. J. Pathol., 168(6), 2036-2053, 2006)。

[0006] 除了抗体，还有可以用于特异性结合目标分子（例如 Binz, H. K., Amstutz, P.

和 Plückthun, A., Nat. Biotechnol. 23, 1257-1268, 2005) 从而作为拮抗剂发挥作用的新的结合蛋白或结合结构域。不具有 Fc 的一个这样的新型结合蛋白或结合结构域是基于设计的重复蛋白或设计的重复结构域 (WO 2002/020565 ; Binz, H. K., Amstutz, P., Kohl, A., Stumpp, M. T., Briand, C., Forrer, P., Grütter, M. G. 和 Plückthun, A., Nat. Biotechnol. 22, 575-582, 2004 ; Stumpp, M. T., Binz, H. K 和 Amstutz, P., Drug Discov. Today 13, 695-701, 2008)。WO2002/020565 描述了可以如何构建大的重复蛋白库以及其一般应用。然而，WO2002/020565 既没有公开对 PDGF-BB 的结合特异性的重复结构域的选择，也没有公开特异性结合 PDGF-BB 的重复结构域的具体重复模块或重复序列基序。并且，WO2002/020565 没有启示具有对 PDGF-BB 的结合特异性的重复结构域可以用于调控 PDGF-BB 介导的信号传导途径以成功治疗疾病。这些设计的重复结构域利用重复蛋白的模块化性质且可以具有 N- 末端和 C- 末端加帽 (capping) 模块以通过屏蔽结构域的疏水核心而防止设计的重复结构域聚集 (Forrer, P., Stumpp, M. T., Binz, H. K. 和 Plückthun, A., FEBS letters 539, 2-6, 2003)。

[0007] 本发明的技术问题是鉴别通过特异性结合 PDGF-BB 来调控 PDGF-BB 介导的信号传导途径从而改善某些癌症、血管疾病（如视网膜疾病）、纤维化疾病和其他病理状况的治疗的新型结合蛋白（例如锚蛋白重复蛋白或结构域）。通过提供权利要求中表征的实施方案获得了这个技术问题的解决方案。

[0008] 发明概述

[0009] 本发明涉及一种包含至少一个锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域在 PBS 中与 PDGF-BB 结合的 Kd 小于 10^{-7}M。

[0010] 更特别地，本发明涉及一种包含至少一个锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域与选自 SEQ ID NO :23-60 的锚蛋白重复结构域竞争结合 PDGF-BB，或其中所述锚蛋白重复结构域选自 SEQ ID NO :23-60，其中所述锚蛋白重复结构域的位置 1 的 G 和 / 或位置 2 的 S 任选地缺失，且所述锚蛋白重复结构域的倒数第二位置的 L 和 / 或最后位置的 N 任选地被 A 置换。

[0011] 在进一步的实施方案中，本发明涉及一种包含至少一个锚蛋白重复结构域的重组 PDGF-BB 结合蛋白，其包含有选自 SEQ ID NO :12、14、15、17、18 和 19 及其中 SEQ ID NO :12、14、15、17、18 和 19 中最多 9 个氨基酸被任意氨基酸置换的序列的氨基酸序列的锚蛋白重复模块。

[0012] 特别地，本发明涉及一种包含序列 SEQ ID NO :12-19 和 23-61 中任一种的多肽的重组 PDGF-BB 结合蛋白。

[0013] 本发明还涉及编码本发明的结合蛋白的核酸分子，和涉及包含一种或多种上述结合蛋白或核酸分子的药物组合物。

[0014] 本发明还涉及用本发明的结合蛋白治疗病理状况的方法。

[0015] 附图简要说明

[0016] 图 1. NHI-3T3 成纤维细胞增殖的抑制

[0017] 显示了各种浓度的具有对 PDGF-BB 的特异性的 DARPin（以 DARPin#49 为例）对 NIH-3T3 成纤维细胞增殖的抑制以及相应的拟合抑制曲线。然后从该拟合抑制曲线计算出 DARPin#49 的 IC_{50} 值是 1.9nM。

[0018] OD :在 450nm 下的光密度 ;C :DARPin#49 的浓度 (nM) ;D1 :DARPin#49。X 轴以对数刻度显示。DARPin#49 的定义如下。

[0019] 图 2. PDGFRβ 的竞争试验

[0020] 对于独特的单一实验显示了各种浓度的具有对 PDGF-BB 的特异性的 DARPin 对 PDGF-BB 与 PDGFRβ 结合的抑制以及相应的拟合抑制曲线。然后对于 DARPin#50(D1) 和 #28(D2) 的 IC_{50} 值分别计算为约 20 和 18pM。OD :在 450nm 下的光密度 ;C :DARPin 的浓度 (pM)。X 轴以对数刻度显示。DARPin#50 和 #28 的定义如下。

[0021] 图 3. 抗 PDGF-BB DARPin 与溶媒对脉络膜血管新生的作用

[0022] 从 0 天直到第 14 天每天对小鼠腹腔内注射溶媒或 DARPin#61-PEG20(即,用标准方法 (例如,如 WO2011/135067 中所述) 通过其 C 末端 Cys 残基与 PEG20 偶联的 DARPin#61)。在第 2 天,用激光烧灼应用于眼睛以诱导脉络膜血管新生 (CNV) 且在第 14 天测量 CNV 的程度。符号代表单个眼睛和代表三个诱导的 CNV 位点各自的平均值。条棒代表单个组的中位值。

[0023] A :CNV 的面积 (mm^2) ;V :溶媒 (即,PBS) ;D1 :PBS 中的 DARPin#61-PEG20,注射的每剂量 10mg/kg ;D2 :PBS 中的 DARPin#61-PEG20,注射的每剂量 1mg/kg。

[0024] 发明详述

[0025] 根据本发明的重组结合蛋白或结构域对哺乳动物 PDGF-BB 是特异性的。优选地,根据本发明的重组结合结构域对于小鼠、大鼠、狗、兔、猴或人来源的 PDGF-BB 是特异性的。更优选地,根据本发明的重组结合结构域对于人来源的 PDGF-BB 是特异性的。

[0026] 术语“蛋白质”是指多肽,其中该多肽的至少部分具有或者能够通过在多肽链内和 / 或之间形成二级、三级或四级结构来获得特定的三维排列。如果蛋白质由两个或更多个多肽组成,单个多肽链可以非共价或共价地连接,例如通过两个多肽之间的二硫键。蛋白质的一部分 (其单独地具有或能够通过形成二级或三级结构来获得特定的三维排列) 被称为“蛋白质结构域”。本领域技术人员熟知此类蛋白质结构域。

[0027] 在重组蛋白质、重组蛋白结构域等中所用的术语“重组”是指该多肽是采用相关领域技术人员熟知的重组 DNA 技术制备的。例如,编码多肽的重组 DNA 分子 (例如通过基因合成制备) 可以被克隆到细菌表达质粒 (例如 pQE30, Qiagen)、酵母表达质粒或哺乳动物表达质粒中。例如,当这样构建的重组细菌表达质粒被插入到合适的细菌 (例如大肠杆菌 (Escherichia coli)) 中,该细菌可以产生由这个重组 DNA 编码的多肽。相应地产生的多肽被称为重组多肽。

[0028] 在本发明的情况中,术语“多肽”涉及由一条或多条含有通过肽键连接的多个 (例如,两个或更多个) 氨基酸的链组成的分子。优选地,多肽由多于 8 个通过肽键连接的氨基酸组成。

[0029] 术语“多肽标签”是指与多肽 / 蛋白质连接的氨基酸序列,其中该氨基酸序列可用于该多肽 / 蛋白质的纯化、检测或靶向,或其中该氨基酸序列促进多肽 / 蛋白质的理化性能,或其中该氨基酸序列有效应子功能。结合蛋白的单个多肽标签、部分和 / 或结构域可直接或通过多肽接头互相连接。这些多肽标签是本领域技术人员熟知的且完全可由本领域技术人员得到。多肽标签的例子是小多肽序列,例如 His(例如 SEQ ID NO :9 的 His 标签)、myc、FLAG 或 Strep- 标签或者部分如酶 (例如像碱性磷酸酶的酶)(其允许检测该多肽 / 蛋

白质），或者可用于靶向的部分（例如免疫球蛋白或其片段）和 / 或作为效应物分子。

[0030] 术语“多肽接头”是指能够连接例如两个蛋白结构域、多肽标签和蛋白结构域、蛋白结构域和非多肽部分（例如聚乙二醇）或两个序列标签的氨基酸序列。本领域技术人员已知此类额外的结构域、标签、非多肽部分和接头。专利申请 WO2002/020565 的说明书中提供了一系列例子。此类接头的特殊例子是可变长度的甘氨酸 - 丝氨酸接头和脯氨酸 - 苏氨酸接头，优选所述接头长度为 2-24 个氨基酸；更优选所述接头长度为 2-16 个氨基酸。SEQ ID NO :10 提供了甘氨酸 - 丝氨酸接头的例子，和 SEQ ID NO :11 提供了脯氨酸 - 苏氨酸接头的例子。优选地，SEQ ID NO :11 的脯氨酸 - 苏氨酸接头前接 GS 和 / 或后接 GS。

[0031] 术语“聚合物部分”是指蛋白质性聚合物部分或非蛋白质性聚合物部分。“蛋白质性聚合物部分”优选是没有形成稳定三级机构的多肽。蛋白质性聚合物部分的例子是 XTEN® (Amunix 的注册商标 ;WO2007/103515) 多肽，或如 WO2008/155134 中所描述的含有脯氨酸、丙氨酸和丝氨酸残基的多肽。这样的蛋白质性聚合物部分可以通过利用标准的 DNA 克隆技术，接着标准表达和纯化来生成遗传融合多肽而与例如，本发明的结合结构域共价连接。“非蛋白质性聚合物部分”是非由多肽构成的聚合物部分。非蛋白质性聚合物部分的例子是羟乙基淀粉 (HES)、聚乙二醇 (PEG)、聚丙二醇或聚氧化烯。术语“PEG 化的”是指 PEG 部分共价连接于，例如，本发明的多肽。本发明的聚合物部分可以在分子量上宽范围地变化。优选地，所述聚合物部分通过多肽接头连接到结合结构域。

[0032] 在特定的实施方案中，PEG 部分或任意其他非蛋白质性聚合物可以，例如，通过马来酰亚胺接头与半胱氨酸巯基偶联，使半胱氨酸通过肽接头与如本文所述的结合结构域的 N- 或 C- 末端偶联。

[0033] 术语“结合蛋白”是指如下进一步详细解释的含有一个或多个结合结构域、一个或多个生物活性化合物和一个或多个聚合物部分的蛋白质。优选地，所述结合蛋白含有多达四个结合结构域。更优选地，所述结合蛋白含有多达两个结合结构域。最优选地，所述结合蛋白只含有一个结合结构域。此外，任意这类结合蛋白可以包含额外的非结合结构域的蛋白质结构域、多聚化部分、多肽标签、多肽接头和 / 或单一 Cys 残基。多聚化部分的例子是配对以提供功能性免疫球蛋白 Fc 结构域的免疫球蛋白重链恒定区及亮氨酸拉链或包含在两个此类多肽之间形成分子间二硫键的游离巯基的多肽。单一 Cys 残基可用于将其他部分与多肽偶联，例如，通过使用本领域技术人员熟知的马来酰亚胺化学作用。优选地，所述结合蛋白是重组结合蛋白。同样优选地，结合蛋白的结合结构域具有不同的靶标特异性。

[0034] 术语“生物活性化合物”是指在应用于患有疾病的哺乳动物时减轻该疾病的化合物。生物活性化合物可具有拮抗性或激动性的性能，且可以是蛋白质性生物活性化合物或非蛋白质性生物活性化合物。这类蛋白质性生物活性化合物可以通过利用标准的 DNA 克隆技术，接着其标准表达和纯化以生成遗传融合多肽而共价连接于，例如，本发明的结合结构域。这类非蛋白质性生物活性化合物可以通过化学手段共价连接于例如本发明的结合结构域，例如通过经由马来酰亚胺接头与半胱氨酸巯基偶联，使半胱氨酸通过肽接头与如本文所述的结合结构域的 N- 或 C- 末端连接。蛋白质性生物活性化合物的例子是具有独特靶标特异性的结合结构域（通过与生长因子的结合中和生长因子）、细胞因子（例如，白细胞介素）、生长因子（例如，人生长激素）、抗体及其片段、激素（例如，GLP-1）和任何可能的蛋白质性药物。非蛋白质性生物活性化合物的例子是毒素（例如，来自 ImmunoGen 的 DM1）、靶

向 GPCR 的小分子、抗生素和任何可能的非蛋白质性药物。

[0035] 术语“结合结构域”是指表现出与蛋白质支架相同的“折叠”（三维结构）且具有预定性质的蛋白质结构域，如下所定义的。这种结构域可通过合理的，或更为普遍地，组合蛋白质工程技术（其为本领域已知的技能）(Binz 等，2005, loc. cit.）获得。例如，具有预定性质的结合结构域可以通过包含以下步骤的方法获得：(a) 提供与如下进一步定义的蛋白质支架表现出相同折叠的蛋白质结构域的多样化集合；和 (b) 筛选所述多样化集合和 / 或从所述多样化集合中选择以获得至少一个具有所述预定性质的蛋白质结构域。蛋白质结构域的多样化集合可以基于所用的筛选和 / 或选择系统通过多种方法提供，且可包括使用本领域技术人员熟知的方法，例如噬菌体展示或核糖体展示。优选地，所述结合结构域是重组结合结构域。

[0036] 术语“蛋白质支架”是指带其中可高度耐受氨基酸插入、置换或缺失的暴露表面区域的蛋白质。可用于生成本发明的结构结构域的蛋白质支架的实例有抗体及其片段（如单链 Fv 或 Fab 片段）、来自金黄色葡萄球菌 (Staphylococcus aureus) 的蛋白质 A、来自菜粉蝶 (Pieris brassicae) 的胆汁三烯结合蛋白或其他脂笼蛋白质、锚蛋白重复蛋白或其他重复蛋白及人纤连蛋白。本领域技术人员熟知蛋白质支架 (Binz 等，2005, loc. cit. ;Binz 等，2004, loc. cit.）。

[0037] 术语“靶标”是指单个分子例如核酸分子、多肽或蛋白质、碳水化合物或任意其他天然存在的分子，包括这类单个分子的任意部分或两个或更多个这类分子的复合物。靶标可以是完整的细胞或组织样品，或者可以是任意非天然分子或部分。优选地，靶标是天然存在的或非天然的多肽或含有化学修饰（例如被天然的或非天然的磷酸化、乙酰化或甲基化修饰）的多肽。在本发明的特殊应用中，靶标是 PDGF-BB。

[0038] 术语“预定性质”是指如与靶标结合、阻断靶标、靶标介导反应的激活、酶促活性的性质，及相关的其他性质。根据所需性质的类型，普通技术人员将能够识别进行筛选和 / 或选择具有所需性质的结合结构域的形式和必要步骤。优选地，所述预定性质是指与靶标的结合。

[0039] 下面对重复蛋白的定义是基于专利申请 WO2002/020565 中的那些。专利申请 WO2002/020565 还包括了对重复蛋白特征、技术和应用的一般描述。

[0040] 术语“重复蛋白”是指包含一个或多个重复结构域的蛋白质。优选地，每一个所述的重复蛋白包括多达四个重复结构域。更优选地，每一个所述的重复蛋白包括多达两个重复结构域。最优选地，每一个所述的重复蛋白只包括一个重复结构域。此外，所述重复蛋白可包含额外的非重复蛋白结构域、多肽标签和 / 或多肽接头。

[0041] 术语“重复结构域”是指包含两个或更多个连续重复单元（模块）作为结构单元的蛋白质结构域，其中所述结构单元有相同折叠，且紧密堆叠以形成具有联合疏水核心的超螺旋结构。优选地，重复结构域还包含 N- 末端和 / 或 C- 末端加帽单元（或模块）。甚至更为优选地，所述 N- 末端和 / 或 C- 末端加帽单元（或模块）是加帽重复。

[0042] 术语“设计的重复蛋白”和“设计的重复结构域”分别是指通过专利申请 WO2002/020565 中所述的发明过程获得的重复蛋白质或重复结构域。设计的重复蛋白和设计的重复结构域是合成的而不是天然的。它们分别是通过表达相应设计的核酸获得的人造蛋白质或结构域。优选地，表达在真核或原核细胞（例如细菌细胞）中或者通过体外无细

胞表达系统完成。因此，设计的锚蛋白重复蛋白（即，DARPin）对应于包含至少一个锚蛋白重复结构域的本发明的重组结合蛋白。

[0043] 术语“结构单元”是指多肽的局部有序部分，其通过两个或更多个沿多肽链彼此靠近的二级结构区段之间的三维相互作用形成。这样的结构单元呈现结构基序。术语“结构基序”是指在至少一个结构单元中存在的二级结构元件的三维排列。本领域技术人员熟知结构基序。结构单元单独不能获得限定的三维结构排列，但是他们的连续排列（例如作为重复结构域中的重复模块）导致相邻单元的互相稳定，从而生成超螺旋结构。

[0044] 术语“重复单元”是指包含一个或多个天然存在的重复蛋白的重复序列基序的氨基酸序列，其中所述“重复单元”以多拷贝存在，且其呈现决定蛋白质折叠的全部所述基序共有的限定折叠拓扑结构。所述重复单元对应于 Forrer 等，2003，loc.Cit 中所述的重复蛋白的“重复结构单元（重复序列）”或 Binz 等，2004，loc.cit 中所述的重复蛋白的“连续同源结构单元（重复序列）”。这样的重复单元包含框架残基和相互作用残基。这类重复单元的例子有犰狳重复单元、富含亮氨酸重复单元、锚蛋白重复单元、三角形四肽（tetratricopeptide）重复单元、HEAT 重复单元和富含亮氨酸变体重复单元。包含两个或更多个这类重复单元的天然存在的蛋白质称为“天然存在的重复蛋白”。当互相比较时，重复蛋白的单个重复单元的氨基酸序列可含有显著数目的突变、置换、添加和 / 或缺失，但仍基本上保持了重复单元的一般模式或基序。

[0045] 因此，术语“锚蛋白重复蛋白”是指作为例如 Forrer 等，2003，loc.cit. 所述的锚蛋白重复的重复单元。本领域技术人员熟知锚蛋白重复。术语“锚蛋白重复结构域”是指包含两个或更多个连续的锚蛋白重复单元（模块）作为结构单元，及优选地 N- 末端和 / 或 C- 末端加帽单元（或模块）的重复结构域。

[0046] 术语“框架残基”是指重复单元的氨基酸残基或对应的重复模块的氨基酸残基，其造成折叠拓扑结构，，其对所述重复单元（或模块）的折叠有贡献或对与相邻单元（或模块）的相互作用有贡献。这种贡献可能是与重复单元（或模块）中的其他残基的相互作用，或对如 α - 螺旋或 β - 折叠片或者形成线性多肽或环的氨基酸延伸中存在的多肽主链构象的影响。

[0047] 术语“靶相互作用残基”是指重复单元的氨基酸残基或对应的重复模块的氨基酸残基，其对与靶物质的相互作用有贡献。这种贡献可能是与靶物质的直接相互作用，或对其他直接相互作用的残基的影响，例如，通过稳定重复单元（或模块）的多肽构象以允许或增强直接相互作用的残基与所述靶标的相互作用。可以通过分析通过物理化学方法（例如 X 射线晶体学、NMR 和 / 或 CD 光谱）或通过与操作者熟知的在结构生物学和 / 或信息生物学的从业者公知的已知和相关结构信息对比而获得的结构数据识别这种框架和靶相互作用残基。

[0048] 优选地，用于重复序列基序推导的重复单元是同源重复单元，其中所述重复单元包含相同的结构基序且其中所述重复单元的超过 70％的框架残基是彼此同源的。优选地，所述重复单元的超过 80％的框架残基是同源的。最优选地，所述重复单元的超过 90％的框架残基是同源的。本领域技术人员已知用于确定多肽之间同源性百分比的计算机程序，例如 Fasta、Blast 或 Gap。更优选地，用于推导重复序列基序的重复单元从在特定靶标上选择的重复结构域获得的同源重复单元。

[0049] 术语“重复序列基序”是指从一个或多个重复单元或重复模块推断的氨基酸序列。优选地，所述重复单元或重复模块来自于具有对相同靶标的结合特异性的重复结构域。这种重复序列基序包含框架残基位置和靶相互作用残基位置。所述框架残基位置对应于重复单元（或模块）的框架残基的位置。同样的，所述靶相互作用残基位置对应于重复单元（或模块）的靶相互作用残基的位置。重复序列基序包含固定位置和随机化位置。术语“固定位置”是指重复序列基序中的氨基酸位置，其中所述位置设置于特定的氨基酸。最常见的是，这种固定位置对应于框架残基的位置和／或对于特定靶标特异性的靶相互作用残基的位置。术语“随机化位置”是指重复序列基序的氨基酸位置，其中所述位置处允许两个或更多个氨基酸，例如，其中允许通常二十种天然存在的氨基酸的任一种，或其中允许二十种天然存在的氨基酸的大部分氨基酸（例如除了半胱氨酸以外的氨基酸，或除了甘氨酸、半胱氨酸和脯氨酸以外的氨基酸）。最常见的是，这种随机化位置对应于靶相互作用残基的位置。但是，框架残基的某些位置也可以随机化。

[0050] 术语“折叠拓扑结构”是指所述重复单元或重复模块的三级结构。折叠拓扑结构通过形成至少 α－螺旋或 β－折叠片的部分的氨基酸延伸，或形成线性多肽或环的氨基酸延伸，或 α－螺旋、β－折叠片和／或线性多肽／环的任意组合确定。例如，锚蛋白重复单元／模块由 β－转角，接着两个反平行的 α－螺旋和达到下一重复单元／模块的转角的环组成。

[0051] 术语“连续的”是指其中重复单元或重复模块串联布置的排列。在设计的重复蛋白质中，存在至少有 2 个，一般约 2-6 个，特别至少约 6 个，经常 20 个或更多个重复单元（或模块）。在大多数情况下，重复结构域的重复单元（或模块）表现高度的序列同一性（在对应位置的相同氨基酸残基）或序列相似性（氨基酸残基不同，但具有相似的理化性质），且某些氨基酸残基可能是高度保守的关键残基。但是，重复结构域的不同重复单元（或模块）之间通过氨基酸插入和／或缺失和／或置换导致的高度序列变异性是可能的，只要保持了重复单元（或模块）的共同折叠拓扑结构。

[0052] 本领域技术人员熟知用于通过理化手段例如 X 射线晶体学、NMR 或 CD 光谱直接确定重复蛋白质的折叠拓扑结构的方法，。用于识别并确定重复单元或重复序列基序或用于鉴定识别包含这种重复单元或基序的相关蛋白家族的方法，例如同源性检索 (BLAST 等)，在生物信息学领域中是良好建立的，且为本领域技术人员熟知。优化初始重复序列基序的步骤可包括迭代过程。

[0053] 术语“重复模块”是指设计的重复结构域的重复氨基酸序列，其原本来源于天然存在的重复蛋白质的重复单元。在重复结构域中包含的每一个重复模块来源于天然存在的重复蛋白质家族或亚家族（例如犰狳重复蛋白或锚蛋白重复蛋白的家族）的一个或多个重复单元。更优选地，重复结构域中包含的每一个重复模块包含从同源重复单元推断的重复序列基序，该同源重复单元获自在靶标上选择的重复结构域，例如如实施例 1 所述的，且具有相同的靶标特异性。

[0054] 因此，术语“锚蛋白重复模块”应当是指原本来源于天然存在的锚蛋白重复蛋白的重复单元的重复模块。本领域技术人员熟知锚蛋白重复蛋白。

[0055] “重复模块”可以包含在对应的重复模块的所有拷贝中存在的氨基酸残基的位置（“固定位置”）和具有不同的或“随机化”氨基酸残基的文职（“随机化位置”）。

[0056] 术语“加帽模块”是指与重复结构域的 N- 或 C- 末端重复模块融合的多肽，其中所述加帽模块与所述重复模块形成紧密的三级相互作用（例如三级结构相互作用）从而提供在不与连续重复模块相接触的侧将所述重复模块的疏水核心与溶剂屏蔽开的帽。所述 N- 和 / 或 C- 末端加帽模块可以是，或可以来源于，与重复单元相邻的天然存在的重复蛋白中存在的加帽单元或其他结构单元。术语“加帽单元”是指天然存在的折叠多肽，其中所述多肽限定了与重复单元 N- 或 C- 末端融合的特定结构单元，其中所述多肽与所述重复单元形成紧密的三级结构相互作用从而提供在一侧将所述重复单元的疏水核心与溶剂屏蔽开的帽。优选地，加帽模块或加帽单元是加帽重复。术语“加帽重复”是指与所述相邻的重复单元（或模块）具有相似或相同折叠和 / 或与所述相邻的重复单元（或模块）具有序列相似性的加帽模块或加帽单元。WO2002/020565 和 Interlandi 等，2008(loc.cit.) 中描述了加帽模块和加帽重复。N- 末端锚蛋白加帽模块（例如 N- 末端加帽重复）的例子是 SEQ ID NO :1-3，和锚蛋白 C- 末端加帽模块（例如 C- 末端加帽重复）的例子是 SEQ ID NO :4-8、13 和 16。

[0057] 例如，SEQ ID NO :49 的 N- 末端锚蛋白加帽模块由 1-32 位的氨基酸编码，且 SEQ ID NO :49 的 C- 末端锚加帽模块由 132-159 位的氨基酸编码。

[0058] 本发明的重组结合蛋白包含至少一个锚蛋白重复结构域，其中所述锚蛋白重复结构域具有对哺乳动物 PDGF-BB 的结合特异性。

[0059] 术语“具有对靶标的结合特异性”、“特异性地结合靶标”或“靶标特异性”等是指结合蛋白或结合域在 PBS 中以比不相关蛋白（例如 E.coli 麦芽糖结合蛋白 (MBP)）更低的解离常数与靶标结合。优选地，在 PBS 中对靶标的解离常数与对 MBP 的相应解离常数相比低至少 10 倍，更优选地至少 10^2 倍，甚至更优选地至少 10^3 倍，或最优选地至少 10^4 倍。

[0060] 实施例中示出了具有对 PDGF-BB 的结合特异性且包含锚蛋白重复结构域的重组结合蛋白。

[0061] 特别地，本发明涉及如在此定义的包含具有与 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，其在 PBS 中以小于 10^{-6}M 的解离常数 (Kd) 与 PDGF-BB 结合。优选地，所述锚蛋白重复结构域在 PBS 中以小于 10^{-7}M 的 Kd 结合 PDGF-BB，更优选地小于 10^{-8}M、10^{-9}M、10^{-10}M，或最优选地小于 10^{-11}M。

[0062] 本领域技术人员熟知测定蛋白 - 蛋白相互作用的解离常数的方法，例如基于表面等离子体共振 (SPR) 的技术（如 SPR 平衡分析）或等温滴定量热法 (ITC)。如果在不同条件（例如盐浓度、pH) 下测量，所测量的特定蛋白 - 蛋白相互作用的 Kd 值可能会变化。因此，Kd 值的测量最好是用标准化蛋白质溶液和标准化缓冲液（如 PBS) 进行。

[0063] 实施例 2 示出了包含在 PBS 中具有以小于 10^{-6}M 的 Kd 结合 PDGF-BB 的锚蛋白重复结构域的重组结合蛋白。

[0064] 优选地是包含具有对人 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白。

[0065] 更优选的是包含含有 70-300 个氨基酸，尤其是 90-200 个氨基酸的锚蛋白重复结构域的重组结合蛋白。

[0066] 本发明的结合结构域是锚蛋白重复结构域或设计的锚蛋白重复结构域，优选如 WO2002/020565 所述。实施例中示出了具有对 PCGF-BB 的结合特异性的设计的锚蛋白重复

结构域的例子。

[0067] 在进一步的实施方案中，本发明涉及包含至少一个具有对哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域以小于 10^{-7}M 的 IC_{50}值抑制在 PBS 中 PDGF-BB 与 PDGFRβ 的结合。优选地，所述锚蛋白重复结构域以小于 10^{-7}M 的 IC_{50}值抑制在 PBS 中 PDGF-BB 与 PDGFRβ 的结合，更优选地，小于 10^{-8}M、10^{-9}M、10^{-10}M，或最优选地小于 10^{-11}M。

[0068] 半最大抑制浓度 (IC_{50}) 是化合物（如本发明的结合结构域）抑制生物、生化或生理功能的效力的量度。本领域技术人员熟知确定抑制蛋白 - 蛋白相互作用的 IC_{50}值的方法，例如竞争 ELISA。如果在不同条件（例如盐浓度、pH）下测量，所测量的蛋白 - 蛋白相互作用的特定抑制剂的 IC_{50}值可以变化。因此，IC_{50}值的测量优选用标准化蛋白质溶液和标准化缓冲液（如 PBS）进行。

[0069] 实施例 4 中示出了包含以小于 10^{-7}M 的 IC_{50}值抑制 PBS 中 PDGF-BB 与 PDGFRβ 结合的锚蛋白重复结构域的重组结合蛋白。

[0070] 在进一步的实施方案中，本发明涉及包含具有对 PDGF-BB 的结合特异性的至少一个锚蛋白重复结构域的重组结合蛋白，其抑制 PDGF-BB 刺激的 NIH-3T3 成纤维细胞 (ATCC，cat 号 :CRL-1658) 增殖的 IC_{50}值小于 10^{-6}M。优选地，所述重复结构域抑制 PDGF-BB 刺激的 NIH-3T3 成纤维细胞增殖的 IC_{50}值小于 10^{-7}M，更优选地小于 10^{-8}M、10^{-9}M、10^{-10}M，或最优选地 10^{-11}M。

[0071] NIH-3T3 细胞响应于 PDGF-BB 而生长，且因此可以用于测量本发明化合物的功能抑制性能力。NIH-3T3 细胞在培养基中生长，且然后在加入 PDGF-BB 和的抗 -PDGF-BB DARPin 滴定之前营养饥饿 7 小时。如采用本领域技术人员熟知的标准测量法测量的，本发明化合物抑制 PDGF-BB 的能力的评估通过 NIH-3T3 细胞的增殖能力确定。实施例 3 中示出了包含以小于 10^{-6}M 的 IC_{50}值抑制 PDGF-BB 刺激的 NIH-3T3 成纤维细胞增殖的锚蛋白重复结构域的重组结合蛋白。

[0072] 本发明涉及包含具有对 PDGF-BB 的结合特异性的至少一个锚蛋白重复结构域的重组结合蛋白，其中所述结合蛋白和 / 或锚蛋白重复结构域在 PBS 中热展开时的中点变性温度 (Tm) 高于 40℃，且当在 PBS 中 37℃下孵育 1 天时以高达 10g/L 的浓度形成低于 5％ (w/w) 的不溶性聚集体。

[0073] 术语“PBS”是指含有 137mM NaCl、10mM 磷酸盐和 2.7mM KCl 且 pH 为 7.4 的磷酸盐缓冲的水溶液。

[0074] 优选地，重组结合蛋白和 / 或结合结构域在 pH 7.4 的 PBS 中热展开时的中点变性温度 (Tm) 高于 45℃，更优选高于 50℃，更优选高于 55℃，且最优选高于 60℃。本发明的结合蛋白或结合结构域在生理条件下具有限定的二级和三级结构。这种多肽的热展开导致其二级和三级结构的丧失，其可以进行接着，例如，圆二色性 (CD) 测量。热展开时结合蛋白或结合结构域的中点变性温度对应于当通过缓慢地将温度从 10℃提高到约 100℃使所述蛋白或结构域热变性时，在生理缓冲液中协同转变的中点处的温度。本领域技术人员熟知确定热展开时中点变性温度的测定。结合蛋白或结合结构域在热展开时的这一中点变性温度指示该多肽的热稳定性。

[0075] 还优选的是当在 PBS 中 37℃下孵育超过 5 天、优选超过 10 天，更优选超过 20 天、

更优选超过 40 天且最优选超过 100 天时，以高达 20g/L、优选高达 40g/L、更优选高达 60g/L、甚至更优选高达 80g/L 和最优选高达 100g/L 的浓度形成低于 5％ (w/w) 的不溶性聚集体的重组结合蛋白和 / 或锚蛋白重复结构域。可以通过可见沉淀的出现、凝胶过滤或动态光散射（在不溶性聚集体形成时急剧增加）检测不溶性聚集体的形成。不溶性聚集体可以通过以 10000xg 离心 10 分钟从蛋白样品除去。优选地，重组结合蛋白和 / 或锚蛋白重复结构域在所述的 PBS 中 37℃的孵育条件下形成低于 2％，更优选低于 1％、0.5％、0.2％、0.1％或最优选低于 0.05％ (w/w) 的不溶性聚集体。可以通过分离不溶性聚集体与可溶性蛋白质，然后用标准定量方法测定可溶性和不溶性部分中的蛋白质量来确定不溶性聚集体的百分比。

[0076] 还优选的是在含有 100mM 二硫苏糖醇 (DTT) 的 PBS 中 37℃下孵育 1 小时或 10 小时时，没有丧失其原始三维结构的重组结合蛋白和 / 或锚蛋白重复结构域。

[0077] 在一个特定实施方案中，本发明涉及包含锚蛋白重复结构域的重组结合蛋白，其特异性结合 PDGF-BB 且具有上述所示的的或优选的中点变性温度和非聚集性质。

[0078] 在进一步的实施方案中，本发明涉及包含至少一个具有对哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域与选自 SEQ ID NO :23-60、优选 SEQ ID NO :24、45 和 50、尤其 SEQ ID NO :24 和 50 的锚蛋白重复结构域竞争结合哺乳动物 PDGF-BB。

[0079] 还优选所述锚蛋白重复结构域与选自 DARPins#23-60 的结合蛋白竞争结合哺乳动物 PDGF-BB。优选地，所述重复结构域与选自 DARPins#24、45 和 50 的结合蛋白竞争结合哺乳动物 PDGF-BB。更优选地，所述锚蛋白重复结构域与结合蛋白 DARPins#24 或 50 竞争结合哺乳动物 PDGF-BB。

[0080] 术语“竞争结合”是指本发明的两个不同结合结构域不能同时与同一靶标结合，而其两者均能单独地与同一靶标结合。因此，这两个结合结构域竞争结合所述靶标。优选地，所述两个竞争的结合结构域与所述靶标上的重叠或相同结合表位结合。本领域技术人员熟知用于确定两个结合结构域是否竞争结合靶标的方法，例如竞争酶联免疫吸附测定法 (ELISA) 或竞争 SPR 测量（如通过采用来自 BioRad 的 Proteon 仪器）。例如，SEQ ID NO :#49 或 SEQ ID NO :#58 的锚蛋白重复结构域与 SEQ ID NO :#50 的锚蛋白重复结构域竞争结合人 PDGF。

[0081] 术语“表位”是指靶蛋白（如 PDGF-BB) 表面上本发明的结合域（如锚蛋白重复结构域）本身连接的特定位点。这个术语类似于本领域技术人员熟知的抗体的表位定义。如果本发明的两个结合域结合同一表位，则其竞争结合 PDGF-BB。可以通过，例如与 PDGF-BB 复合的本发明结合结构域的蛋白质 X 射线晶体学（一种本领域技术人员熟知的方法）来阐明表位的准确分子排列。

[0082] 在进一步的实施方案中，本发明涉及包含至少一个具有对哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域包含与选自 SEQ ID NO :23-60 的一个锚蛋白重复结构域有至少 70％氨基酸序列同一性的氨基酸序列，

[0083] 其中所述锚蛋白重复结构域的位置 1 的 G 和 / 或位置 2 的 S 任选地缺失，且

[0084] 所述锚蛋白重复结构域的倒数第二位置的 L 和 / 或最后位置的 N 任选地被 A 置换。

[0085] 优选地，本发明的重组结合蛋白中的这种锚蛋白重复结构域包含与选自 SEQ ID

NO:24、45 和 50,更优选 24 和 50 的一个锚蛋白重复结构域有至少 70%氨基酸序列同一性的氨基酸序列。

[0086] 优选地,本发明的重组结合蛋白中的这种锚蛋白重复结构域包含与在选自 SEQ ID NOs:23-60 的锚蛋白重复结构域的 N- 末端和 C- 末端加帽模块之间存在的一个、两个或三个锚蛋白重复结构域具有至少 70%,例如 70%、71%、72%、73%、74%、75%、76%、77%、78%、79%、80%、81%、82%、83%、84%、85%、86%、87%、88%、89%、90%、91%、92%、93%、94%、95%、96%、97%、98%、99%或 100%氨基酸序列同一性的氨基酸序列。

[0087] 优选地,不是具有 70%的氨基酸序列同一性,这种锚蛋白重复结构域或这种本发明重组结合蛋白的锚蛋白重复结构域中的 N- 末端和 C- 末端加帽模块之间的一个、两个或三个重复模块包含有至少 75%氨基酸序列同一性,更优选至少 76%、更优选至少 80%、更优选至少 85%、更优选至少 90%或最优选至少 95%的氨基酸序列。优选地,所述的氨基酸序列同一性的百分比是在框架位置中。

[0088] 优选地,SEQ ID NO:23-60 的重复结构域中最多 30 个氨基酸,例如 30、29、28、27、26、25、24、23、22、21、20、19、18、17、16、15、14、13、12、11、10、9、8、7、6、5、4、3、2、1 个或没有氨基酸被另一氨基酸置换。特别的,SEQ ID NO:23-60 中最多 25 个氨基酸、更优选最多 20 个氨基酸、更优选最多 15 个氨基酸、甚至更优选最多 11 个氨基酸、更优选最多 8 个氨基酸、更优选最多 5 个氨基酸、更优选最多 2 个氨基酸和最优选没有氨基酸被置换。

[0089] 优选地,当 SEQ ID NO:13 或 16 的加帽模块、SEQ ID NO:12、14、15、17、18 或 19 的重复模块或者 SEQ ID NO:23-60 的重复结构域中有氨基酸被置换时,这些氨基酸选自 A、D、E、F、H、I、K、L、M、N、Q、R、S、T、V、W 和 Y,更优选选自 A、D、E、H、I、K、L、Q、R、S、T、V 和 Y。还优选地,氨基酸被同源氨基酸置换,即,氨基酸被包含有相似生物物理性质的侧链的氨基酸置换。例如,带负电荷的氨基酸 D 可以被带负电荷的氨基酸 E 置换,或疏水氨基酸如 L 可以被 A、I 或 V 置换。本领域技术人员熟知在多肽中用一个氨基酸置换另一个氨基酸的技术。

[0090] 在进一步的实施方案中,本发明涉及包含至少一个具有对哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白,其中所述锚蛋白重复结构域选自 SEQ ID NO:23-60,

[0091] 其中所述锚蛋白重复结构域的位置 1 的 G 和 / 或位置 2 的 S 任选地缺失,且

[0092] 所述锚蛋白重复结构域的倒数第二位置的 L 和 / 或最后位置的 N 任选地被 A 置换。

[0093] 优选地,这种锚蛋白重复结构域选自 SEQ ID NO:24、45 和 50,更优选 24 和 50。

[0094] 在进一步的实施方案中,本发明涉及重组结合蛋白,其中锚蛋白重复结构域与选自 SEQ ID NOs:23-60 的锚蛋白重复结构域结合相同的表位。优选地,这种锚蛋白重复结构域选自 SEQ ID NO:24、45 和 50,更优选 24 和 50。

[0095] 在进一步的实施方案中,本发明涉及包含至少一个具有对哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白,其中所述锚蛋白重复结构域包含具有选自 SEQ ID NO:12、14、15、17、18 和 19 及 SEQ ID NO:12、14、15、17、18 和 19 中最多 9 个氨基酸被任何氨基酸置换的序列的氨基酸序列的锚蛋白重复模块。

[0096] 优选地,所述锚蛋白重复结构域的这种锚蛋白重复模块选自 SEQ ID NO:12、14 和 17,更优选 12 和 17。

[0097] 优选地，SEQ ID NO：12、14、15、17、18 和 19 的重复模块中最多 8 个氨基酸、更优选最多 7 个氨基酸、更优选最多 6 个氨基酸、更优选最多 5 个氨基酸、甚至更优选最多 4 个氨基酸、更优选最多 3 个氨基酸、更优选最多 2 个氨基酸和最优选 1 个氨基酸被另一氨基酸置换。优选地，所述的氨基酸置换在框架位置中。因此，SEQ ID NO：12、14、15、17、18 和 19 的框架位置中最多 8 个氨基酸被任何氨基酸置换，优选最多 7、6、5、4、3 或 2 个氨基酸，且最优选 1 个氨基酸。

[0098] 在进一步的实施方案中，本发明涉及重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有锚蛋白重复序列 KDEEGTTPLHYAAVWGHLEIVEVLLKAGADVNA(SEQ ID NO:12) 和其中 SEQ ID NO:12 中最多 9 个氨基酸被任何氨基酸置换的序列的重复模块，其中

[0099] 位置 3 的 E 任选地被选自 D、W、Q、I 和 Y，优选选自 D 和 W 的氨基酸置换；

[0100] 位置 4 的 E 任选地被选自 T、D、Y 和 S，优选选自 T 和 D 的氨基酸置换；

[0101] 位置 6 的 T 任选地被选自 S 和 F 的氨基酸，优选被 S 置换；

[0102] 位置 11 的 Y 任选地被 F 置换；

[0103] 位置 14 的 V 任选地被选自 A、Y 和 T 的氨基酸，优选被 A 置换；以及

[0104] 位置 15 的 W 任选地被选自 F、K、V 和 Y，优选选自 F 和 Y 的氨基酸置换。

[0105] 在进一步的实施方案中，本发明涉及包含至少一个具有哺乳动物 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，其中所述锚蛋白重复结构域包含具有选自 SEQ ID NO：13 和 16 或 SEQ ID NO：13 和 16 中最多 9 个氨基酸被任何其他氨基酸所置换的序列的氨基酸序列的加帽模块。

[0106] 优选地，所述锚蛋白重复结构域中包含的 SEQ ID NO：13 和 16 加帽模块中最多 8 个氨基酸被其他氨基酸置换，更优选最多 7 个氨基酸、更优选最多 6 个氨基酸、更优选最多 5 个氨基酸、甚至更优选最多 4 个氨基酸、更优选最多 3 个氨基酸、更优选最多 2 个氨基酸、更优选最多 1 个氨基酸和最优选 SEQ ID NO：13 和 16 中没有氨基酸被置换。优选地，所述置换的氨基酸在框架位置。

[0107] 在再另一个实施方案中，本发明涉及重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有序列 QDIYGATPADLAALVGHEDIAEVLQKLN(SEQ ID NO:13) 或 SEQ ID NO:13 中最多 9 个氨基酸被任何氨基酸置换的序列的 C- 末端加帽模块，其中

[0108] 位置 3 的 I 任选地被选自 K、L、A 和 V 的氨基酸置换，优选 L、A 和 V；

[0109] 位置 4 的 Y 任选地被选自 W、F 和 S，优选选自 W 和 F 的氨基酸置换；

[0110] 位置 6 的 A 任选地被 K 置换；

[0111] 位置 14 的 L 任选地被选自 F、Y 和 D，优选选自 F 和 Y 的氨基酸置换；

[0112] 位置 15 的 V 任选地被选自 L、I、A 和 N 的氨基酸置换，优选 L 和 I；以及

[0113] 位置 23 的 V 任选地被选自 I 和 L 的氨基酸置换。

[0114] 优选的是其中锚蛋白重复结构域包含 SEQ ID NO:12 的锚蛋白重复模块和 C- 末端加帽模块 SEQ ID NO:13 的重组结合蛋白。优选地，所述 C- 末端加帽模块直接接着所述锚蛋白重复结构域中的所述锚蛋白重复模块。

[0115] 在再另一个实施方案中，本发明涉及一种重组结合蛋白，其中具有对 PDGF-BB 的

结合特异性的锚蛋白重复结构域包含具有锚蛋白重复序列 KDQEGTTPLHFAASVGHLEIVEVLLKA GADVNA(SEQ ID NO:15) 或 SEQ ID NO:15 中最多 9 个氨基酸被任何氨基酸置换的序列的重复模块，且其中

[0116] 位置 3 的 Q 任选地被 A 置换；

[0117] 位置 4 的 E 任选地被 D 置换；

[0118] 位置 6 的 T 任选地被 E 置换；

[0119] 位置 11 的 F 任选地被 Y 置换；

[0120] 位置 14 的 S 任选地被 V 置换；以及

[0121] 位置 15 的 V 任选地被 W 置换。

[0122] 在再另一个实施方案中，本发明涉及一种重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有序列 QDHYGATPADLAALIGHEDIAEVLQKLN(SEQ ID NO:16) 或 SEQ ID NO:16 中最多 9 个氨基酸被任何氨基酸置换的序列的 C- 末端加帽模块，且

[0123] 其中

[0124] 位置 3 的 H 任选地被 I 置换；以及

[0125] 位置 4 的 Y 任选地被 W 置换。

[0126] 在再另一个实施方案中，本发明涉及一种重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有锚蛋白重复序列 KDLNGQTPLHLAADIGHLEIVEVLLKA GADVNA(SEQ ID NO:17) 或 SEQ ID NO:17 中最多 9 个氨基酸被任何氨基酸置换的序列的重复模块，且其中

[0127] 位置 1 的 K 任选地被 Q 或 I 置换；

[0128] 位置 3 的 L 任选地被 N 置换；以及

[0129] 位置 27 的 A 任选地被 H 置换。

[0130] 在再另一个实施方案中，本发明涉及一种重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有锚蛋白重复序列 KDYAGSTPLRLAAWAGHLEIVEVLLKA GADVNA(SEQ ID NO:18) 或 SEQ ID NO:18 中最多 9 个氨基酸被任何氨基酸置换的序列的重复模块，且其中

[0131] 位置 1 的 K 任选地被 Q 置换；

[0132] 位置 14 的 W 任选地被 H 置换；

[0133] 位置 15 的 A 任选地被 V 置换；以及

[0134] 位置 27 的 A 任选地被 N 或 Y 置换。

[0135] 在再另一个实施方案中，本发明涉及一种重组结合蛋白，其中具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域包含具有锚蛋白重复序列 KDYFGYTPLHLAAYFGHLEIVEVLLKA GADVNA(SEQ ID NO:19) 或 SEQ ID NO:19 中最多 9 个氨基酸被任何氨基酸置换的序列的重复模块，且其中

[0136] 位置 1 的 K 任选地被 N 置换；

[0137] 位置 12 的 A 任选地被 T 置换；

[0138] 位置 13 的 A 任选地被 T 置换；

[0139] 位置 22 的 E 任选地被 D 置换；以及

[0140] 位置 27 的 A 任选地被 H 或 Y 置换。

[0141] 进一步优选的是分别包含 N- 末端或 C- 末端锚蛋白加帽重复的 N- 末端或 C- 末端锚蛋白加帽模块，其中所述加帽重复中的一个或多个氨基酸残基被在对应的锚蛋白加帽单元或锚蛋白重复单元比对时对应位置存在的氨基酸残基取代。

[0142] 可以用 20 种最常见的天然存在的氨基酸中的任意一种进行氨基酸置换，优选用选自 A、D、E、F、H、I、K、L、M、N、Q、R、S、T、V、W 和 Y 的氨基酸置换，更优选用选自 A、D、E、H、I、K、L、Q、R、S、T、V 和 Y 的氨基酸置换。还优选地，氨基酸被同源氨基酸置换，即，氨基酸被包含有相似生物物理性质的侧链的氨基酸置换。例如，带负电荷的氨基酸 D 可以被带负电荷的氨基酸 E 置换，或疏水氨基酸如 L 可以被 A、I 或 V 置换。本领域技术人员熟知用一个同源氨基酸置换另一个氨基酸。

[0143] 还优选的是包含基于 SEQ ID NO :4-18、13 和 16 的任一上述 C- 末端加帽模块的位置 27 和 28 处的氨基酸 A 的 C- 末端加帽模块。

[0144] 还优选的是包含基于 SEQ ID NO :4-18、13 和 16 的任一上述 C- 末端加帽模块的位置 1-26 或位置 1-27 的氨基酸的 C- 末端加帽模块。

[0145] SEQ ID NO :1-3 的位置 1 的氨基酸 G 和 / 或位置 2 的 S 可以从 N- 末端锚蛋白加帽模块移除而不对其性质具有任何明显影响。这两个氨基酸用作连接锚蛋白重复结构域与其他氨基酸和蛋白质的接头。本发明还包含其中移除位置 1 的 G 和 / 或位置 2 的 S 的含有 N- 末端锚蛋白加帽模块的这种锚蛋白重复结构域。可以理解，如在此定义的锚蛋白重复结构域中的氨基酸位置（如“位置 33”）可以相应地适应，从而造成编号偏移。例如，如果缺失了一个氨基酸，“位置 33”将变成“位置 32”，或如果缺失了两个氨基酸，“位置 33”将变成“位置 31”。

[0146] 本发明的锚蛋白重复结构域的锚蛋白加帽模块可以通过组合本领域技术人员已知的技术（如氨基酸序列的比对、诱变和基因合成）被锚蛋白加帽模块取代。例如，SEQ ID NO :49 的 C- 末端加帽重复可以被 SEQ ID NO :8 的 C- 末端加帽重复取代，其通过 (i) 通过与 SEQ ID NO :8 的序列比对确定 SEQ ID NO :49（即，序列位置 132-159）的 C- 末端加帽重复，(ii) 用 SEQ ID NO :8 的序列取代确定的 SEQ ID NO :49 的 C- 末端加帽重复，(iii) 生成编码重复结构域的基因，其编码取代的 C- 末端加帽模块，(iv) 在大肠杆菌的细胞质中表达修饰的重复结构域，以及 (v) 通过标准方法纯化修饰的重复结构域而进行。作为进一步的例子，SEQ ID NO :49 的 N- 末端加帽重复可以被 SEQ ID NO :2 的 N- 末端加帽重复取代，其通过 (i) 通过与 SEQ ID NO :2 进行序列比对确定 SEQ ID NO :49（即，序列位置 1-32）的 N- 末端加帽重复，(ii) 用 SEQ ID NO :2 的序列取代确定的 SEQ ID NO :49 的 N- 末端加帽重复，(iii) 生成编码重复结构域的基因，其编码取代的 N- 末端加帽模块，(iv) 在大肠杆菌细胞质中表达修饰的重复结构域，以及 (v) 用标准方法纯化修饰的重复结构域而进行。

[0147] 此外，本发明的锚蛋白重复结构域可以通过组装 N- 末端锚蛋白加帽模块（如 SEQ ID NO :2 的 N- 末端加帽重复），接着一个或多个重复模块（如包含 SEQ ID NO :49 的位置 33-131 的氨基酸残基的三个锚蛋白重复模块）以及 C- 末端加帽模块（如 SEQ ID NO :8 的 C- 末端加帽重复）来遗传地构建。所述遗传组装的重复结构域基因然后可以如上所述在大肠杆菌中表达。

[0148] 进一步优选的是含有缺乏氨基酸 C、M 或 N 的氨基酸序列的重组结合蛋白、重复结

构域、重复模块、N- 末端加帽模块或 C- 末端加帽模块。

[0149] 进一步优选的是含有缺乏氨基酸 N 接着 G 的氨基酸序列的重组结合蛋白、重复结构域、重复模块、N- 末端加帽模块或 C- 末端加帽模块。

[0150] 进一步优选的是包含任一这种 N- 末端或 C- 末端加帽模块的重组结合蛋白或重复结构域。

[0151] 在根据本发明的包含锚蛋白重复结构域的重组结合蛋白的进一步优选实施方案中，所述重复结构域的 N- 末端加帽模块的一个或多个氨基酸残基被在 N- 末端加帽单元的比对时对应位置存在的氨基酸残基置换。优选地，最多 30％的氨基酸残基被置换，更优选最多 20％，甚至更优选最多 10％的氨基酸残基被置换。最优选地，这种 N- 末端加帽单元是天然存在的 N- 末端加帽单元。

[0152] 在根据本发明的包含锚蛋白重复结构域的重组结合蛋白的进一步优选实施方案中，所述重复结构域的 C- 末端加帽模块的一个或多个氨基酸残基被在 C- 末端加帽单元序列的比对时对应位置存在的氨基酸残基置换。优选地，最多 30％的氨基酸残基被置换，更优选最多 20％，甚至更优选最多 10％的氨基酸残基被置换。最优选地，这种 C- 末端加帽单元是天然存在的 C- 末端加帽单元。

[0153] 在再另一个特定实施方案中，最多 30％氨基酸残基，更优选最多 20％，甚至更优选最多 10％的氨基酸残基被未在重复单元、N- 末端加帽单元或 C- 末端加帽单元的对应位置中发现的氨基酸置换。

[0154] 术语“共有序列”是指氨基酸序列，其中所述共有序列通过多个重复单元的结构和 / 或序列比对获得。利用两个或更多个结构和 / 或序列比对的重复单元并在比对中允许空位，有可能确定在每个位置上最常见的氨基酸残基。共有序列是指在每个位置上都是最常表现的氨基酸的序列。在单一位置上具有高于平均出现水平的两个或更多个氨基酸的情况中，共有序列可以包括这些氨基酸的子集。所述两个或更多个重复单元可以从单一重复蛋白中包含的重复单元，或从两个或更多个不同的重复蛋白获取。

[0155] 本领域技术人员熟知共有序列及其确定方法。

[0156] “共有氨基酸残基”是在共有序列中某个位置上存在的氨基酸。如果在所述两个或更多个重复单元中发现有两个或更多个，如三个、四个或五个氨基酸残基具有相似机率，则共有氨基酸可以是最常见的氨基酸之一或所述两个或更多个氨基酸残基的组合。

[0157] 进一步优选的是非天然存在的加帽模块、重复模块、结合蛋白或结合结构域。

[0158] 术语“非天然存在”是指合成的或不是来自于自然的，更具体的，该术语是指人工制造的。术语“非天然存在的结合蛋白”或“非天然存在的结合结构域”是指所述结合蛋白或所述结合结构域是合成的（即，通过化学合成由氨基酸制备）或重组的且不是来自于自然的。“非天然存在的结合蛋白”或“非天然存在的结合结构域”分别是通过表达对应设计的核酸获得的人造的蛋白质或结构域。优选的，表达在真核或细菌细胞中或通过使用体外无细胞表达系统完成。另外，该术语是指所述结合蛋白或所述结合结构域的序列在序列数据库（如 GenBank、EMBL-Bank 或 Swiss-Prot）中不是作为非人工序列存在。本领域技术人员熟知这些数据库和其他相似的序列数据库。

[0159] 在一个特定的实施方案中，本发明涉及包含与 PDGF-BB 特异性结合的锚蛋白重复结构域和进一步包含与血管内皮生长因子 A(VEGF-A) 特异性结合的锚蛋白重复结构域的

重组结合蛋白。具有对 PDGF-BB 的特异性的锚蛋白重复结构域的例子在本文中给出，且具有对 VEGF-A 的特异性的锚蛋白重复结构域的例子描述于其全文通过引用结合于此的 WO 2010/060748(US 2011/0207668) 和 WO 2011/135067(US 2013/0116197) 中。这样两个重复结构域可以通过本领域技术人员已知的方法利用遗传手段通过多肽接头连接。在本发明的一个实施方案中，包含与 PDGF-BB 特异性结合的锚蛋白重复结构域和与血管内皮生长因子 A(VEGF-A) 特异性结合的锚蛋白重复结构域的重组结合蛋白可用于治疗视网膜疾病和脉络膜新生血管疾病，如渗出型年龄相关性黄斑变性、息肉状脉络膜血管新生和病理性近视。

[0160] 另一个优选的实施方案是包含具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白，所述锚蛋白重复结构域包含参与与 PDGF-BB 结合的一个、两个、三个或更多个内部重复模块。优选地，这种锚蛋白重复结构域包含 N- 末端加帽模块，两个到四个内部重复模块和 C- 末端加帽模块。优选的，所述加帽模块是加帽重复。还优选的，所述加帽模块参与与 PDGF-BB 的结合。

[0161] 进一步优选的是包含两个或更多个所述具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域的重组结合蛋白。优选的，所述结合蛋白包含 2 个或 3 个所述重复结构域。所述两个或更多个重复结构域有相同或不同的氨基酸序列。

[0162] 在根据本发明的包含锚蛋白重复结构域的重组结合蛋白的进一步优选实施方案中，所述锚蛋白重复结构域的重复模块的一个或多个氨基酸残基被重复单元序列比对时对应位置存在的氨基酸残基置换。优选地，最多 30％的氨基酸残基被置换，更优选最多 20％，甚至更优选最多 10％的氨基酸残基被置换。最优选地，这种重复单元是天然存在的重复单元。

[0163] 在再另一个特定实施方案中，最多 30％的氨基酸残基，如 29％、28％、27％、26％、25％、24％、23％、22％、21％、20％、19％、18％、17％、16％、15％、14％、13％、12％、11％、10％、9％、8％、7％、6％、5％、4％、3％、2％、1％或 0％的氨基酸残基被未在重复单元的对应位置中发现的氨基酸置换。更优选最多 20％，甚至更优选 10％的氨基酸残基被未在重复单元的对应位置中发现的氨基酸置换。

[0164] 在进一步的实施方案中，如在此所述的任意重组 PDGF-BB 结合蛋白或结构域可以共价结合一个或多个另外的部分，包括例如，结合不同靶标以产生双特异性结合剂的部分、生物活性化合物、标记部分（例如，荧光标记如荧光素、或放射性示踪剂）、促进蛋白纯化的部分（例如，小肽标签如 His- 或 strep- 标签）、提供具有提高的疗效的效应子功能的部分（如，抗体的 Fc 部分以提供抗体依赖性细胞介导细胞毒性、毒性蛋白部分如铜绿假单胞菌 (Pseudomonas aeruginosa) 外毒素 A(ETA) 或小分子毒性剂如美登木素生物碱或 DNA 烷化剂）或提供改进的药代动力学的部分。改进的药代动力学可以根据认识到的治疗需要评估。通常需要提高生物利用度和 / 或增加剂量之间的时间，可能通过增加给药后在血清中蛋白质保持可用的时间。在某些情况下，需要提高蛋白质的血清浓度随时间的连续性（如，减少在给药后短时间的浓度和紧接下次给药前的浓度之间的蛋白质血清浓度差异）。倾向于减缓蛋白质从血液的清除的部分包括羟乙基淀粉 (HES)、聚乙二醇 (PEG)、糖（例如，唾液酸）、良好耐受的蛋白质部分（例如，Fc 片段或血清白蛋白）和对丰富的血清蛋白有特异性和亲和力的结合结构域或肽，如抗体 Fc 片段或血清白蛋白。WO2012/069654 中提供了具有对血清白蛋白的亲和力的这种结合结构域的例子。本发明的重组结合蛋白可以连接于将哺

乳动物（如小鼠、大鼠或人）中多肽的清除率相对于未修饰多肽降低超过 3 成的部分。

[0165] 在进一步的实施方案中，本发明涉及编码特定重组结合蛋白、特定锚蛋白重复结构域、特定锚蛋白重复模块和特定加帽模块的核酸分子。此外，考虑包含所述核酸分子的载体。

[0166] 进一步地，考虑包含一个或多个上述重组结合蛋白，尤其是包含重复结构域的重组蛋白，或编码特定结合蛋白的核酸分子，及任选的药学上可接受的载体和 / 或稀释剂的药物组合物。药学上可接受的载体和 / 或稀释剂是本领域技术人员已知的，并如以下更详细地解释。更进一步地，考虑包含一个或多个上述重组结合蛋白，尤其是包含重复结构域的结合蛋白的诊断组合物。

[0167] 药物组合物包含如上所述的重组结合蛋白和药学上可接受的载体、赋形剂或稳定剂，例如，如 Remington's Pharmaceutical Sciences16th edition, Osol, A. Ed. [1980] 所描述的。技术人员已知的合适的载体、赋形剂或稳定剂是盐水、Ringer 溶液、葡萄糖溶液、Hank 溶液、非挥发油、油酸乙酯、盐水中的 5％葡萄糖、提高等渗性和化学稳定性的物质、缓冲剂和防腐剂。其他合适的载体包括其本身不能诱导对接受该组合物的个体有害的抗体的产生的任何载体，如蛋白质、多糖、聚乳酸、聚乙醇酸、聚氨基酸和氨基酸共聚物。药物组合物也可以是包含额外活性剂如抗癌剂或抗血管生成剂的组合制剂。

[0168] 用于体内给药的制剂必须是无菌的或灭菌的。这可以通过用无菌过滤膜过滤轻易实现。

[0169] 药物组合物可以通过本领域技术人员技能范围内的任意合适的方法给药。

[0170] 进一步地，任意上述药物组合物被认为用于疾病的治疗。

[0171] 本发明进一步提供治疗方法。该方法包括向需要的患者施用治疗有效量的，即足够对患者产生所需效果的量的，本发明的重组结合蛋白。

[0172] 进一步地，考虑治疗哺乳动物包括人的病理状况的方法，包括向需要的患者施用有效量的上述药物组合物。

[0173] 此类病理状况的例子是动脉粥样硬化、再狭窄、肺动脉高血压、眼和视网膜疾病和纤维化疾病，包括肺间质纤维化、肝硬化、硬皮病、肾小球硬化和心肌纤维化。此外，抗 PDGF-BB 疗法可用于肿瘤病理状况，如胶质瘤、肉瘤、白血病、淋巴瘤和上皮癌。

[0174] 本发明的重组结合蛋白或锚蛋白重复结构域可以通过几种方法获得和 / 或进一步进化，如在噬菌体 (WO 1990/002809、WO 2007/006665) 或细菌细胞 (WO 1993/010214) 的表面上展示、核糖体展示 (WO 1998/048008)、质粒上展示 (WO 1993/008278) 或通过使用共价 RNA- 重复蛋白杂合构建体 (WO 2000/032823)、或胞内表达和选择 / 筛选如通过蛋白质互补试验 (WO 1998/341120)。本领域技术人员熟知此类方法。

[0175] 可以根据本领域技术人员已知的方案 (WO 2002/020565、Binz, H. K. 等，J. Mol. Biol., 332, 489-503, 2003 和 Binz 等，2004, loc. cit) 获得用于选择 / 筛选本发明的重组结合蛋白或锚蛋白重复结构域的锚蛋白重复蛋白文库。实施例 1 举例说明了用此类文库选择具有对 PDGF-BB 的特异性的锚蛋白重复结构域。再者，本发明的锚蛋白重复结构域可以由根据本发明的锚蛋白重复模块和合适的加帽模块或加帽重复 (Forrer, P. 等，FEBS letters 539, 2-6, 2003) 通过标准的重组 DNA 技术（例如 WO 2002/020565、Binz 等，2003, loc. cit. 和 Binz 等，2004, loc. cit) 模块化组装。

[0176] 本发明不限制于实施例中所述的具体实施方案。可以根据以下描述的简要大纲使用和处理其他源。

实施例

[0177] 以下描述的所有原料和试剂都是本领域技术人员已知的，且是市售的或可以使用已知技术制备。

[0178] 材料

[0179] 化学品从 Fluka（瑞士）购买。寡核苷酸来自 Microsynth（瑞士）。除非另有说明，DNA 聚合酶、限制性酶和缓冲剂来自 New England Biolabs（美国）或 Fermentas（立陶宛）。克隆和蛋白生产菌株是大肠杆菌 XL1-blue(Stratagene，美国）或 BL21(Novagen，美国）。重组人和鼠 PDGF-BB 从 Reliatech（德国，产品号分别是 200-055 和 M10-125）购买。生物素化的 PDGF-BB 使用标准的生物素化试剂和方法 (Pierce，美国）将生物素部分与蛋白质的伯胺偶联而通过化学方式获得。

[0180] 分子生物学

[0181] 除非另有说明，方法根据所描述的方案 (Sambrook J.，Fritsch E.F. and Maniatis T.，Molecular Cloning:A Laboratory Manual，Cold Spring Harbor Laboratory 1989，New York) 进行。

[0182] 设计的锚蛋白重复蛋白文库

[0183] 描述了产生设计的锚蛋白重复蛋白文库的方法 (WO2002/020565、Binz 等 2003，loc.cit. 和 Binz 等 2004，loc.Cit)。通过这些方法可以构建具有随机化锚蛋白重复模块和 / 或随机化加帽模块的设计的锚蛋白重复蛋白文库。例如，此类文库可以相应地基于固定的 N- 末端加帽模块（例如 SEQ ID NO :2 的 N- 末端加帽模块）或根据 SEQ ID NO :64 的随机化 N- 末端加帽模块、一个或多个根据 SEQ ID NO :20、62 或 63 的序列基序的随机化重复模块和固定的 C- 末端加帽模块（例如 SEQ ID NO :8 的 C- 末端加帽模块）或按照 SEQ ID NO :65 的随机化 C- 末端加帽模块组装而成。优选地，此类文库组装为在重复或加帽模块的随机位置上没有氨基酸 C、G、M、N（在 G 残基之前）或 P。此外，按照 SEQ ID NO :20、62 或 63 的序列基序的随机化重复模块可以进一步在位置 10 和 / 或位置 17 处随机化；按照 SEQ ID NO :64 的序列基序的随机化 N- 末端加帽模块可以进一步在位置 7 和 / 或位置 9 处随机化；以及按照 SEQ ID NO :65 的序列基序的随机化 C- 末端加帽模块可以进一步在位置 10、11 和 / 或 17 处随机化。

[0184] 此外，所述文库中的这种随机化模块可以包含额外的具有随机化氨基酸位置的多肽环插入。此类多肽环插入的例子是抗体的互补决定区 (CDR) 环文库或从头产生的肽文库。例如，可以用人核糖核酸酶 L 的 N- 末端锚蛋白重复结构域的结构 (Tanaka，N.，Nakanishi，M，Kusakabe，Y，Goto，Y.，Kitade，Y，Nakamura，K.T.，EMBO J.23(30)，3929-3938，2004) 作为指导设计这种环插入。类似于其中在靠近两个锚蛋白重复的边界存在的 β - 转角中插入十个氨基酸的这种锚蛋白重复结构域，锚蛋白重复蛋白文库可以包含在锚蛋白重复结构域的一个或多个 β - 转角中插入的不同长度（例如 1-20 个氨基酸）的随机化环（具有固定的和随机化的位置）。

[0185] 锚蛋白重复蛋白文库的任何此类 N- 末端加帽模块优选具有 RELLKA 或 RILKAA 基

序而不是 RILLAA 基序（例如，在 SEQ ID NO :64 的位置 21-26 存在）且锚蛋白重复蛋白文库的任何此类 C- 末端加帽模块优选具有 KAA 或 KLA 基序而不是 KLN 基序（例如，在 SEQ ID NO :65 的最后三个氨基酸）。

[0186] 这种锚蛋白重复蛋白文库的设计可以通过与靶标相互作用的锚蛋白重复结构域的已知结构作为指导。此类结构的例子（通过它们蛋白质数据库 (PDB) 的独特登录或识别码 (PDB-ID) 确认）是 1WDY、3V31、3V30、3V2X、3V2O、3UXG、3TWQ-3TWX、1N11、1S70 和 2ZGD。

[0187] 描述了设计的锚蛋白重复蛋白文库的例子，如 N2C 和 N3C 设计的锚蛋白重复蛋白文库 (WO 2002/020565、Binz 等 . 2003, loc. cit. 、Binz 等 . 2004, loc. cit.)。N2C 和 N3C 中的数字描述在 N- 末端和 C- 末端加帽模块之间存在的随机化重复模块的数目。

[0188] 用于定义重复单元和模块内的位置的命名法是基于 Binz 等 2004, loc. cit. ，具有锚蛋白重复模块和锚蛋白重复单元的边界平移一个氨基酸位置的修饰。例如 Binz 等 2004(loc. cit.) 的锚蛋白重复模块的位置 1 对应于本公开的锚蛋白重复模块的位置 2，且结果是 Binz 等 2004(loc. cit.) 的锚蛋白重复模块的位置 33 对应于本公开中的下一个锚蛋白重复模块的位置 1。

[0189] 所有 DNA 序列均由测序确认，且所有所述蛋白质的计算分子量由质谱确认。

[0190] 实施例 1. 选择包含具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域的结合蛋白

[0191] 使用核糖体展示 (Hanes, J. 和 Plückthun, A., PNAS 94, 4937-42, 1997)，从 Binz 等 2004(loc. cit.) 所述的 DARPin 文库选择许多具有对 PDGF-BB 的结合特异性的设计的锚蛋白重复蛋白 (DARPin)。通过粗提取物 ELISA 评估选择的克隆与特异性 (PDGF-BB) 和非特异性 (MBP，大肠杆菌麦芽糖结合蛋白）靶标的结合，其表明成功选择了数百个 PDGF-BB 结合蛋白。例如，SEQ ID NO:23-61 的锚蛋白重复结构域构成包含具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域的所选择结合蛋白的氨基酸序列。SEQ ID NO :12、14、15、17、18 和 19 提供了来自这种具有对 PDGF-BB 的特异性的锚蛋白重复结构域的单个锚蛋白重复模块。SEQ ID NO :13 和 16 提供这种具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域的单个加帽模块。

[0192] 通过核糖体展示选择 PDGF-BB 特异的锚蛋白重复蛋白

[0193] 通过核糖体展示 (Hanes 和 Plückthun, loc. cit.) 利用人和小鼠 PDGF-BB 为靶标蛋白、如上所述的设计的锚蛋白重复蛋白文库和确立的方案 (Zahnd, C., Amstutz, P. 和 Plückthun, A., Nat. Methods 4, 69-79, 2007) 进行 PDGF-BB 特异的锚蛋白重复蛋白的选择。各轮选择后逆转录 (RT)-PCR 循环数从 40 恒定地减少到 30，从而由于结合物的富集调节到产量。前四轮的选择采用标准核糖体展示选择，采用降低的靶标浓度和提高洗涤严格性来从第 1 轮到第 4 轮增加选择压力 (Binz 等 . 2004, loc. cit.)。为了富集高亲和力抗 -PDGF-BBDARPin，第四轮标准核糖体展示选择（以上）的结果经历具有提高的选择严格性的解离速率选择轮 (Zahnd, 2007, loc. cit.)。进行最后标准选择轮以扩增并回收解离速率选择的结合蛋白。

[0194] 如通过粗提取 ELISA 所示选择的克隆与 PDGF-BB 特异性结合

[0195] 采用标准方案使用 DARPin 表达细胞的粗大肠杆菌提物通过酶联免疫吸附测定法 (ELISA) 来鉴定与 PDGF-BB 特异性结合的单个选择的 DARPin。将通过核糖体展示选择的

DARPin 克隆到 pQE30(Qiagen) 表达载体中，转化到大肠杆菌 XL1-Blue(Stratagene) 中，且然后在含有 1ml 生长培养基（含有 1％葡萄糖和 100μg/ml 氨苄青霉素的 2YT) 的 96 孔深孔板中（各克隆在单一孔中）37℃下生长过夜。在新的 96 孔深孔板中用 100μl 的过夜培养液接种 1ml 新鲜的含有 50μg/ml 氨苄青霉素的 2YT。37℃孵育 2 小时后，用 IPTG（最终浓度为 1mM) 诱导表达并继续 3 小时。收获细胞，重悬浮于 100μlB-PERII(Pierce) 中并在室温下伴随振摇孵育 15 分钟。然后加入 900μl PBS-TC（补充有 0.25％酪蛋白水解物、0.1％ Tween 20® 的 PBS，pH 7.4)，通过离心去除细胞碎片。应用 100μl 的各裂解克隆到包含通过其生物素部分固定的 PDGF-BB 或不相关 MBP 的 Neutravidin 包被 MaxiSorp 板的孔中，并在室温下孵育 1 小时。在用 PBS-T（补充有 0.1％ Tween 20® 的 PBS，pH 7.4) 充分洗涤后，板用单克隆辣根标记的抗 RGS(His)$_4$ 抗体 (34650，Qiagen) 通过标准 ELISA 过程显影。然后通过 POD 底物 (Roche) 检测结合。405nm 下测量显色。通过这样的粗细胞提取物 ELISA 筛选数百个克隆发现了具有对 PDGF-BB 的特异性的超过百种不同 DARPin。选择这些结合蛋白用于进一步分析。选择的与 PDGF-BB 特异性结合的锚蛋白重复结构域的氨基酸序列的示例提供于 SEQ ID NO：23-61 中。

[0196] 将这些与具有对 PDGF-BB 的结合特异性的锚蛋白重复结构域和不具有对 PDGF-BB 的结合特异性的阴性对照 DARPin（即，DARPin#21 和 22) 克隆到基于 pQE(QIAgen，德国）的表达载体中，提供了如下所述促进简单的蛋白质纯化的 N- 末端 His- 标签。因此，构建了编码以下 DARPin 的表达载体：

[0197] DARPin#21（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:21)；

[0198] DARPin#22（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:22)；

[0199] DARPin#23（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:23)；

[0200] DARPin#24（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:24)；

[0201] DARPin#25（具有与其 N 末端融合的 His 标签 (SEQ ID NO:9) 的 SEQ ID NO:25)；

[0202] DARPin#26（具有与其 N 末端融合的 His 标签 (SEQ ID NO:9) 的 SEQ ID NO:26)；

[0203] DARPin#27（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:27)；

[0204] DARPin#28（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:28)；

[0205] DARPin#29（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:29)；

[0206] DARPin#30（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:30)；

[0207] DARPin#31（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:31)；

[0208] DARPin#32（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:32)；

[0209] DARPin#33（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:33)；

[0210] DARPin#34（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:34)；

[0211] DARPin#35（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:35)；

[0212] DARPin#36（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:36)；

[0213] DARPin#37（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:37)；

[0214] DARPin#38（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:38)；

[0215] DARPin#39（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:39)；

[0216] DARPin#40（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:40)；

[0217] DARPin#41（具有与其 N 末端融合的 His- 标签 (SEQ ID NO:9) 的 SEQ ID NO:41)；

[0218] DARPin#42(具有与其N末端融合的His标签(SEQ ID NO:9)的SEQ ID NO:42);

[0219] DARPin#43(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:43);

[0220] DARPin#44(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:44);

[0221] DARPin#45(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:45);

[0222] DARPin#46(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:46);

[0223] DARPin#47(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:47);

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[0225] DARPin#49(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:49);

[0226] DARPin#50(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:50);

[0227] DARPin#51(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:51);

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[0229] DARPin#53(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:53);

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[0233] DARPin#57(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:57);

[0234] DARPin#58(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:58);

[0235] DARPin#59(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:59);

[0236] DARPin#60(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:60);

[0237] DARPin#61(具有与其N末端融合的His-标签(SEQ ID NO:9)的SEQ ID NO:61);

[0238] DARPin的高水平和可溶性表达

[0239] 为了进一步分析,将上述粗细胞提取物ELISA中显示特异性PDGF-BB结合的所选择克隆在大肠杆菌BL21或XL1-Blue细胞中表达并利用其His-标签用标准方案纯化。用50ml的静止过夜培养物(TB,1%葡萄糖、100μg/ml氨苄青霉素;37℃)接种1l培养液(不含葡萄糖的相同培养基)。在600nm下吸光度为0.7(对于BL21是1)时,培养物用0.5mM IPTG诱导并于37℃孵育4-5小时。离心该培养物,且所得沉淀在40ml TBS500(50mM Tris-HCl、500mM NaCl、pH 8)中重悬浮并超声处理。裂解物再离心,并将甘油(最终浓度10%(v/v))和咪唑(最终浓度20mM)加入所得上清液中。蛋白质用Ni-次氮基三乙酸柱(2.5ml柱体积)按照生产商(QIAgen,德国)的指示纯化。或者,缺乏6xHis-标签的DARPin或选择的重复结构域根据本领域技术人员已知的标准树脂和方案通过阴离子交换色谱法接着尺寸排阻色谱进行纯化。如从SDS-15% PAGE估计的,可以从纯度大于95%的1L大肠杆菌培养液中纯化最多200mg具有对PDGF-BB的结合特异性的的高度可溶性DARPin。这样的纯化DARPin用于进一步表征。

[0240] 实施例2.通过表面等离子体共振分析对与PDGF-BB特异性结合的DARPin的表征

[0241] 将来自人和小鼠的生物素化PDGF-BB分子通过结合包被的Streptavidin固定在流动池中,并分析其与各种选择的DARPin的相互作用。

[0242] 表面等离子体共振(SPR)分析

[0243] 用ProteOn仪器(BioRad)测定SPR,并根据本领域技术人员已知的标准过程进行测量。运行缓冲液是含有0.005% Tween 20®的PBS,pH 7.4。将中性亲和素(Neutravidin)

共价固定在 GLC 芯片 (BioRad) 上以达到约 8000 共振单元 (RU) 的水平。然后将 PDGF-BB 固定在中性亲和素包被的芯片上。然后通过注射 100μl 含有浓度为 12.5、6.26、3.13 和 1.67nM(结合速率测量) 的 DARPin 系列稀释的运行缓冲液 (含有 0.005% Tween 20® 的 PBS),接着以 30μl/ 分钟的恒定流速的 10 分钟到最多 3 小时的运行电泳缓冲液 (解离速率测量) 测量 DARPin PDGF-BB 的相互作用。从 PDGF-BB 注入后的 RU 痕迹中提取未包被参照细胞和参照注射 (即,仅运行缓冲液的注射) 的信号 (即,共振单元 (RU) 值) 从 PDGF-BB 注射后获得的 RU 迹线减去 (双参照)。从由结合速率以及解离速率测量获得的 SRP 迹线,可以确定相应 DARPin PDGF-BB 相互作用的结合速率和解离速率。

[0244] 表 1 总结了所得结果。解离常数 (Kd) 用本领域技术人员已知的标准过程从估计的结合速率和解离速率计算。

[0245] 表 1. 通过 SPR 确定的 DARPin PDGF-BB 相互作用 (人和小鼠) 的解离常数

[0246]

DARPin#	Kd [M] (人)	Kd [M] (小鼠)
23	2.14E-11	1.72E-11
24	3.01E-11	n.d.
25	1.47E-11	1.28E-11
26	1.77E-11	1.74E-11

[0247]

28	1.71E-11	n.d.
29	1.05E-10	n.d.
30	1.10E-10	n.d.
31	1.09E-10	n.d.
32	6.38E-11	8.34E-11
33	8.06E-11	9.04E-11
34	7.75E-11	5.92E-11
35	9.56E-11	9.81E-11
36	2.42E-11	5.30E-11
37	1.52E-10	8.28E-11
38	9.41E-11	5.83E-11
39	1.72E-10	3.82E-10
40	3.44E-11	6.08E-11
42	8.05E-11	9.74E-11
43	1.29E-06	1.51E-06
44	7.68E-11	9.02E-11
45	1.08E-10	n.d.
46	1.12E-10	n.d.
47	9.37E-11	n.d.
48	1.13E-10	1.21E-10
49	7.69E-11	1.02E-10
50	1.15E-10	n.d.
51	1.21E-10	n.d.
53	1.28E-10	n.d.
54	2.45E-10	n.d.
55	5.55E-11	n.d.
56	1.50E-10	n.d.
57	1.23E-10	n.d.
58	2.57E-10	n.d.
59	1.71E-10	n.d.

[0248]

[0249] n.d.：未测定

[0250] 实施例 3. 具有对 PDGF-BB 的结合特异性的 DARPin 对成纤维细胞增殖的抑制

[0251] NIH-3T3 成纤维细胞是用于涉及 PDGF-BB 的分析的标准细胞系。在第 1 天 70-80％汇合时，收获细胞并以生长培养基中 5000 细胞 / 孔的密度接种到 96 孔培养板中，接着饥饿细胞约 7-8 小时，随后更换培养基为分析培养基并孵育 24 小时。所有的孵育条件均为 37℃，5％ CO_2 流。细胞饥饿之后，在第 2 天，更换培养基为含有稀释系列的生长因子人 PDGF-BB（用于增殖分析）或 20ng/mL 人 PDGF-BB 与 2.5 倍稀释系列的 DARPin（200nM 到 0.05nM）的抑制混合物（用于抑制分析）的新鲜的分析培养基。在加入 20μL WST-1 试剂（Roche 产品编号 11644807001）后，细胞在这个条件下再孵育 48 小时。该试剂使分析活细胞数的比色分析成为可能。在加入 WST-1 后 2、4 和 6 小时的几个时间点，于 A_{450} 处读取信号，具有 A_{600} 的校正背景。

[0252] 表 2 总结了实施例结果。利用 GraphPad Prism 软件以及本领域技术人员已知的标准过程从上述所得的滴定曲线计算 IC_{50} 值。图 1 中给出了 DARPin#49 的滴定曲线的例子。

[0253] 表 2. 各种 DARPin 对 PDGF-BB 诱导的 NIH-3T3 细胞增殖的抑制效力

[0254]

DARPin#	IC_{50} [nM]
24	1.4
28	1.6
30	3.2
49	1.9
59	2.0

[0255] 实施例 4. 通过受体竞争试验对与 PDGF-BB 特异性结合的 DARPin的表征

[0256] 在受体竞争 ELISA（基于 PDGF-BB Quantikine，R&D 系统）中确定 PEG 化的抗 -PDGF-BB DARPin 抑制人 PDGF-BB 结合其受体 PDGFRβ 的效力。PDGFRβ /Fc 嵌合体预包被于微板上。DARPin 在具有确定量的 PDGF-BB 的 PDGF-BB Quankinine 试剂盒（R&D 系统）的分析稀释液中预孵育并在室温下 750rpm 振摇孵育 2 小时。这些预孵育混合物然后转移到预包被的孔中，且没有被 DARPin 阻断的任何 PDGF-BB 被固定的受体结合。在洗掉任何未结合的物质后，对 PDGF-BB 特异性的辣根过氧化物酶连接的多克隆抗体加入孔中。在洗涤以除去任何未结合的抗体 - 酶试剂后，在孔中加入底物溶液，并与结合的 PDGF-BB 的量成比例地显色。停止显色，且在 405nm 下测量颜色强度。在这个试验中，如表 3 中总结的，测试的 DARPin 显示高 PDGF-BB 抑制效力。图 2 中给出了对于一组 DARPin 的示例滴定曲线。利用 GraphPad Prism 软件以及本领域技术人员已知的标准过程从如上所述获得的这种滴定曲线计算 IC_{50} 值。

[0257] 表 3. DARPin 对 PDGF-BB 与其受体 PDGFRβ 相互作用的抑制（给出平均 IC_{50} 值）

[0258]

DARPin#	IC_{50} [pM]
23	22
24	15
28	16
29	15
30	490
31	480
34	210
37	> 400
38	85
44	130
45	160

[0259]

46	150
47	140
49	66
50	32
51	8
52	68
53	36
54	210
55	14
56	170
57	204
58	470

[0260] 实施例 5. 具有对 PDGF-BB 的结合特异性的 DARPin 对小鼠中激光诱导的脉络膜血管新生的抑制

[0261] 在体内测试对新生血管生长的效果。选择小鼠激光脉络膜血管新生模型并按照发表所述进行 (Takahashi, K., Saishin, Y., Saishin, Y., King, A. G., Levin, R. 和 Campochiaro, P. A., Arch. Ophthalmol. 127(4), 494-499, 2009)。

[0262] 如前所述，通过激光光凝诱导的 Bruch 膜破裂诱导脉络膜血管新生 (CNV)。在第

2 天，用盐酸氯胺酮（100mg/kg 体重）麻醉成年 C57BL/6 小鼠，并用 1％托吡卡胺扩瞳。用 OcuLight GL 二极管激光器中的狭缝灯递送系统对每个视网膜递送三次 532nm 二极管激光光凝（75μm 光斑大小，持续 0.1 秒，120mW）烧伤，用手持的盖片作为接触透镜来观察视网膜。在视网膜后极的 9、12 和 3 点钟方向进行烧伤。在激光的同时产生的气泡（其意味着 Bruch 膜的破裂）是获得脉络膜血管新生的重要因素，并因此在本实验中只包括其中产生气泡的烧伤。如图 3 所示，每天分别施用浓度为 10 或 1mg/kg 的 DARPin#61-PEG20。在第 14 天，小鼠用荧光素标记的葡聚糖灌注心脏，如所描述的（Takahashi 等，loc cit）制备视网膜平面装备（flat-mounts），并通过图像分析定量血管新生的面积。用 1 元 ANOVA 和 Dunnett 后检验比较所有 DARPin 组与溶媒组进行统计分析。本领域技术人员熟知这些技术。图 3 示出了结果。

[0001]

SEQUENCE LISTING

<110> Molecular Partners AG
Baumann, Michael

<120> 设计的与血小板源生长因子结合的锚蛋白重复蛋白

<130> P2414

<150> EP12174020
<151> 2012-06-28

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20 25 30

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20 25 30

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20 25 30

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Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp Asn Gly
1 5 10 15

Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
20 25

[0003]

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Gln Asp Lys Phe Gly Lys Thr Pro Phe Asp Leu Ala Ile Arg Glu Gly
1 5 10 15

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20 25

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Gln Asp Lys Phe Gly Lys Thr Pro Phe Asp Leu Ala Ile Asp Asn Gly
1 5 10 15

Asn Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala
20 25

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Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala Asp Ala Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Ala Ala
20 25

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1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

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<210> 10
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[0005]

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Gly Gly Gly Ser
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Pro Thr Pro Thr
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1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
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Ala

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Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val Gly
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His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25

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1 5 10 15

[0007]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

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20 25 30

Ala

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1 5 10 15

[0008]

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20 25

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1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

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1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

[0009]

Ala

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1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 20
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[0010]

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Xaa Asp Xaa Xaa Gly Xaa Thr Pro Leu His Leu Ala Ala Xaa Xaa Gly
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His Leu Glu Ile Val Glu Val Leu Leu Lys Xaa Gly Ala Asp Val Asn
20 25 30

Ala

<210> 21
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Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

[0011]

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Thr Asp Asn Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Ser Asn Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val Asn
50 55 60

Ala Ser Asp Leu Thr Gly Ile Thr Pro Leu His Leu Ala Ala Ala Thr
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val
85 90 95

Asn Ala Tyr Asp Asn Asp Gly His Thr Pro Leu His Leu Ala Ala Lys
100 105 110

Tyr Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
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1 5 10 15

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20 25 30

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35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Lys Asp Gly Tyr Thr Pro Leu His Leu Ala Ala Arg Glu
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile Asp
100 105 110

Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
115 120 125

<210> 23
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<220>
<223> 合成构建体

<400> 23

[0013]

Gly Ser Asp Leu Gly Trp Lys Leu Leu Gln Ala Ala Lys Phe Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 24
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 24

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

[0014]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 25
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 25

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

[0015]

Gly His Glu Asp Ile Ala Glu Ile Leu Gln Lys Leu Asn
85 90

<210> 26
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 26

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Ile Leu Gln Lys Leu Asn
85 90

<210> 27
<211> 93
<212> PRT
<213> 人工序列

<220>

[0016]

<223> 合成构建体

<400> 27

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 28
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 28

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

[0017]

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 29
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 29

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0018]

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 30
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 30

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Tyr Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 31
<211> 93

[0019]

<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 31

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Phe Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 32
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 32

Gly Ser Asp Leu Gly His Lys Leu Leu Gln Ala Ala Lys His Gly Gln
1 5 10 15

[0020]

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Asp Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ala Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Phe Leu
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 33
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 33

Gly Ser Asp Leu Gly His Lys Leu Leu Gln Ala Ala Glu Gln Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Tyr Gly Thr Thr Pro Leu His Phe Ala Ala Val Trp Gly
35 40 45

[0021]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Val Phe Gly Ala Thr Pro Ala Asp Leu Ala Ala Tyr Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 34
<211> 102
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 34

Gly Ser Asp Leu Gly Asp Lys Leu Leu Gln Ser Asp Leu Gly Arg Lys
1 5 10 15

Leu Leu His Ala Ala Arg Ser Gly Gln Asp Asp Glu Val Arg Ile Leu
20 25 30

Leu Ala Ala Gly Ala Asp Val Asn Ala Lys Asp Glu Asp Gly Thr Thr
35 40 45

Pro Leu His Tyr Ala Ala Ala Trp Gly His Leu Glu Ile Val Glu Val
50 55 60

Leu Leu Lys Ala Gly Ala Asp Val Asn Ala Gln Asp Ile Tyr Gly Ala
65 70 75 80

Thr Pro Ala Asp Leu Ala Ala Tyr Ile Gly His Glu Asp Ile Ala Glu
85 90 95

[0022]

Val Leu Gln Lys Leu Asn
100

<210> 35
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 35

Gly Ser Asp Leu Gly Gln Lys Leu Leu Tyr Ala Ala Glu His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Gln Thr Gly Ser Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Met Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Leu Trp Gly Ala Thr Pro Ala Asp Leu Ala Ala Phe Leu
65 70 75 80

Gly His Glu Asp Ile Ala Val Val Leu Gln Lys Leu Asn
85 90

<210> 36
<211> 93
<212> PRT
<213> 人工序列

[0023]

<220>
<223> 合成构建体

<400> 36

Gly Ser Asp Leu Gly Ser Lys Leu Leu Thr Ala Ala Leu Asp Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Glu Glu Gly Thr Thr Pro Leu His Tyr Ala Ala Val Val Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Trp Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 37
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 37

Gly Ser Asp Leu Gly Trp Lys Leu Leu Glu Ala Ala Arg Thr Gly Gln
1 5 10 15

[0024]

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Ile Thr Gly Thr Thr Pro Leu His Tyr Ala Ala Ala Trp Gly
35 40 45

His Met Glu Ile Val Glu Val Leu Leu Lys Thr Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Leu Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Leu
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 38
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 38

Gly Ser Asp Leu Gly Asp Lys Leu Leu Trp Ala Ala Lys His Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Asp Glu Gly Ser Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Thr Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0025]

Ala Gln Asp Ile Trp Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Leu
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 39
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 39

Gly Ser Asp Leu Gly Asn Lys Leu Leu Ser Ala Ala Arg Leu Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Trp Asp Gly Ser Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Leu Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Ile
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

[0026]

<210> 40
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 40

Gly Ser Asp Leu Gly Tyr Lys Leu Leu Ser Ala Ala Gln Tyr Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Tyr Ser Gly Thr Thr Pro Leu His Tyr Ala Ala Thr Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Ile
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 41
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 41

[0027]

Gly Ser Asp Leu Gly Arg Lys Leu Leu Tyr Ala Ala Trp Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Val Asp Val Asn Ala
20 25 30

Lys Asp Trp Thr Gly Phe Thr Pro Leu His Tyr Ala Ala Tyr Lys Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ala Trp Gly Ala Thr Pro Ala Asp Leu Ala Ala Phe Val
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

<210> 42
<211> 127
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 42

Gly Ser Asp Leu Gly Tyr Lys Leu Leu Phe Ala Ala Tyr Val Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Arg His Gly Arg Thr Pro Leu His Leu Ala Ala Trp Glu Gly
35 40 45

[0028]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Asp Glu Gly Thr Thr Pro Leu His Leu Leu Ala Ala Trp
65 70 75 80

Glu Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
85 90 95

Val Asn Ala Gln Asp Val Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala
100 105 110

Tyr Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 43
<211> 126
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 43

Gly Ser Asp Leu Gly Leu Lys Leu Leu Glu Ala Ala Gln Arg Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Arg Glu Gly Trp Thr Pro Leu His Val Ala Ala Tyr Glu Gly
35 40 45

[0029]

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Thr Gly Leu Thr Pro Leu His Val Ala Ala Val Trp
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp Ilc Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Tyr
100 105 110

Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 44
<211> 126
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 44

Gly Ser Asp Leu Gly Ala Lys Leu Leu His Ala Ala Val Val Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Ser Gly His Thr Pro Leu His Leu Ala Ala Tyr Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0030]

Ala Lys Asp Gln Glu Gly Thr Thr Pro Leu His Phe Ala Ala Ser Val
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp His Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu
100 105 110

Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 45
<211> 126
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 45

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Ser Gly His Thr Pro Leu His Leu Ala Ala Tyr Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

[0031]

Ala Lys Asp Gln Glu Gly Thr Thr Pro Leu His Phe Ala Ala Ser Val
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp His Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu
100 105 110

Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 46
<211> 126
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 46

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Ser Gly His Thr Pro Leu His Leu Ala Ala Tyr Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Gln Glu Gly Thr Thr Pro Leu His Phe Ala Ala Ser Val
65 70 75 80

[0032]

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp His Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu
100 105 110

Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 47
<211> 126
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 47

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Lys Ser Gly His Thr Pro Leu His Leu Ala Ala Tyr Ser Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Gln Glu Gly Thr Thr Pro Leu His Phe Ala Ala Ser Val
65 70 75 80

[0033]

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Gln Asp His Tyr Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu
100 105 110

Ile Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
115 120 125

<210> 48
<211> 93
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 48

Gly Ser Asp Leu Gly Gln Lys Leu Leu Val Ala Ala Lys Glu Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Ala Asp Gly Glu Thr Pro Leu His Tyr Ala Ala Val Trp Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Ile Trp Gly Ala Thr Pro Ala Asp Leu Ala Ala Leu Ile
65 70 75 80

Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
85 90

[0034]

<210> 49
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 49

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Gln Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
85 90 95

Asn Ala Asn Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Asp Val Leu Leu Lys His Gly Ala Asp
115 120 125

[0035]

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 50
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 50

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

[0036]

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 51
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 51

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

[0037]

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 52
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 52

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Gln Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

[0038]

Ala Gln Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
85 90 95

Asn Ala Asn Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Asp Val Leu Leu Lys His Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala Ala
130 135 140

Asp Asn Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

<210> 53
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 53

Gly Ser Asp Leu Asp Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

[0039]

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala Ala
130 135 140

Asp Asn Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

<210> 54
<211> 159
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 54

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

[0040]

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala
130 135 140

Asp Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

<210> 55
<211> 159
<212> PRT
<213> 人工序列

<220>

[0041]

<223> 合成构建体

<400> 55

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

[0042]

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Gly Ser Asp Leu Asp Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
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Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Pro Ala Asp Ile Ala Ala
130 135 140

[0043]

Asp Asn Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

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<400> 57

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

[0044]

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala
130 135 140

Asp Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

<210> 58
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<212> PRT
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<400> 58

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Glu Leu Leu Lys Ala Gly Ala Asp Val Asn Ala
20 25 30

Lys Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
50 55 60

Ala Lys Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val
85 90 95

[0045]

Asn Ala Lys Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Ser Gly Lys Thr Pro Ala Asp Leu Ala Ala
130 135 140

Asp Ala Gly His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
145 150 155

<210> 59
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<400> 59

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
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Gly Asp Glu Val His Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Gln Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

[0046]

Ala Gln Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
85 90 95

Asn Ala Asn Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

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<400> 60

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

Asp Asp Glu Val His Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Ile Asp Asn Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

[0047]

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala His Val
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Tyr Gly Ala Asp Val
85 90 95

Asn Ala Asn Asp Tyr Phe Gly Phe Thr Pro Leu His Leu Thr Thr Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Tyr Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Asn
145 150 155

<210> 61
<211> 167
<212> PRT
<213> 人工序列

<220>
<223> 合成构建体

<400> 61

Gly Ser Asp Leu Gly Lys Lys Leu Leu Glu Ala Ala Arg Ala Gly Gln
1 5 10 15

[0048]

Asp Asp Glu Val Arg Ile Leu Met Ala Asn Gly Ala Asp Val Asn Ala
20 25 30

Gln Asp Leu Asn Gly Gln Thr Pro Leu His Leu Ala Ala Asp Ile Gly
35 40 45

His Leu Glu Ile Val Glu Val Leu Leu Lys His Gly Ala Asp Val Asn
50 55 60

Ala Gln Asp Tyr Ala Gly Ser Thr Pro Leu Arg Leu Ala Ala Trp Ala
65 70 75 80

Gly His Leu Glu Ile Val Glu Val Leu Leu Lys Asn Gly Ala Asp Val
85 90 95

Asn Ala Asn Asp Tyr Phe Gly Tyr Thr Pro Leu His Leu Ala Ala Tyr
100 105 110

Phe Gly His Leu Glu Ile Val Asp Val Leu Leu Lys His Gly Ala Asp
115 120 125

Val Asn Ala Gln Asp Lys Phe Gly Lys Thr Ala Phe Asp Ile Ser Ile
130 135 140

Asp Asn Gly Asn Glu Asp Leu Ala Glu Ile Leu Gln Lys Leu Gly Gly
145 150 155 160

Gly Ser Gly Gly Gly Ser Cys
165

<210> 62
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[0049]

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<222> (3)..(4)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
<221> 杂项特征
<222> (6)..(6)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
<221> 杂项特征
<222> (14)..(15)
<223> Xaa 可以是任意天然存在的氨基酸

<400> 62

Lys Asp Xaa Xaa Gly Xaa Thr Pro Leu His Leu Ala Ala Xaa Xaa Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 63
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<212> PRT
<213> 人工序列

<220>
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<220>

[0050]

<220>
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<223> Xaa 可以是任意天然存在的氨基酸

<220>
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<222> (6)..(6)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
<221> 杂项特征
<222> (11)..(11)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
<221> 杂项特征
<222> (14)..(15)
<223> Xaa 可以是任意天然存在的氨基酸

<400> 63

Lys Asp Xaa Xaa Gly Xaa Thr Pro Leu His Xaa Ala Ala Xaa Xaa Gly
1 5 10 15

His Leu Glu Ile Val Glu Val Leu Leu Lys Ala Gly Ala Asp Val Asn
20 25 30

Ala

<210> 64
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<220>
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<220>
<221> 杂项特征

[0051]

<222> (6)..(6)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
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<222> (10)..(10)
<223> Xaa 可以是任意天然存在的氨基酸

<220>
<221> 杂项特征
<222> (13)..(14)
<223> Xaa 可以是任意天然存在的氨基酸

<400> 64

Gly Ser Asp Leu Gly Xaa Lys Leu Leu Xaa Ala Ala Xaa Xaa Gly Gln
1 5 10 15

Asp Asp Glu Val Arg Ile Leu Leu Ala Ala Gly Ala Asp Val Asn Ala
20 25 30

<210> 65
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<220>
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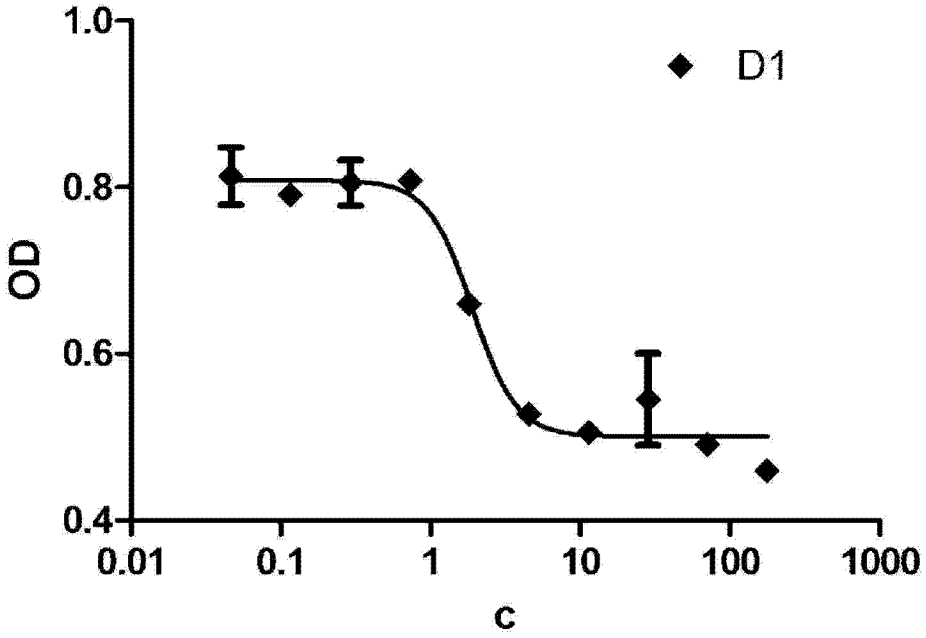
[0052]

<222> (14)..(15)
<223> Xaa 可以是任意天然存在的氨基酸

<400> 65

Gln Asp Xaa Xaa Gly Xaa Thr Pro Ala Asp Leu Ala Ala Xaa Xaa Gly
1 5 10 15

His Glu Asp Ile Ala Glu Val Leu Gln Lys Leu Asn
20 25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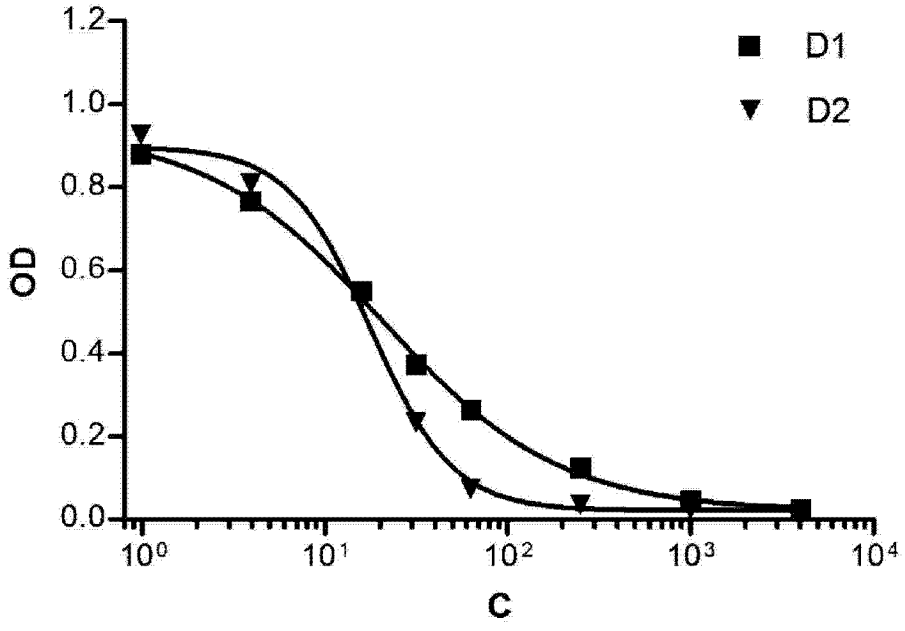
D1
0.01
0.1
1
10
100
1000
C

图 1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0.4
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0.0
OD
D1
D2
10^0
10^1
10^2
10^3
10^4
C

图 2

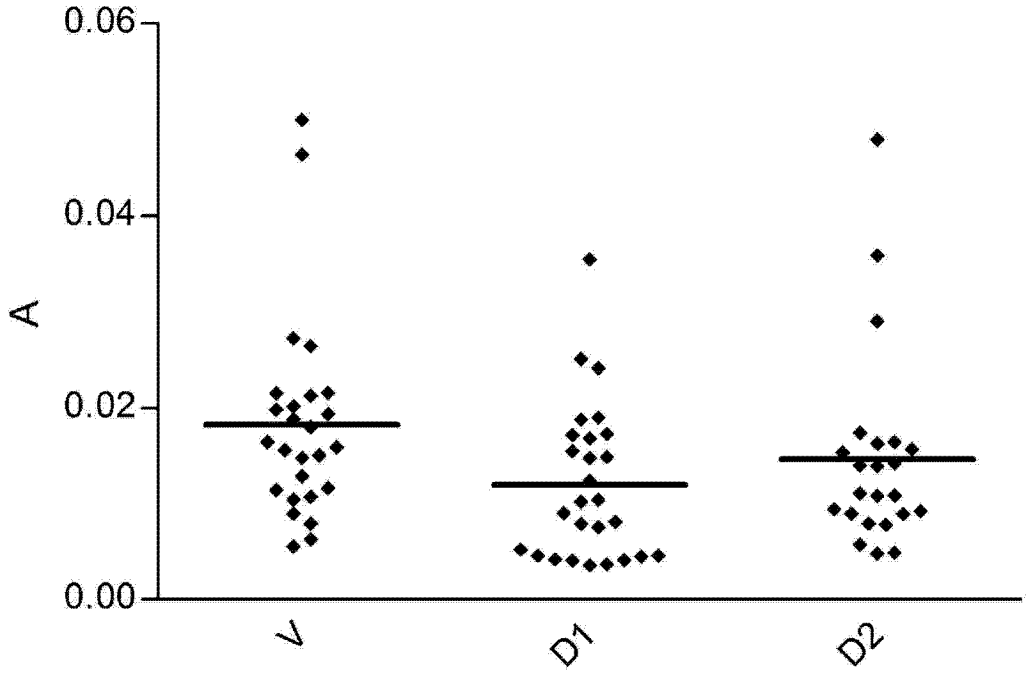

图 3