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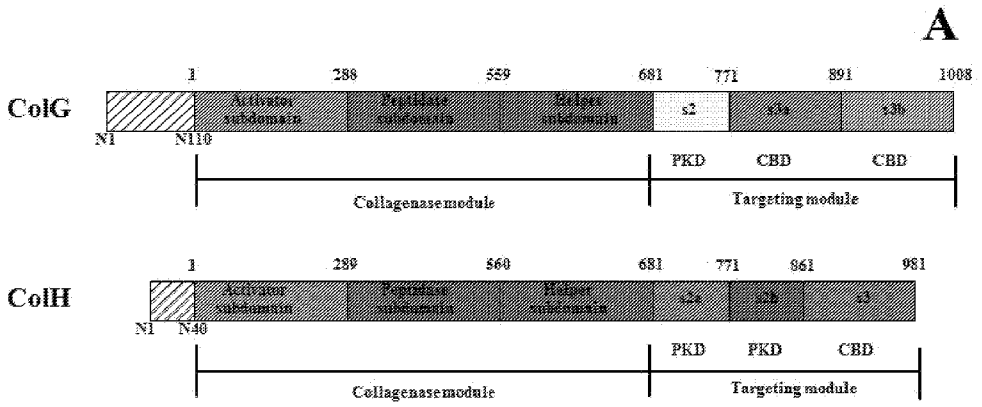

Fig. 1A

(57) Abstract: The invention generally relates to collagen-binding agent compositions and methods of using the same. More specifically, the invention relates in part to new collagen-binding agent compositions and methods that may be used to treat damaged collagen within tissues or used to specifically target therapeutics to tissues containing undamaged or damaged collagen.

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COLLAGEN-BINDING AGENT COMPOSITIONS AND METHODS OF USING THE SAME

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

This invention was made with government support under grant number GM103450 and GM103429 awarded by the National Institute of Health. The United States government has certain rights in the invention.

CROSS-REFERENCE TO RELATED PATENT APPLICATIONS

The present application claims the benefit of priority to United States Provisional Patent Application No. 62/457,410, filed on February 10, 2017, the content of which is incorporated herein by reference in its entirety.

SEQUENCE LISTING

This application is being filed electronically via EFS-Web and includes an electronically submitted Sequence Listing in .txt format. The .txt file contains a sequence listing entitled "2018-02-09_5965-00084_ST25.txt" created on Febraury 9, 2018 and is 72,312 bytes in size. The Sequence Listing contained in this .txt file is part of the specification and is hereby incorporated by reference herein in its entirety.

INTRODUCTION

Collagen is the primary structural protein found in the extracellular space of various animal tissues. Collagen, for example, can be found in fibrous tissues such as skin, ligaments, and tendons. It is also abundant in bones, cartilage, corneas, blood vessels, and muscle tissues.

Abnormal or damaged collagen underlies several medical conditions including wounding and collagenopathies. Collagenopathies represent a large number of diseases in which collagen structure or formation is not normal. This group of diseases results in a broad spectrum of symptoms including bone defects, vascular defects, and skin defects. Many of these diseases have no or only ineffective treatments available.

Collagen-targeting agents are also attractive agents for delivering therapeutics to different animal tissues or subsites within a particular tissue. Delivery of therapeutic agents to sites within the body of a subject where a particular therapeutic agent is needed in order to be effective is a developing area. Targeted delivery systems allow the therapeutic agents to be most active at the sites where the agent is needed while minimizing the off-site effects of the therapeutic agent which may lead to unwanted toxicity and side effects. While use of targeted liposomes or polypeptides,

such as antibodies, to target therapeutic agents to particular sites within the body has proved successful, additional delivery agents are needed. There thus is a need in the art for new collagen-targeting agents that may be used to treat tissues with damaged or normal collagen or to deliver appropriate therapeutics to such tissues.

SUMMARY

In one aspect, collagen-binding agents are provided. The collagen-binding agents may include two collagen-binding domains linked by a domain linker. The two collagen-binding domains may be selected from any one of the polypeptides of SEQ ID NOs: 1-47, a variant of the polypeptides of SEQ ID NOs: 1-47, or a fragment of the polypeptides of SEQ ID NOs: 1-47. SEQ ID NOs: 1-39 specify individual collagen-binding domains (i.e., ColG s3a, ColG s3b, ColH s3) from ColG and ColH proteins from various bacterial species. SEQ ID NOs: 40-47 specify tandem collagen-binding domains found in ColG proteins from various bacterial species. *See, also*, **Fig. 5**. The collagen-binding agents of the present invention may further include a therapeutic agent linked to the collagen-binding agent by a therapeutic agent linker. The thereapeutic agent may be selected from FGF, parathyroid hormone (PTH), PTH/PTHrP receptor agonist, PTH/PTHrP receptor antagonist, bone morphogenic protein (BMP), G-CSF, BMP-2, BMP-3, anti-sclerostin antibody, growth hormone, IGF-1, VEGF, TGF-β, KGF, TGF-α, TGF-β1, TGF-β receptor, CT, GH, GM-CSF, EGF, PDGF, celiprolol, activins and connective tissue growth factors.

In another aspect, pharmaceutical compositions are provided. The pharmaceutical compositions may include any of the collagen-binding agents described herein and a pharmaceutical carrier.

In a further aspect, methods of treating a condition are also provided. The methods may include administering any of collagen-binding agents or pharmaceutical compositions described herein to a subject in an amount effective to treat the condition.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows a schematic diagram of the collagenases secreted by *C. histolyticum*. **Fig. 1B** shows pseudo-twofold symmetry in the structure of the tandem collagen-binding domain. **Fig. 1C** shows the two-fold axis is perpendicular to the plane of the page. The key residues for binding to collagen are highlighted: yellow for ColGS3a, blue for ColGS3b.

Fig. 2A shows the apparent molecular weights of the tandem CBD as a function of pCa determined by analytical size exclusion chromatography. **Fig. 2B** shows molecular envelopes of the tandem CBD at pCa 3, 4, and 5 based on SAXS scattering profiles.

Fig. 3 shows the influence of collagen binding on self-assembly of collagen type I.

Fig. 4A shows the molecular envelopes of the tandem CBD to mini-collagen based on SAXS scattering profiles. **Figs. 4B and 4C** show models of the binding of tandem collagen-binding domain to collagen fibril.

Fig. 5 shows a sequence alignment of bacterial tandem collagen binding domains (See SEQ ID NOs: 40-47). Residues binding to collagen are highlighted in blue. Calcium-binding residues are in red, structurally important residues are shown in green.

Fig. 6 shows a scheme for the preparation of the four collagen binding-basic fibroblast growth factor constructs used in Example 2.

Fig. 7 shows the *in-vitro* proliferation activity of bFGF and CB-bFGFs. Dose-dependent induction of periosteal mesenchymal cell proliferation by bFGF and CB-bFGFs. Cell numbers were quantified three days after the treatment. Data are presented as the mean ± S.E. (n=8).

Figs. 8A-8F show 3D micro-CT analysis of femurs after injection of poly(Pro-Hyp-Gly)$_{10}$ loaded with bFGF and CB-bFGFs. 3D micro-CT images of fractured mouse femurs treated with (**Fig. 8A**) PBS, (**Fig. 8B**) bFGF, (**Fig. 8C**) bFGF-CBD(H), (**Fig. 8D**) bFGF-PKD-CBD, (**Fig. 8E**) bFGF-CBD-CBD (**Fig. 8F**) after 4 weeks of recovery. Red: newly formed bone; gray: existing bone. The scale bars indicate 3 mm.

Figs. 9A-9B shows 3D micro-CT analysis of rat femurs four weeks after the injection of poly(Pro-Hyp-Gly)$_{10}$ loaded with bFGF and CB-bFGFs at femoral fracture sites. Fig. 9A is a graph showing callus volume (mm^3) and Fig. 9B is a graph showing bone mineral content. Data are presented as the mean ± S.E. (n=8). Lower case letters above the bars in the graph are defined as follows: a, $P<0.05$ compared with the control group. b, $P<0.05$ compared with the bFGF group. c, $P<0.05$ compared with the bFGF-CBD(H) group. d, $P<0.05$ compared with the bFGF-PKD-CBD group. e, $P<0.05$ compared with the bFGF-CBD(G) group.

Figs. 10A-10F show representative hematoxylin and eosin-stained tissue sections on day 14 of fracture healing. Histological images of fractured mouse femurs treated with (**Fig. 10A**) PBS, (**Fig. 10B**) bFGF, (**Fig. 10C**) bFGF-CBD (ColH s3), (**Fig. 10D**) bFGF-PKD-CBD (ColH s2b-s3),

(**Fig. 10E**) bFGF-CBD (ColG s3b), and (**Fig. 10F**) bFGF-CBD-CBD (s3a-s3b) after 14 days of recovery. Scale bar = 500 μm.

Fig. 11 shows an HPLC profile of H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 (SEQ ID NO: 60). HPLC gradient: 10–40% CH_3CN in 0.05% TFA over 30 min at 60 °C.

Fig. 12 is a sequence alignment showing the alignment of several M9B bacterial collagenases from the Bacillus and Clostridium families (See SEQ ID NOs: 1-39). The residues shown in blue are important for collagen binding activity, those shown in green are important for maintaining the architecture or protein folding. Both of these are also underlined for the top and bottom sequences. Residues shown in red are critical for Ca2+ binding and those in orange are critical for positioning the Ca2+ binding residues.

DETAILED DESCRIPTION

In the non-limiting Examples, the present inventors report the high-resolution structure of a bacterial tandem collagen-binding agent for the first time. The pseudo-symmetrical arrangement of the tandem collagen-binding agent, resulting from gene duplication and fusion, could allow it to recognize a unique niche in collagen fibril to facilitate degradation of collagen. The structure of the tandem collagen-binding agent also reveals that it could wedge between parallel collagen molecules. This structure combined with the previously identified collagen-binding domain preference for under-twisted regions of collagen suggests the tandem collagen-binding agent targets the bacterial collagenase, ColG, to damaged regions of the collagen fibril. Based on this new understanding of how tandem collagen-binding agent interacts with collagen, the present inventors disclose new collagen-binding agents that may be used to treat damaged collagen within tissues or used to specifically target therapeutics to tissues containing undamaged or damaged collagen. The tandem collagen binding agents provided herein may allow for tighter binding to collagen by bridging two collagen fibrils. This novel binding interaction of the collagen binding agents described herein will provide novel and unexpected therapeutic uses. The increased binding affinity and cross-linking of collagen molecules may make these tandem collagen binding agents more effective for certain applications than their single binding counterparts. Applications may include skin applications.

In one aspect, collagen-binding agents are provided. The collagen-binding agents may include two collagen-binding domains linked by a domain linker. As used herein, a "collagen-binding domain" refers to a polypeptide that binds collagen. In some embodiments, the collagen-

binding domain may bind collagen with a K_d of less than 500 μM, 100 μM, 10 μM, 1 μM, 500 nM, 100 nM, 10 nM, 1 nM, or 0.1 nM. Determination of whether a collagen-binding domain binds collagen can be made, for example, as described in U.S. Patent Publication No. 2010/0129341, which is incorporated herein by reference in its entirety. Briefly, the collagen-binding domain may be incubated with collagen in binding buffer, and the mixture is then filtered through a filter that would otherwise allow the collagen-binding domain to pass through but that blocks the collagen and therefore holds back materials that bind to the collagen. The filtrate is then assayed for the presence of the collagen-binding domain. Suitably, at least 80%, 85%, 90%, 95%, 98% or more suitably at least 99% of the collagen-binding domain is retained by the filter in this assay, as compared to when the filtration is performed without collagen.

The collagen-binding domain may be a bacterial collagen-binding domain. Among other proteins, the collagen-binding domain may be derived from a bacterial ColG protein (Matsushita et al., (1999) J. Bacteriol. 181:923-933) or a bacterial ColH protein (Yoshihara et al., (1994) J. Bacteriol. 176: 6489-6496). As shown in **Fig. 1**, ColG is a class I collagenase found in various bacterial species including *Clostridium* species. ColH is a class II collagenase also found in various bacterial species including *Clostridium* species. The collagen-binding domain may also be any one of the polypeptides provided in SEQ ID NOS: 1-39, which specify collagen-binding domains from various bacterial species such as *Clostridium* and *Bacillus* species. Those of skill in the art will appreciate that other members of these collagen-binding protein families (i.e, ColG and ColH) may be useful in the compositions and methods described herein.

Suitably, the collagen-binding agents lack collagenase activity. "Collagenase activity" refers to the ability of a polypeptide to degrade or breakdown collagen. For example, as shown in **Fig. 1A**, ColG and ColH proteins have collagenase modules that include several subdomains that enable these proteins to degrade or breakdown collagen. In some embodiments, the collagen-binding agents lack these or similar collagenase modules.

The collagen-binding agents may include two collagen-binding domains that may be the same type of collagen-binding domain or may be different types of collagen-binding domains. For example, as exemplified in **Fig. 1A**, bacterial ColG proteins have two types of collagen-binding domains: a s3a collagen-binding domain and a s3b collagen-binding domain. Bacterial ColH proteins have a single collagen-binding domain known as an s3 domain. Thus, the two collagen-binding domains of the presently disclosed collagen-binding agents may be both ColG s3a domains,

both ColG s3b domains, both ColH s3 domains, or any combinations thereof. Furthermore, the two collagen-binding domains of the presently disclosed collagen-binding agents may be from the same bacterial species or different species. For example, one of the collagen-binding domains may be a ColG s3a domain from *Clostridium histoliticum* while the other collagen-binding domain may be a ColG s3b domain from *Brevibacillus brevis*.

The two collagen-binding domains of the presently disclosed collagen-binding agents may be selected from any one of the polypeptides of SEQ ID NOs: 1-47 or those polypeptides shown in **Figs. 5 and 12.** SEQ ID NOs: 1-39 specify individual collagen-binding domains (i.e., ColG s3a, ColG s3b, ColH s3) from ColG and ColH proteins from various bacterial species. SEQ ID NOs: 40-47 specify tandem collagen-binding domains found in ColG proteins from various bacterial species. *See, also*, **Fig. 5**.

The collagen-binding domains of the present invention may be variants of the polypeptides of SEQ ID NOs: 1-47. As used herein the term "wild-type" is a term of the art understood by skilled persons and means the typical form of a polypeptide as it occurs in nature as distinguished from "variant" or "mutant" forms. As used herein, a "variant, "mutant," or "derivative" refers to a polypeptide molecule having an amino acid sequence that differs from a reference protein or polypeptide molecule. A variant may have one or more insertions, deletions, or substitutions of an amino acid residue relative to a reference molecule. A variant or mutant may include a fragment of a reference molecule. For example, a collagen-binding domain variant may have one or more insertions, deletions, or substitution of at least one amino acid residue relative to the "wild-type" collagen-binding domain polypeptide. The polypeptide sequences of "wild-type" collagen-binding domains from various bacterial species are presented as SEQ ID NOS: 1-47. These sequences may be used as reference sequences.

A "deletion" in a polypeptide refers to a change in the amino acid sequence resulting in the absence of one or more amino acid residues. A deletion may remove at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, 100, or more amino acids residues. A deletion may include an internal deletion and/or a terminal deletion (e.g., an N-terminal truncation, a C-terminal truncation or both of a reference polypeptide).

"Insertions" and "additions" in a polypeptide refer to changes in an amino acid sequence resulting in the addition of one or more amino acid residues. An insertion or addition may refer to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 or more amino acid residues. A variant

of a collagen-binding domain may have N-terminal insertions, C-terminal insertions, internal insertions, or any combination of N-terminal insertions, C-terminal insertions, and internal insertions.

Regarding variant collagen-binding domains and agents, the phrases "% sequence identity," "percent identity," or "% identity" refer to the percentage of residue matches between at least two amino acid sequences aligned using a standardized algorithm. Methods of amino acid sequence alignment are well-known. Some alignment methods take into account conservative amino acid substitutions. Such conservative substitutions, explained in more detail below, generally preserve the charge and hydrophobicity at the site of substitution, thus preserving the structure (and therefore function) of the polypeptide. Percent identity for amino acid sequences may be determined as understood in the art. (*See, e.g.*, U.S. Patent No. 7,396,664, which is incorporated herein by reference in its entirety). A suite of commonly used and freely available sequence comparison algorithms is provided by the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), which is available from several sources, including the NCBI, Bethesda, Md., at its website. The BLAST software suite includes various sequence analysis programs including "blastp," that is used to align a known amino acid sequence with other amino acids sequences from a variety of databases.

Polypeptide sequence identity may be measured over the length of an entire defined polypeptide sequence, for example, as defined by a particular SEQ ID number, or may be measured over a shorter length, for example, over the length of a fragment taken from a larger, defined polypeptide sequence, for instance, a fragment of at least 15, at least 20, at least 30, at least 40, at least 50, at least 70 or at least 150 contiguous residues. Such lengths are exemplary only, and it is understood that any fragment length supported by the sequences shown herein, in the tables, figures or Sequence Listing, may be used to describe a length over which percentage identity may be measured.

As described herein, variant collagen-binding domains and agents may have at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 1-47.

The polypeptide sequences of the variant collagen-binding domains and agents as contemplated herein may include conservative amino acid substitutions relative to a reference amino acid sequence. For example, variant collagen-binding domains and agents may include

conservative amino acid substitutions relative to a reference molecule. "Conservative amino acid substitutions" are those substitutions that are a substitution of an amino acid for a different amino acid where the substitution is predicted to interfere least with the properties of the reference polypeptide. In other words, conservative amino acid substitutions substantially conserve the structure and the function of the reference polypeptide. Conservative amino acid substitutions generally maintain (a) the structure of the polypeptide backbone in the area of the substitution, for example, as a beta sheet or alpha helical conformation, (b) the charge or hydrophobicity of the molecule at the site of the substitution, and/or (c) the bulk of the side chain.

The disclosed variant collagen-binding domains and agents described herein may have one or more functional or biological activities exhibited by a reference polypeptide (e.g., one or more functional or biological activities exhibited by wild-type collagen-binding domains and agents (i.e, SEQ ID NOs: 1-47, See **Figs. 5 and 12**). Suitably, the disclosed variant collagen-binding domains and agents retain at least 20%, 40%, 60%, 80%, or 100% of the collagen binding activity of the reference polypeptide (i.e., SEQ ID NOs: 1-47, See **Figs. 5 and 12**).

Fig. 5 shows a sequence alignment of several bacterial tandem collagen-binding domains included as SEQ ID NOs: 40-47. As can be seen from the sequence alignment, these proteins have a relatively small amount of sequence identity, but they all are expected to bind to collagen in a similar fashion and are believed to have similar conformations. Thus any of the polypeptides shown in **Fig. 5** (i.e., SEQ ID NOs: 40-47) or the collagen-binding domains, variants, or fragments thereof can be used in the compositions and methods described herein. In **Figs. 5 and 12**, the amino acid residues important for collagen-binding activity are highlighted in blue. Calcium-binding residues are in red and structurally important residues are shown in green. Based on this alignment it becomes immediately apparent to a person of ordinary skill in the art that various amino acid residues may be altered (i.e. substituted, deleted, etc.) without substantially affecting the collagen-binding activity of the polypeptide. For example, a person of ordinary skill in the art would understand that amino acid residues shown in black likely could be modified without substantially affecting the collagen-binding activity of the polypeptide. A person of ordinary skill in the art would also appreciate that substitutions in a reference collagen-binding domain or agent could be based on alternative amino acid residues that occur at the same position in other collagen-binding domains or agents from other bacterial species. At position 996, for example, the collagen-binding domains shown have either a tyrosine (Y) or phenylalanine (F) residue. Thus, one exemplary

modification that is apparent from the sequence alignment in **Fig. 5** is a Y996F substitution in a collagen-binding domain or agent. Similar modifications could be made at each position of the sequence alignment shown in **Fig. 5**.

SEQ ID NOs: 1-39 also include collagen-binding domains that are not shown in **Fig. 5**, several of these are included in **Fig. 12**. A person of ordinary skill in the art, however, could easily align these polypeptide sequences with the polypeptide sequences shown in **Figs. 5 and 12** to determine what additional variants could be made to these additional collagen-binding domains. Thus polypeptides having 85%, 88%, 90%, 92%, 94%, 95%, 96%, 97%, 98% or 99% amino acid identity to the collagen-binding polypeptides provided herein. The variant polypeptides are still able to bind collagen.

The collagen-binding domains of the present invention may be full-length versions of SEQ ID NOs: 1-47 or fragments of SEQ ID NOs: 1-47 having at least 8, 16, 32, 64, 100 or more consecutive amino acids of any one of SEQ ID NOs: 1-47. As used herein, a "fragment" is a portion of an amino acid sequence which is identical in sequence to, but shorter in length than, a reference sequence. A fragment may comprise up to the entire length of the reference sequence, minus at least one amino acid residue. In some embodiments, a fragment may comprise at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more contiguous amino acid residues of a reference polypeptide (i.e., SEQ ID NOs: 1-47). Fragments may be preferentially selected from certain regions of a molecule. The term "at least a fragment" encompasses the full length polypeptide. A fragment of a collagen-binding domain or agent may comprise or consist essentially of a contiguous portion of an amino acid sequence of the full-length collagen-binding domain or agent (i.e., SEQ ID NOs: 1-47). A fragment may include an N-terminal truncation, a C-terminal truncation, or both truncations relative to the full-length collagen-binding domain or agent. The N-terminal and/or C-terminal truncations may include removal of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues from a reference polypeptide (i.e., SEQ ID NOs: 1-47).

The collagen-binding domains and agents contemplated herein may be further modified *in vitro* or *in vivo* to include non-amino acid moieties. These modifications may include but are not limited to acylation (e.g., O-acylation (esters), N-acylation (amides), S-acylation (thioesters)), acetylation (e.g., the addition of an acetyl group, either at the N-terminus of the protein or at lysine residues), formylation, lipoylation (e.g., attachment of a lipoate, a C8 functional group), myristoylation (e.g., attachment of myristate, a C14 saturated acid), palmitoylation (e.g., attachment

of palmitate, a C16 saturated acid), alkylation (e.g., the addition of an alkyl group, such as an methyl at a lysine or arginine residue), isoprenylation or prenylation (e.g., the addition of an isoprenoid group such as farnesol or geranylgeraniol), amidation at C-terminus, glycosylation (e.g., the addition of a glycosyl group to either asparagine, hydroxylysine, serine, or threonine, resulting in a glycoprotein). Distinct from glycation, which is regarded as a nonenzymatic attachment of sugars, polysialylation (e.g., the addition of polysialic acid), glypiation (e.g., glycosylphosphatidylinositol (GPI) anchor formation, hydroxylation, iodination (e.g., of thyroid hormones), and phosphorylation (e.g., the addition of a phosphate group, usually to serine, tyrosine, threonine or histidine) represent other possible modifications.

The two collagen-binding domains may be a ColG or ColH collagen-binding domain from a bacterial species. In some embodiments, the two collagen-binding domains may be any one of the polypeptides of SEQ ID NOs: 1-39, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 1-39, or a fragment of at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more consecutive amino acids of any one of SEQ ID NOs: 1-39.

The two collagen-binding domains may be a bacterial ColG s3b domain and a bacterial ColG s3a domain, s3c domain, or ColH s3 domain. In some embodiments, the one of the collagen-binding domains may be any one of the polypeptides of SEQ ID NOs: 15-30, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 15-30, or a fragment of at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more consecutive amino acids of any one of SEQ ID NOs: 15-30 and wherein the other collagen-binding domain may be any one of the polypeptides of SEQ ID NOs: 1-14 and 31-39, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 1-14 and 31-39, or a fragment of at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more consecutive amino acids of any one of SEQ ID NOs: 1-14 and 31-39.

The two collagen-binding domains may both be a bacterial ColG s3b domain. In some embodiments, the two collagen-binding domains may be any one of the polypeptides of SEQ ID NOs: 15-30, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 15-30, or a fragment of at least 8, 10,

15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more consecutive amino acids of any one of SEQ ID NOs: 15-30.

The two collagen-binding domains may be selected from a bacterial ColG s3a domain, a s3b domain, or a ColH s3 domain. In some embodiments, the two collagen-binding domains may be any one of the polypeptides of SEQ ID NOs: 1-14 and 31-39, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 1-14 and 31-39, or a fragment of at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, or more consecutive amino acids of any one of SEQ ID NOs: 1-14 and 31-39.

The two collagen-binding domains may be tandem collagen-binding domains from bacterial ColG proteins. In some embodiments, the collagen-binding agent may be any one of the polypeptides of of SEQ ID NOs: 40-47, a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of SEQ ID NOs: 40-47, or a fragment of at least 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, 90, 100, 150 or more consecutive amino acids of any one of SEQ ID NOs: 40-47.

The collagen-binding agents may include two collagen-binding domains linked by a domain linker. As used herein, a "domain linker" may include a covalent bond and/or a linker or spacer moiety. For instance, the two collagen-binding domains may be linked directly through, e.g., a peptide bond or chemical cross-linking, or indirectly, through, e.g., a linker or spacer polypeptide. Useful domain linkers include polypeptides, amino acids, nucleic acids, as well as homofunctional linkers or heterofunctional linkers. Particularly useful conjugation reagents that can facilitate formation of a covalent bond between the two collagen-binding domains may include a N-hydroxysuccinimide (NHS) ester and/or a maleimide.

A domain linker may also include a spacer polypeptide. The spacer polypeptide may be any length and may include traditional or non-traditional amino acids. For example, the spacer polypeptide may be 1-100 amino acids long, suitably it is at least 2, 3, 5, 10, 15, 20, 25 or more amino acids long such that the two collagen-binding domains of the collagen-binding agent can mediate collagen binding. Spacer polypeptides may include, without limitation, any one of the polypeptides of SEQ ID NOs: 48-55, a GST tag, a His-tag, a Ser linker, or a Gly linker.

In some embodiments, the domain linker may include a tag system. A tag system includes any group of agents capable of binding one another with a high affinity. Several tag systems are well-known in the art and include, without limitation, biotin/avidin, biotin/streptavidin, or

digoxigenin (DIG) systems. In some embodiments, said tag system comprises biotin/avidin or biotin/streptavidin.

The domain linker may include any one of the polypeptides of SEQ ID NOs: 48-55 or a polypeptide having at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% 98%, or 99% sequence identity to any one of the polypeptides of SEQ ID NOs: 48-55.

The collagen-binding agents of the present invention may further include a therapeutic agent linked to the collagen-binding agent by a therapeutic agent linker. As used herein, a "therapeutic agent" may include any suitable pharmaceutical or other active agent, including, without limitation, osteogenic promoters, antimicrobials, anti-inflammatory agents, polypeptides such as recombinant proteins, cytokines or antibodies, small molecule chemicals, hormones, growth factors, polynucleotides, carbohydrates, lipids, or any combination thereof. Suitably the therapeutic agent(s) are capable of promoting bone growth, decreasing inflammation, promoting collagen stability. Suitably, the therapeutic agent is one whose therapeutic effect is in the region of collagen or damaged collagen. The therapeutic agent may include, without limitation, FGF, parathyroid hormone (PTH), PTH/PTHrP receptor agonist, PTH/PTHrP receptor antagonist, bone morphogenic protein (BMP), G-CSF, FGF, BMP-2, BMP-3, anti-sclerostin antibody, growth hormone, IGF-1, VEGF, TGF-β, KGF, TGF-α, TGF-β1, TGF-β receptor, CT, GH, GM-CSF, EGF, PDGF, celiprolol, activins and connective tissue growth factors. Alternatively, the invention may also aid cell therapy. Cell therapy focuses on the administration of exogenous stem cells. One of the challenges is to deliver these cells at the lesion. Laminin could be attached to CBD. Cells decorated with anchoring collagen binder may seek a lesion and reside at injury site longer to aid in repair. PTH/PTHrP receptor agonists and PTH/PTHrP receptor antagonists have been described in WO 2013/090770, which is incorporated by reference in its entirety. Suitable FGF proteins include, without limitation, bFGF (FGF-2), FGF-4, or FGF-10 (See SEQ ID NO: 56).

The PTH/PTHrP receptor agonist polypeptide segment may be a synthetic polypeptide or a naturally occurring polypeptide. Such polypeptides may be a portion of a polypeptide or may comprise one or more mutations. The mutations may make the PTH/PTHrP receptor agonist a better or worse agonist as compared to the wild-type PTH/PTHrP. Agonist activity with the PTH/PTHrP receptor can be assayed as described in WO2013/090770 and known to those skilled in the art by a cAMP stimulation assay. An agonist will stimulate cAMP synthesis in the assay described. Suitably, an agonist can activate receptor activity at least 10%, 20%, 30%, 40%, 50%,

60%, 70%, 80%, 90%, 100% or even 110% or 120% as much as wild-type PTH(1-34). The PTH/PTHrP receptor agonist polypeptide segment is a PTH or PTHrP polypeptide segment. One human isoform of PTH is SEQ ID NO: 62. One human isoform of PTHrP is SEQ ID NO:57. While the human isoforms are provided, those of skill in the art will appreciate that other non-human-derived isoforms may be used as well. Such non-human-derived isoforms may be able to interact with human PTH/PTHrP receptor and vice versa. The PTH/PTHrP receptor agonist polypeptide segment may be or may include residues 1-33 of SEQ ID NO:62 (residues 1-33 of PTH (SEQ ID NO: 57)). The PTH/PTHrP receptor agonist polypeptide segment may be or may include residues 1-34 of PTH (SEQ ID NO: 62). In other embodiments, it is a fragment of residues 1-34 of PTH (SEQ ID NO: 62). In other embodiments, the PTH/PTHrP receptor agonist polypeptide segment may be or may include residues 1-84 of PTH (SEQ ID NO: 62). In other embodiments, the PTH/PTHrP receptor agonist polypeptide segment may be or may include residues 1-14 of PTH (SEQ ID NO: 62) or residues 1-7 of PTH (SEQ ID NO: 62). The key amino acids for binding to the PTH receptor as an agonist are amino acids 1, 2 and 5 of PTH. In still other embodiments, the PTH/PTHrP receptor agonist is a PTH or PTHrP polypeptide segment for any other species.

The PTH/PTHrP receptor antagonist can include in one embodiment PTH(7-34), i.e., residues 7-34 of PTH (SEQ ID NO: 62). In another embodiment, it is or includes residues 7-33 of PTH (SEQ ID NO: 62). In other embodiments, it is a fragment of residues 7-34 of SEQ ID NO: 57. In another embodiment, the PTH/PTHrP receptor antagonist includes PTH(7-14), i.e., residues 7-14 of PTH (SEQ ID NO: 62). In another embodiment, the PTH/PTHrP receptor antagonists include ((-1)-33) of PTH/PTHrP. In another embodiment, the PTH/PTHrP receptor antagonists include residues 1-14 of PTH with an N-terminal extension. Adding an N-terminal extension to PTH or active N-terminal fragments of PTH converts the PTH peptides to antagonists. The N-terminal extension can be 1, 2, 3, 4, 5, or more amino acids in length. The identity of the amino acids in the N-terminal extension is typically not important. In one embodiment, the PTH/PTHrP receptor antagonist includes residues 1-33 of PTH with a Gly-Ser extension at the N-terminus (SEQ ID NO:58). In another embodiment, the PTH/PTHrP receptor antagonist includes PTHrP(7-34), i.e., residues 7-34 of SEQ ID NO:57, or a fragment of residues 7-34 of SEQ ID NO:57. In another embodiment, the PTH/PTHrP receptor antagonist includes mouse TIP(7-39) (See Hoare S R, Usdin T B. 2002. Specificity and stability of a new PTH1 receptor antagonist, mouse TIP(7-39). Peptides 23:989-98.). Other PTH/PTHrP receptor antagonists that may be used in the fusion proteins are also

disclosed in Hoare et al. The PTH/PTHrP receptor antagonist may be a fragment of at least 8, 10, 12 or more amino acids from residues 1-34 of SEQ ID NO:62. In other embodiments the PTH/PTHrP receptor antagonist may be PTH/PTHrP receptor antagonist polypeptide from another species.

In one embodiment, the therapeutic agent or PTH/PTHrP receptor agonist or antagonist polypeptide segment is N terminal to the collagen-binding polypeptide in a fusion protein. That is, the two polypeptide segments each have an N-terminal and a C-terminal, and the N-terminal of the collagen-binding polypeptide is linked directly or indirectly, e.g., through a therapeutic agent linker polypeptide segment (such as PKD, a Glycine or Serine linker) to the C-terminal of the therapeutic agent or PTH/PTHrP agonist or antagonist polypeptide.

A "therapeutic agent linker" may include a covalent bond and/or a linker or spacer moiety. For instance, the collagen-binding agent and the therapeutic agent may be linked directly through, e.g., a peptide bond or chemical cross-linking, or indirectly, through, e.g., a linker or spacer polypeptide. Useful therapeutic agent linkers include polypeptides, amino acids, nucleic acids, as well as homofunctional linkers or heterofunctional linkers. Particularly useful conjugation reagents that can facilitate formation of a covalent bond between the two collagen-binding domains may include a N-hydroxysuccinimide (NHS) ester and/or a maleimide.

A therapeutic agent linker may also include a spacer polypeptide. The spacer polypeptide may be any length and may include traditional or non-traditional amino acids. For example, the spacer polypeptide may be 1-100 amino acids long, suitably it is at least 2, 3, 5, 10, 15, 20, 25 or more amino acids long such that the two collagen-binding domains of the collagen-binding agent can mediate collagen binding. Spacer polypeptides may include, without limitation, a PKD (polycystic kidney disease) domain from a collagenase, a GST tag, a His-tag, a Ser linker, or a Gly linker. In some embodiments, the therapeutic agent linker may include a tag system. A tag system includes any group of agents capable of binding one another with a high affinity. Several tag systems are well-known in the art and include, without limitation, biotin/avidin, biotin/streptavidin, or digoxigenin (DIG) systems. In some embodiments, said tag system comprises biotin/avidin or biotin/streptavidin. The therapeutic agent(s) may be linked to the N-terminus or C-terminus of the collagen-binding agent by the therapeutic agent linker. In embodiments where the therapeutic agent includes a polypeptide, either the C-terminus or N-terminus of the therapeutic agent may be linked to the N-terminus or C-terminus of the collagen-binding agent by the therapeutic agent linker.

Furthermore, either the same of different therapeutic agents may be linked to both the N-terminus and C-terminus of the collagen-binding agent by two or more therapeutic agent linkers.

Pharmaceutical compositions including any of the collagen-binding agents described herein are provided. The pharmaceutical compositions may include a pharmaceutical carrier, excipient, or diluent (i.e., agents), which are nontoxic to the cell or animal being exposed thereto at the dosages and concentrations employed. Often a pharmaceutical agent is in an aqueous pH buffered solution. Examples of pharmaceutical carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™ brand surfactant, polyethylene glycol (PEG), and PLURONICS™ surfactant.

Methods of treating a condition are also provided. The methods may include administering any of collagen-binding agents or pharmaceutical compositions described herein to a subject in an amount effective to treat the condition. The "condition" may include a wound (chronic and acute), hyperparathyroidism, a hair condition (either excessive hair growth or hair loss), a collagenopathy, and a bone condition. Bone conditions include fractures, osteoporosis, periodontal defects, or other bone defects. Collagenopathies include, without limitation, osteogenesis imperfecta (OI), Stickler's syndrome, Ehlers-Danlos syndrome, Alport's syndrome, Caffey's disease, and localized collagen or cartilage damage. Many of these diseases are caused by genetic defects that result in the collagen in certain tissues being under twisted or partially untwisted.

Bone loss due to, for example, a collagenopathy such as osteogenesis imperfecta, Stickler's syndrome, osteoporosis or others which put an individual at higher risk for a bone fracture due to a collagen defect could be treated by administration of a collagen-binding agent linked to a bone anabolic peptide. The collagen-binding agent may target the bone anabolic agents to sites where the collagen is malformed and thus may prevent fracture.

Vascular fragility due to defects such as Ehlers-Danlos syndrome type IV, Alport's syndrome or other diseases where blood vessel rupture is more likely due to a defect in collagen formation may be administered collagen-binding agents including peptides that stimulate vascular

growth or repair. The collagen binding agents will target the peptide to the areas having collagen damage and these areas are likely to have damaged vessels. The therapeutic agents will stimulate growth and repair at the site of damage and prevent vessel rupture.

Skin fragility due to disorders such as Ehlers-Danlos syndrome, Caffey's disease or other diseases where weakening of the skin due to a collagen defect leads to hyperelasticity, easy bruising or poor wound healing. Dermal and epidermal growth factors may serve as therapeutic agents which when linked to collagen-binding agents and delivered to areas of damaged collagen will stimulate growth and repair of the skin, preventin striae and improving healing.

Also provided herein are methods of treating hyperparathyroidism by administering, for example, collagen-binding agents linked to PTH to a subject in need of treatment for hyperparathyroidism. In one embodiment the PTH administered to the subject may be a PTH from a different species. The effects of PTH agonists and antagonists on hair growth have been studied for over almost 15 years. PTH has a common receptor with PTH-related peptide (PTHrP), which is normally produced by dermal fibroblasts. PTHrP affects keratinocyte proliferation/differentiation and modulates the hair cycle. Most of the testing on hair growth effects has been performed with PTH antagonists, as indications from initial testing were that these were the most effective agents. Both injected and topical formulations have been tested in animal models of chemotherapy-induced alopecia and in the SKH-1 hairless mouse. Part of the effect of PTH antagonists on hair growth is to transition the hair follicles into a dystrophic catagen stage, which protects them from chemotherapeutic damage. However, clinical trials of topical PTH antagonists for chemotherapy-induced alopecia by IGI Pharmaceuticals were discontinued in phase 2 because of limited efficacy. Thus new compositions for treating alopecia are needed.

In another aspect, methods of treating a hair condition (excess hair loss or hair growth) The methods may include administering a collagen-binding agent linked to a PTH/PTHrP receptor agonist to a subject in need of treatment to induce hair growth or stop hair loss. The method is applicable to individuals with alopecia, including chemotherapy induced alopecia, but also alopecia areata, alopecia caused by male pattern baldness, polycystic ovarian syndrome or other hair loss. The compositions may be administered locally or topically to treat hair loss.

In another aspect, methods of slowing hair growth or regrowth after a hair removal procedure by administering a collagen-binding agent linked to a PTH/PTHrP receptor antagonist to a subject are provided. In one embodiment, the collagen-binding agent + PTH antagonist

composition is applied locally, topically. The collagen-binding agent + PTH antagonist may be applied after a hair removal procedure to prevent or slow hair regrowth.

The subject of the present invention may be any mammal, suitably a human, domesticated animal such as a dog, cat, horse, cow, pig, or a mouse or rat. Treating the condition or treatment includes but is not limited to ameliorating at least one symptom of the condition, reducing or slowing further progression of the condition, reducing or slowing the spread of the condition to unaffected areas. Treating a subject refers to any type of treatment that imparts a benefit to a subject afflicted with a disease or at risk of developing the disease, including improvement in the condition of the subject (e.g., in one or more symptoms), delay in the progression of the disease, delay the onset of symptoms or slow the progression of symptoms, etc.

An effective amount or a therapeutically effective amount as used herein means the amount of a composition that, when administered to a subject for treating a state, disorder or condition is sufficient to effect a treatment (as defined above). The therapeutically effective amount will vary depending on the compound, formulation or composition, the disease and its severity and the age, weight, physical condition and responsiveness of the subject to be treated.

The compositions (i.e. collagen-binding agents and pharmaceutical compositions) described herein may be administered by any means known to those skilled in the art, including, but not limited to, oral, topical, intranasal, intraperitoneal, parenteral, intravenous, intramuscular, subcutaneous, intrathecal, transcutaneous, nasopharyngeal, intra-lesional, intra-tumoral, intradermal, or transmucosal absorption. Thus the compositions may be formulated as an ingestable, injectable, topical or suppository formulation. The compositions may also be delivered with in a liposomal or time-release vehicle. Administration of the compositions to a subject in accordance with the invention may exhibit beneficial effects in a dose-dependent manner. Thus, within broad limits, administration of larger quantities of the compositions is expected to achieve increased beneficial biological effects than administration of a smaller amount. Moreover, efficacy is also contemplated at dosages below the level at which toxicity is seen.

It will be appreciated that the specific dosage administered in any given case will be adjusted in accordance with the composition or compositions being administered, the disease to be treated or inhibited, the condition of the subject, and other relevant medical factors that may modify the activity of the compositions or the response of the subject, as is well known by those skilled in the art. For example, the specific dose for a particular subject depends on age, body weight, general

state of health, diet, the timing and mode of administration, the rate of excretion, medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the compositions described herein and of a known agent, such as by means of an appropriate conventional pharmacological protocol.

The maximal dosage for a subject is the highest dosage that does not cause undesirable or intolerable side effects. The number of variables in regard to an individual treatment regimen is large, and a considerable range of doses is expected. The route of administration will also impact the dosage requirements. It is anticipated that dosages of the compositions will improve the condition being treated by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100% or more as compared to no treatment.

The effective dosage amounts described herein refer to total amounts administered, that is, if more than one composition is administered, the effective dosage amounts correspond to the total amount administered. The compositions can be administered as a single dose or as divided doses. For example, the composition may be administered two or more times separated by 4 hours, 6 hours, 8 hours, 12 hours, a day, two days, three days, four days, one week, two weeks, or by three or more weeks.

The collagen-binding agents or pharmaceutical compositions described herein may be administered one time or more than one time to the subject to effectively improve the condition being treated. Suitable dosage ranges are of the order of several hundred micrograms effective ingredient with a range from about 0.01 to 10 mg/kg/day, preferably in the range from about 0.1 to 1 mg/kg/day. Precise amounts of effective ingredient required to be administered depend on the judgment of the practitioner and may be peculiar to each subject. It will be apparent to those of skill in the art that the therapeutically effective amount of the collagen-binding agents and pharmaceutical compositions described herein will depend, inter alia, upon the administration schedule, whether the composition is administered in combination with other therapeutic agents, the status and health of the recipient, and the therapeutic activity of the particular composition.

The present disclosure is not limited to the specific details of construction, arrangement of components, or method steps set forth herein. The compositions and methods disclosed herein are capable of being made, practiced, used, carried out and/or formed in various ways that will be apparent to one of skill in the art in light of the disclosure that follows. The phraseology and

terminology used herein is for the purpose of description only and should not be regarded as limiting to the scope of the claims. Ordinal indicators, such as first, second, and third, as used in the description and the claims to refer to various structures or method steps, are not meant to be construed to indicate any specific structures or steps, or any particular order or configuration to such structures or steps. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to facilitate the disclosure and does not imply any limitation on the scope of the disclosure unless otherwise claimed. No language in the specification, and no structures shown in the drawings, should be construed as indicating that any non-claimed element is essential to the practice of the disclosed subject matter. The use herein of the terms "including," "comprising," or "having," and variations thereof, is meant to encompass the elements listed thereafter and equivalents thereof, as well as additional elements. Embodiments recited as "including," "comprising," or "having" certain elements are also contemplated as "consisting essentially of" and "consisting of" those certain elements.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended, and all possible combinations of numerical values between and including the lowest value and the highest value enumerated are to be considered to be expressly stated in this disclosure. Use of the word "about" to describe a particular recited amount or range of amounts is meant to indicate that values very near to the recited amount are included in that amount, such as values that could or naturally would be accounted for due to manufacturing tolerances, instrument and human error in forming measurements, and the like. All percentages referring to amounts are by weight unless indicated otherwise.

No admission is made that any reference, including any non-patent or patent document cited in this specification, constitutes prior art. In particular, it will be understood that, unless otherwise stated, reference to any document herein does not constitute an admission that any of these

documents forms part of the common general knowledge in the art in the United States or in any other country. Any discussion of the references states what their authors assert, and the applicant reserves the right to challenge the accuracy and pertinence of any of the documents cited herein. All references cited herein are fully incorporated by reference in their entirety, unless explicitly indicated otherwise. The present disclosure shall control in the event there are any disparities between any definitions and/or description found in the cited references.

Unless otherwise specified or indicated by context, the terms "a", "an", and "the" mean "one or more." For example, "a protein" or "an RNA" should be interpreted to mean "one or more proteins" or "one or more RNAs," respectively.

As used herein, "about," "approximately," "substantially," and "significantly" will be understood by persons of ordinary skill in the art and will vary to some extent on the context in which they are used. If there are uses of these terms which are not clear to persons of ordinary skill in the art given the context in which they are used, "about" and "approximately" will mean plus or minus ≤10% of the particular term and "substantially" and "significantly" will mean plus or minus >10% of the particular term.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Example 1 - Activation and binding mechanism of tandem collagen-binding domains with pseudo-two fold-symmetry.

Clostridium histoliticum secrets virulence factors, including highly active collagenases CollG (class I) and ColH (class II), to penetrate animal tissues. After the multi-domain ColG utilizes its tandem collagen-binding domain (CBD) to anchor itself onto insoluble collagen, subsequent degradation of the hierarchical substrate involves processive cleavage and rearrangement of fibrils. In this work, the structure of the calcium bound tandem CBD is presented at 1.9 Å resolution (R_{work} = 15.0%; R_{free} = 19.6%). The pseudo-two-fold arrangement of CBD would allow ColG to wedge between collagen molecules that are 55 Å apart in order to potentially aid in fibril rearrangement and processive cleavage. Indeed, between 0.1:1 and 0.5:1 molar ratios of tandem CBD and collagen, it accelerated collagen fiber formation. At 1:1 molar ratios and above, the tandem CBD retarded fibril formation. To toggle between collagen molecules, a tighter binding C-side CBD, may initiate binding as demonstrated by small angle X-ray scattering (SAXS) of tandem CBD forming a 1:1

complex with $[(Pro\text{-}Hyp\text{-}Gly)_{10}]_3$. Subsequently, the weaker binding N-side CBD can latch onto a prone collagen molecule to provide the tightest known fibril binding. The conformational change of the tandem CBD is calcium dependent and cooperative as measured by size exclusion chromatography and by SAXS at pCa in the range of 3-6. At pCa > 5, the tandem CBD adopts an extended structure that is easier to be secreted from the bacterium. In the host pCa^{2+} >3, the compact structure seen in the crystal structure is adopted. The binding and activation mode described here will help guide site-directed drug delivery vehicle development.

Abbreviations used: CBD, collagen binding domain; MALDI -TOF –MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; DLS, dynamic light scattering; SAXS, small angle X-ray scattering.

Clostridium histolyticum, which was recently proposed to be reclassified as *Hathewaya histolyticum* based on 16S rRNA gene comparison, produces collagenases that cause extensive tissue damage during myonecrosis (Lawson and Rainey 2015). The most significant of these enzymes, ColG (class I) and ColH (class II), are multi-domain enzymes that include a N-terminal collagenase module (s1), one or two polycystic kidney disease (PKD)-like domains, and one to three C-terminal collagen-binding domains (CBDs) (**Fig. 1A**), and division of roles in collagenolysis were suggested (Fields 2013).

The C-terminal domains of ColG (s3a, s3b), and ColH (s3), are homologs consisting of approximately 120 amino acids. The domains bind to soluble collagenous peptides and insoluble collagen fibril. Their role in binding to collagen fibril is essential in dismantling its hierarchical structure (Matsushita, Jung et al. 1998, Matsushita, Koide et al. 2001). Truncation of CBD from either full-length ColG or ColH incapacitates their abilities to degrade collagen fibril. Such enzymes can only degrade solubilized collagen or denatured collagen (gelatin). Mutagenesis and collagen-binding studies mapped the binding surface of s3b, while NMR and SAXS studies showed that s3b unidirectionally binds to under-twisted regions of mini-collagen collagen (Philominathan, Koide et al. 2009, Philominathan, Koide et al. 2012). High-speed atomic force microscopy has recently revealed ColG's ability to dismantle collagen in real time (Watanabe-Nakayama, Itami et al. 2016). During degradation, ColG moves processively along collagen fibril from C-terminus to N-terminus to dismantle the fibril. ColG also preferentially targeted less ordered region of collagen fibril. Structural insight for ColG's processsivity and preference for disordered region are described.

Bacterial collagenases require calcium to attain both full catalytic activity and collagen-

binding function. The activation of bacterial collagenase involves domain rearrangement triggered by the Ca^{2+} binding to the linker from α-helix to β-sheet (Wilson, Matsushita et al. 2003, Philominathan, Matsushita et al. 2009, Spiriti and van der Vaart 2010, Sides, Liyanage et al. 2012, Bauer, Wilson et al. 2013, Bauer, Janowska et al. 2015). The full-length *apo*-ColG is expected to be relatively flexible inside the bacteria where Ca^{2+} concentration is low ($0.2\text{-}0.3x10^{-6}$ M) allowing the enzyme to be secreted more easily (Wilson, Matsushita et al. 2003). Upon secretion, the linker chelates to Ca^{2+} (~1.2 mM) in the ECM to adopt a rigid structure. Though it has not been shown for ColG, Ca^{2+} chelation indeed triggers a full-length ColH to adopt a less flexible and compact structure as demonstrated using SAXS and limited proteolysis (Ohbayashi, Matsumoto et al. 2013).

The clostridial collagenases have been successfully used for years as a wound debridement. Recently the mixture of ColG and ColH was approved for use in the treatment of excessive connective tissue build up found, for example, in Dupuytren's disease (Gaston, Larsen et al. 2015). Furthermore, the enzymes showed potential to treat different types of connective tissue build ups (Duarte, Correia et al. 2014) and to isolate pancreatic islets for transplantation (McCarthy, Breite et al. 2011). In addition to therapeutic use of full-length collagenase for removal of connective tissue, the non-catalytic segments are used for targeted drug-delivery to reduce dosage and to minimize side effects. Initially, Nishi *et al.* developed fusion proteins of the targeting segment (s2b-s3) and growth factors. When injected, the fusion proteins remained active at the site of injection for up to 10 days (Nishi, Matsushita et al. 1998). A fusion protein of s3 and parathyroid hormone (PTH-CBD) is being developed to treat osteoporosis (Ponnapakkam, Katikaneni et al. 2011, Ponnapakkam, Katikaneni et al. 2011, Ponnapakkam, Katikaneni et al. 2012), to prevent and to treat alopecia (Katikaneni, Ponnapakkam et al. 2012, Katikaneni, Ponnapakkam et al. 2014, Katikaneni, Ponnapakkam et al. 2014, Katikaneni, Seymour et al. 2015). The systemic application is based on the apparent targeting ability of s3 to blood accessible and regenerating collagen (Stratford, Vu et al. 2014). Although the lower affinity s3 is efficacious as a systemic drug delivery vehicle *in vivo*, the tighter collagen binder s2b-s3 is more efficacious in wound healing applications when it is applied at the site of injury with collagen-based bone graft material (An, Lin et al. 2015, Fujimaki, Inoue et al. 2015, Saito, Uchida et al. 2015, Uchida, Matsushita et al. 2015). Here we report crystal structures of tandem CBD in the presence of calcium. We also propose its mode of binding and interaction with the collagen substrate and the role of calcium based on gel filtration and SAXS results.

Methods and Materials

Production, Purification, Crystallization and Structure Determination

Individual s3a, s3b, as well as tandem CBD derived from the *C. histolyticum* ColG were expressed as glutathione S-transferase (GST)-fusion proteins using method as described previously (Matsushita, Jung et al. 1998). Initial conditions suitable to grow crystals of tandem CBD were identified by high-throughput screen (Hampton Research Crystal Screen HT). Subsequent crystallization trials using the initial conditions were carried out using the hanging-drop method. Crystals of tandem CBD, obtained in the presence of 3 mM calcium (21-26% PEG3350, HEPES pH 7.5 at 37°C), were orthorhombic (space group, $P2_12_12_1$), with cell parameters $a = 51.5$ Å, $b = 54.7$ Å, $c = 92.0$ Å. The crystals grew within 24 hours in 37°C but not at 4, 16 and 24°C. The crystals were temperature sensitive and could not withstand cryogenic temperatures. Therefore diffraction data were collected by means of in–house X-ray facility at room temperature to 1.9 Å resolution using a Rigaku 007 generator with Cu $K\alpha$ radiation. The data sets were processed with d*TREK (Pflugrath 1999) (Table 1). The structure was solved with the molecular replacement program MolRep from the CCP4 package, by using s3b (PDB code 2o8o) as the search model (Murshudov, Vagin et al. 1997). One tandem CBD was found in an asymmetric unit; and therefore, V_M was 2.5 $Å^3$/Da and solvent content was 50% (Matthews 1968). Refinement of the tandem CBD was carried out using Refmac_6.1.13 (Murshudov, Vagin et al. 1997). TLS restraints were applied to main chain atoms with each CBD acting as a TLS group. Babinet scaling was used for bulk solvent refinement. Five percent of the data were set aside to monitor R_{free}. The models were manually adjusted between each refinement cycle using MIFit (McRee 1999). Alternate confirmations were built for Lys818, Glu945, Tyr970, and Arg1005. The Ramachandran plot for the final structure obtained with the program Procheck (Laskowski, Macarthur et al. 1993) showed 91% of the residues in the core region and 9% in the additionally allowed region, with none in the generously allowed or disallowed regions. The final refinement statistics are shown in Table 1.

Table 1: Data collection statistics

Data collection statistic	
Wavelength (Å)	1.5419
Temperature (K)	298
Resolution range (Å)[a]	19.7-1.90 (1.97-1.90)
Space group	$P2_1\ 2_1\ 2_1$

Unit cell dimension (Å)	
a (Å)	51.5
b (Å)	54.7
c (Å)	92.0
Unit cell angle (°)	$\alpha, \beta, \gamma = 90$
Total reflections	89,473
Redundancy	4.47 (4.27)
Completeness (%)[a]	94.8 (94.2)
R_{meas} (%)[a, b]	7.1 (49.2)
$I/\sigma I$[b]	11.2 (2.9)

Refinement statistic	
Unique reflections	18,987
Solvent molecules	211
R_{work} (%)	15.0 (25.5)
R_{free} (%)[b, c]	19.6 (26.8)
Average *B* value (Å^2)	37.68
Coordinates ESU based on R_{free} (Å)	0.13
Root mean square deviations	
Bond distance (Å)	0.011
Bond angles (°)	1.87
Chiral centers (Å^3)	0.17
Planar groups (Å)	0.01
B-factor restrains	
Main - chain bond (Å^2)	3.81
Main-chain angle (Å^2)	4.72
Side- chain bond (Å^2)	6.84
Long range B-factor (Å^2)	12.3
Ramachandran statistic	
Most favored region (%)	90.9
Allowed region (%)	9.1

[a] $R_{meas} = \Sigma_{hkl} \{N_{(hkl)}/N_{(hkl)}-1\}^{1/2} \Sigma_i | I_{i(hkl)} - \langle I_{(hkl)} \rangle | / \Sigma_{(hkl)} \Sigma_i I_{i(hkl)}$

[b] Data for highest resolution shell are given in parentheses

[c] 5% of data excluded from refinement

Small Angle X-ray Scattering

Suitable buffer conditions for small angle X-ray scattering (SAXS) measurements were identified using discontinuous Native-PAGE. For the pCa analysis the tandem CBD was equilibrated into 10 mM HEPES-Na (pH 7.5), 100 mM NaCl, and 2% glycerol. The pCa was maintained with 0.2 mM total EGTA with $CaCl_2$ added to bring pCa values to 3, 4, 5 and 6. The amount of Ca^{2+} needed to reach a given pCa was determined using MAXCHELATOR (Bers, Patton et al. 2010). For the tandem CBD:$[(POG)_{10}]_3$ complex analysis, the complex was equilibrated into 50 mM HEPES-Na (pH 7.5), 100 mM NaCl, and 2 mM $CaCl_2$. $[(POG)_{10}]_3$ was dissolved in 5 mM acetic acid to a concentration of 7.5 mg/mL and stored at 4°C for 24 h. The peptide was then mixed with tandem CBD in a 1:2 molar ratio of tandem CBD to mini-collagen. Measurements were completed for three concentration series per sample. For the pCa series, the concentration of tandem CBD used at pCa 4 and 5 and 6 was 1, 3, and 5 mg/mL. At pCa 3, the concentration series used was 2, 4, and 6 mg/mL. For the complexes with $[(POG)_{10}]_3$, the concentration series used was 1, 3, and 5 mg/mL. All SAXS data were collected at 10°C at the Advanced Light Source at Berkley National Lab (SIBYLS beamline, 12.3.1). All data processing was accomplished using primusqt from the ATAS 2.6.1 software package. For CBD at each pCa, exposure data from the concentration gradient that were not affected by either aggregation or detector saturation were extrapolated to infinite dilution. Determination of the radius of gyration (R_g), maximum diameter (D) as well as *ab initio* shape reconstruction of the extrapolated data was carried out using the dammif function in primusqt. The χ values calculated at the end of each run indicated the agreement between the calculated scattering curve and the experimental scattering curve. For the pCa series, the χ values for each shape ranges between 0.8 And 1.2. For the complex with $([POG]_{10})_3$, the χ value is 0.8.

Collagen Fibril Formation

The impact of addition of tandem CBD on self-assembly of collagen molecules was monitored by measuring turbidity as an increase in optical density at 450 nm, at 37ºC. On ice, a solution of 2 mg/ml of rat collagen was diluted with 40 mM HEPES buffer pH 7.5, with addition of 300 mM NaCl, 2 mM $CaCl_2$, to final concentration of 0.5 mg/ml (2.4 μM). In the next step collagen binding was added in ratios: 0.1 to 1; 0.2 to 1; 0.3:1; 0.5 to 1; 1 to 1; 2 to 1; 3 to 1; 5 to 1 of molar concentration. The turbidity measurement was taken in 96 well plates with 1-minute intervals with spectrophotometer Filter Max F5 (Molecular Devices). From the turbidity curve the following

parameters were estimated t_{lag}-time at the end of lag phase, maximum turbidity, and V the maximum fibril growth rate.

Analytical Size Exclusion Chromatography

Size exclusion chromatography was performed at room temperature on a HPLC system equipped with a Superdex 75 column (1 × 30 cm, Pharmacia) at a flow rate of 0.5 ml/min as described (Wilson, Matsushita et al. 2003).. The following proteins were used as molecular mass standards: bovine serum albumin, 67.0 kDa; chicken ovalbumin, 43.0 kDa; and ribonuclease A, 13.7 kDa (Pharmacia). The measurement was carried out in triplicate.

Quantitative analysis of the binding to a collagenous peptide

Binding of various CBD proteins to a collagenous peptide was measured by surface plasmon resonance using a BIACORE apparatus (Biacore, Uppsala, Sweden) with a sensor chip (CM5, Biacore) on which a peptide, Gly-(Pro-Hyp-Gly)$_{12}$, was covalently immobilized as described (Wilson, Matsushita et al. 2003).

Collagen Fibril Binding Assay

Binding affinities for three CBDs, s3a, s3b, and s3as3b, to collagen fiber were analyzed. Initially, 10 mg of porcine skin fiber (Nippi,Inc; Japan) was placed on a 0.2 nm spin column and washed with 50 mM Tris- HCl (pH 7.5), supplemented with of 200 mM NaCl and 5 mM $CaCl_2$. Then, 20 µL of a protein mixtures containing 0.5 mg/mL BSA (internal control), and a varied concentration (between 2.5 and 20 mg/mL) of tandem CBD, equilibrated in the buffer above, was added and thoroughly mixed with the fiber. After a 30 min incubation at room temperature, the supernatant containing unbound tandem CBD was collected and quantified using SDS-PAGE. For this step, an equal volume of unused protein mixture was used as a control. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250, and their relative amounts were estimated by ImageJ software (version 1.4.2; National Institutes of Health). Based on these estimations a calibration curve was constructed for each CBD and was used to quantify CBD amounts in each supernatant. The results obtained by triplicate assay were analyzed on a Scatchard plot to obtain the dissociation constant (K_d) and the number of binding sites (B_{max}) for each CBD.

Results and Discussion

Structure Description of tandem CBD

Crystal structure of tandem CBD consisting of s3a and s3b was solved at 1.9Å resolution. Although the s3b segment has been described before, the s3a segment is described for the first time

in this paper. Both s3a and s3b adopt a very similar β-sandwich 'jelly-roll' composed of ten β-strands. The CBDs are related by a pseudo two-fold rotational symmetry that is stabilized by salt-bridges and hydrogen-bonding interactions. The pseudo symmetry axis, which is perpendicular to the plane of the page in **Fig. 1B**, positions the collagen-binding pockets in the tandem CBD to be 55 Å apart. Each domain chelates to two Ca^{2+} *i.e.*, one Ca^{2+} is bound with pentagonal bipyramidal geometry and the other is bound with square antiprismatic geometry as described (Bauer, Wilson et al. 2013). The electron density allowed us to observe a prolyl-*cis*-peptide bond between Glu792 and Pro793 and non-prolyl-*cis*-peptide bond between Glu899 and Asn992. Overlay based on 110 equivalent C_α atoms shows that the s3a and s3b share r.m.s.d. of 0.9Å and only significantly deviate at loops (r.m.s.d of 1.1-2.5 Å). Loops in both CBDs exhibited the highest B-factor values, while β-sheet residues exhibited low B-factor values. As expected, B-factor values for residues that interact with Ca^{2+} were amongst the lowest. The average temperature factor for s3a is lower than s3b due to crystal packing. In the previously reported structure of *holo*-s3b (PDB code 4HPK), the protease sensitive and highly dynamic loop 960-968 was not observed (Philominathan, Koide et al. 2009, Sides, Liyanage et al. 2012, Bauer, Wilson et al. 2013). In the structure presented here, the dynamics of this loop are suppressed by crystal packing contacts. Otherwise the s3b alone and s3b in the tandem CBD are virtually identical to each other (C_α r.m.s.d. 0.6 Å).

Ca^{2+}-induced transformation of the tandem CBD

The difference in Ca^{2+} concentration inside bacterium and ECM in host could be taken advantage of to efficiently secrete bacterial collagenases into the host. The intracellular Ca^{2+} concentration of *Clostridium* is likely to be similar to that of *Escherichia coli* ($0.2–0.3 \times 10^{-6}$ M) (Holland, Jones et al. 1999), which is well below the tandem CBD's apparent K_d for Ca^{2+} (**Fig. 2A**). However, extracellular tissue fluids contain Ca^{2+} concentrations at ~1.2 mM (Maurer and Hohenester, 1997). Monitored by size exclusion chromatography, the domain reorientation of s3a-s3b is a cooperative event induced by Ca^{2+}, and SAXS data corroborated the magnitude of the structural change (**Fig. 2B**). The SAXS derived envelope for tandem CBD at pCa less than or equal to 5 (10 μM) adopts a rod like shape (**Fig. 2B**). A bump in the envelope resembles how α-helical linker appeared in SAXS (Sides, Liyanage et al. 2012). At pCa=6, the β-strand A' of s3b also unfolds to greatly increase the linker's dynamic (Sides, Liyanage et al. 2012). Upon increasing Ca^{2+} concentrations, the tandem CBD steadily adopts more compact shapes (**Fig. 2B**). At pCa=4 (100 μM) the shape resembles the crystal structure with the exception of a bulge that suggests the linker

of s3a remained dynamic. At pCa=3 (1 mM) it agrees well with the crystal structure of tandem CBD. The domain rearrangement modeled in the SAXS derived envelope is consistent with the observations made for s3b with its 12-residue-long linker. The linker between s3a and s3b has been shown to undergo secondary structure transformation from α-helix to β-sheet that is induced by Ca^{2+} binding and results in tighter contact between the domains (Wilson, Matsushita et al. 2003, Sides, Liyanage et al. 2012). The β-strand A' of s3b also unfolds to greatly increase the linker's dynamic (Sides, Liyanage et al. 2012). Free energy simulations have determined that the calcium ions not only stabilize the *cis*-peptide bond thermodynamically but also catalyze its formation (Spiriti and van der Vaart 2010). Calcium dependent structural change was monitored for full length ColH by size exclusion chromatography and by SAXS (Ohbayashi, Matsumoto et al. 2013). Expanding upon the findings for tandem CBD, the full length ColG and ColH is also likely to undergo Ca^{2+}-induced domain rearrangement. Dynamic ColG and ColH inside bacterium should allow for rapid secretion inducing maximum damage to host.

Mini collagen-tandem CBD interaction

Our results suggest a unique interaction between tandem CBD and either collagen fibril or mini-collagen. Collagen fibril is built by a staggered array of triple-helical tropocollagen, and is insoluble in water. Meanwhile, synthetic collagenous peptide, or mini-collagen, which mimics the structure of native tropocollagen and is soluble in water, has been used to investigate the individual collagen–protein interactions. Use of mini-collagen also allows quantitative analysis of CBD-collagen interaction. The K_d values have been evaluated for various forms of CBDs to insoluble collagen fibril and to mini-collagen, and they come to a good agreement (Matsushita, Koide et al. 2001). Tandem CBD binds to the collagen fibril the tightest (K_d~) among CBDs tested and much tighter than the sum of s3a or s3b alone (K_d~100μM). However, tandem CBD binds to mini-collagen only as tightly as s3b does. Tandem CBD did show cooperative binding when the immobilized mini-collagen density was high. SAXS results of tandem CBD:mini-collagen also revealed a 1:1 complex. Given the binding affinities of s3a and s3b to mini-collagen, s3b segment of tandem CBD bound unidirectionally to the C-terminus of $[(POG)_{10}]_3$ (Philominathan, Koide et al. 2009, Philominathan, Koide et al. 2012, Bauer, Wilson et al. 2013), thus it was modeled in the envelope as such (**Fig. 2B**).

The s3a binds less tightly than s3b to collagen possibly because it is missing one of the conserved tyrosine residues. When CBD sequences were aligned, three Tyr residues (970, 994 and

996 in s3b) are well conserved, and mutagenesis of any of these residues diminished binding to mini-collagen (Wilson, Matsushita et al. 2003). When the sequences of tandem CBDs were aligned instead difference in N-side CBD and C-side CBD emerged. The equivalent to Tyr 996 in the N-side CBD was not well conserved, but all three Tyr residues were well conserved in the C-side CBD (**Fig. 2B**; **Fig. 5**). The s3a also lacks conserved Tyr970 equivalent and Ser851 occupies the position instead. Neighboring His848 of s3a does occupy approximately the same space near this residue and could, to a lesser extent, fulfill its role. The gene duplication of CBD apparently required a loss of functionally importance at position 877 in order to prevent the domain form becoming stuck at the surface of the fibril. The CBD domains are positioned by an extra β-strand (β-strand A') and interdomain interactions. While β-strand A' is present in both s3a and s3b, it is absent in s3, and hence, could be unique to collagenases with multiple CBDs. The extra β-strand is stabilized by unconserved side-chain interactions, which suggests that gene duplication was a relatively recent event.

The observed 1:1 complex of CBD to mini-collagen indicates that the tighter binding s3b initiates binding. This proposed mechanism is supported by the observation that the tandem CBD binds to mini-collagen just as tightly as s3b alone. Nevertheless, the tandem CBD binds tighter to collagen fibril than s3b alone (Toyoshima, Matsushita et al. 2001). While s3b initiates binding and serves the central role, s3a plays an auxiliary, yet pivotal role in intercalating into a space created between collagen molecules that are 55 Å apart. Although it was suggested that CBDs could lie side by side to bind to one tropocollagen molecule (Eckhard and Brandstetter 2011), our structure suggests this mode is less likely.

ColH, unlike ColG, possesses two PKD-like domains (s2a and s2b) but only a single CBD, s3. Though not a collagen-binder by itself, s2b does enhance s3's affinity to collagen (Matsushita, Jung et al. 1998). While s3a is a collagen-binder, it may work similarly to s2b by serving as the source of weak interactions that allows the segment to scan a single tropocollagen for opportunistic binding sites as well as contribute to an overall tighter collagen-binder. Development of the tandem PKD and tandem CBD segments allowed ColG and ColH to potentially seek different niches in collagen fiber.

Collagen Fibril-Tandem CBD Interaction

The psuedo-two fold symmetry orients the collagen-binding surface at the opposite ends of the molecule and allows the tandem CBD to bind to parallelly oriented two collagen molecules

(**Fig. 1B**). Collagen self assembles to form collagen fibril in a process that can be monitored by change in absorbance at 450nm. Collagen-binding molecules, such as s3b, bind to collagen and retard fibril formation (Okano-Kosugi, Matsushita et al. 2009). However, since the tandem CBD appears to be able to wedge itself between two collagen molecules, we tested its ability to promote fibril self-assembly. To investigate the influence tandem CBD has on collagen fibril formation, the turbidity of mixtures of tandem CBD and collagen was monitored (**Fig. 3**). Remarkably, the ratio of tandem CBD to collagen has contrasting influences on the lag time prior to fiber formation. In the absence of tandem CBD (control), the lag time for fibril formation is 19 min and is consistent with previous observations (Okano-Kosugi, Matsushita et al. 2009). At a 0.1:1 ratio of tandem CBD to collagen, the lag time is reduced to 15 min. When the concentration of tandem CBD is relatively high compared to the ratio of collagen the lag time increases. At a tandem CBD to collagen ratio of 5:1, the lag time is 31 min. The fibril growth for lower ratios of CBD resulted in a much higher absorption of 0.0217 compared to samples with higher ratios of tandem CBD, where the absorption was 0.005. Influence on lag time inversely correlated to collagen fibril thickness. The 0.1:1 and 0.2:1 mixtures of tandem CBD to collagen resulted in fibril that is about 14% thicker than the collagen fibril control. At a 5:1 ratio, the thickness is 2% less than the control. At low concentrations, tandem CBD aids in collagen alignment, and thus accelerates the formation of nuclei for collagen self-assembly. The seemingly opposite effects of this two ratio ranges of CBD to collagen on self-assembly may provide an important clue to understanding how ColG disassemble and breakdown collagen fibril as recently being revealed (Watanabe-Nakayama, Itami et al. 2016).

Collagenolysis by ColG

Hydrolysis of collagen fibril by ColG monitored in real time by high-speed atomic force microscopy revealed as following: (1) the interactions of inter-fibril collagen molecules prevented collagenase molecules from engaging; (2) collagen molecules were rearranged onto other fibrils when subjected to ColG; (3) disordered D-periodicity made the collagen fibril susceptible to degradation by ColG; (4) ColG moves from C-terminus to N-terminus processively; (5) At every pass, ColG evenly trimmed the thickness of the collage fibril. The structure of tandem CBD sheds some light into the ColG's action.

Given the binding clefts' positions on opposite faces of the domain, we propose the following collagen-binding modes: (i) tandem CBD wedges into interfibrous space (**Fig. 4B**), (ii) tandem CBD wedges into crevices found in damaged or remodeling collagen fiber (**Fig. 4C**). The

effort necessary to toggle into interfibrous space may appear as though ColG was stalled. The tandem CBD's ability to facilitate fibril formation could explain how ColG rearranged collagen fibrils. Mechanically disrupting D-periodicity in collagen fibril may introduce pockets for ColG binding. If the fibril is damaged by removing the outermost tropocollagen, the CBD could wedge itself between the exposed tropocollagen. Such action raises the interesting possibility that the tandem CBD could be used to target drugs to damaged collagen. Alternatively, the tandem CBD could toggle between two fibrils. The median of the surface-to-surface distance of one fibril to another is ~3.2 nm in skin (Kuwaba, Kobayashi et al. 2001). Nearly 15% of the interfibrous space in skin should be in the order of ~6 nm. The results suggest that the tandem CBD could be useful to anchor drugs to damaged tissues. Once toggled between collagen molecules, ColG's processive C→N-terminal movement is likely driven by the collagenase module, and thus, evenly trimmed collagen fibril is produced (Eckhard and Brandstetter 2011, Eckhard, Schonauer et al. 2011).

The high-resolution structure of bacterial tandem collagen binding domain is reported for the first time. The pseudo-symmetrical arrangement of CBD, resulting from gene duplication and fusion, could allow it to recognize unique niche in collagen fibril to facilitate degradation of collagen. The structure of tandem CBD reveals that it could wedge between parallelly oriented collagen molecules. The structure combined with previously identified CBD preference for under-twisted regions of collagen suggest the tandem CBD targets ColG to damaged regions of the collagen fibril. Such targeting also opens new drug targeting avenues in which the tandem CBD tightly anchors drugs to the injury site.

References for Example 1

An, B., Y. S. Lin and B. Brodsky (2015). "Collagen interactions: Drug design and delivery." Adv Drug Deliv Rev.

Bauer, R., K. Janowska, K. Taylor, B. Jordan, S. Gann, T. Janowski, E. C. Latimer, O. Matsushita and J. Sakon (2015). "Structures of three polycystic kidney disease-like domains from Clostridium histolyticum collagenases ColG and ColH." Acta Crystallogr D Biol Crystallogr **71**(Pt 3): 565-577.

Bauer, R., J. J. Wilson, S. T. L. Philominathan, D. Davis, O. Matsushita and J. Sakon (2013). "Structural Comparison of ColH and ColG Collagen-Binding Domains from Clostridium histolyticum." Journal of Bacteriology **195**(2): 318-327.

Bers, D. M., C. W. Patton and R. Nuccitelli (2010). "A Practical Guide to the Preparation of Ca2+ a Buffers." Calcium in Living Cells **99**: 1-26.

Duarte, A. S., A. Correia and A. C. Esteves (2014). "Bacterial collagenases - A review." Crit Rev Microbiol.

Eckhard, U. and H. Brandstetter (2011). "Polycystic kidney disease-like domains of clostridial collagenases and their role in collagen recruitment." Biol Chem **392**(11): 1039-1045.

Eckhard, U., E. Schonauer, D. Nuss and H. Brandstetter (2011). "Structure of collagenase G reveals a chew-and-digest mechanism of bacterial collagenolysis." Nat Struct Mol Biol **18**(10): 1109-1114.

Fields, G. B. (2013). "Interstitial collagen catabolism." J Biol Chem **288**(13): 8785-8793.

Fujimaki, H., G. Inoue, K. Uchida, M. Miyagi, W. Saito, A. Sato and M. Takaso (2015). "Elevation of Microglial Basic Fibroblast Growth Factor Contributes to Development of Neuropathic Pain after Spinal Nerve Ligation in Rats." Spine (Phila Pa 1976).

Gaston, R. G., S. E. Larsen, G. M. Pess, S. Coleman, B. Dean, B. M. Cohen, G. J. Kaufman, J. P. Tursi and L. C. Hurst (2015). "The Efficacy and Safety of Concurrent Collagenase Clostridium Histolyticum Injections for 2 Dupuytren Contractures in the Same Hand: A Prospective, Multicenter Study." J Hand Surg Am **40**(10): 1963-1971.

Holland, I. B., H. E. Jones, A. K. Campbell and A. Jacq (1999). "An assessment of the role of intracellular free Ca2+ in E. coli." Biochimie **81**(8-9): 901-907.

Katikaneni, R., T. Ponnapakkam, O. Matsushita, J. Sakon and R. Gensure (2014). "Treatment and prevention of chemotherapy-induced alopecia with PTH-CBD, a collagen-targeted parathyroid hormone analog, in a non-depilated mouse model." Anticancer Drugs **25**(1): 30-38.

Katikaneni, R., T. Ponnapakkam, A. Seymour, J. Sakon and R. Gensure (2014). "Parathyroid hormone linked to a collagen binding domain promotes hair growth in a mouse model of chemotherapy-induced alopecia in a dose-dependent manner." Anticancer Drugs **25**(7): 819-825.

Katikaneni, R., T. Ponnapakkam, H. Suda, S. Miyata, J. Sakon, O. Matsushita and R. C. Gensure (2012). "Treatment for chemotherapy-induced alopecia in mice using parathyroid hormone agonists and antagonists linked to a collagen binding domain." Int J Cancer **131**(5): E813-821.

Katikaneni, R., A. W. Seymour, R. Gulati, T. Ponnapakkam and R. C. Gensure (2015). "Therapy for Alopecia Areata in Mice by Stimulating the Hair Cycle with Parathyroid Hormone Agonists Linked to a Collagen-Binding Domain." J Investig Dermatol Symp Proc **17**(2): 13-15.

Kuwaba, K., M. Kobayashi, Y. Nomura, S. Irie and Y. Koyama (2001). "Elongated dermatan sulphate in post-inflammatory healing skin distributes among collagen fibrils separated by enlarged interfibrillar gaps." Biochem J **358**(Pt 1): 157-163.

Laskowski, R. A., M. W. Macarthur, D. S. Moss and J. M. Thornton (1993). "Procheck - a Program to Check the Stereochemical Quality of Protein Structures." Journal of Applied Crystallography **26**: 283-291.

Lawson, P. A. and F. A. Rainey (2015). "Proposal to restrict the genus Clostridium (Prazmowski) to Clostridium butyricum and related species." Int J Syst Evol Microbiol.

Matsushita, O., C. M. Jung, J. Minami, S. Katayama, N. Nishi and A. Okabe (1998). "A study of the collagen-binding domain of a 116-kDa Clostridium histolyticum collagenase." J Biol Chem **273**(6): 3643-3648.

Matsushita, O., T. Koide, R. Kobayashi, K. Nagata and A. Okabe (2001). "Substrate recognition by the collagen-binding domain of Clostridium histolyticum class I collagenase." J Biol Chem **276**(12): 8761-8770.

Matthews, B. W. (1968). "Solvent content of protein crystals." J Mol Biol **33**(2): 491-497.

McCarthy, R. C., A. G. Breite, M. L. Green and F. E. Dwulet (2011). "Tissue dissociation enzymes for isolating human islets for transplantation: factors to consider in setting enzyme acceptance criteria." Transplantation **91**(2): 137-145.

McRee, D. E. (1999). "XtalView/Xfit--A versatile program for manipulating atomic coordinates and electron density." J Struct Biol **125**(2-3): 156-165.

Murshudov, G. N., A. A. Vagin and E. J. Dodson (1997). "Refinement of macromolecular structures by the maximum-likelihood method." Acta Crystallogr D Biol Crystallogr **53**(Pt 3): 240-255.

Nishi, N., O. Matsushita, K. Yuube, H. Miyanaka, A. Okabe and F. Wada (1998). "Collagen-binding growth factors: production and characterization of functional fusion proteins having a collagen-binding domain." Proc Natl Acad Sci U S A **95**(12): 7018-7023.

Ohbayashi, N., T. Matsumoto, H. Shima, M. Goto, K. Watanabe, A. Yamano, Y. Katoh, K. Igarashi, Y. Yamagata and K. Murayama (2013). "Solution structure of clostridial collagenase h and its calcium-dependent global conformation change." Biophys J **104**(7): 1538-1545.

Okano-Kosugi, H., O. Matsushita, S. Asada, A. B. Herr, K. Kitagawa and T. Koide (2009). "Development of a high-throughput screening system for the compounds that inhibit collagen-protein interactions." Anal Biochem **394**(1): 125-131.

Pflugrath, J. W. (1999). "The finer things in X-ray diffraction data collection." Acta Crystallogr D Biol Crystallogr **55**(Pt 10): 1718-1725.

Philominathan, S. T., O. Matsushita, R. Gensure and J. Sakon (2009). "Ca2+-induced linker transformation leads to a compact and rigid collagen-binding domain of Clostridium histolyticum collagenase." FEBS J **276**(13): 3589-3601.

Philominathan, S. T. L., T. Koide, K. Hamada, H. Yasui, S. Seifert, O. Matsushita and J. Sakon (2009). "Unidirectional Binding of Clostridial Collagenase to Triple Helical Substrates." Journal of Biological Chemistry **284**(16): 10868-10876.

Philominathan, S. T. L., T. Koide, O. Matsushita and J. Sakon (2012). "Bacterial collagen-binding domain targets undertwisted regions of collagen." Protein Science **21**(10): 1554-1565.

Ponnapakkam, T., R. Katikaneni, E. Miller, A. Ponnapakkam, S. Hirofumi, S. Miyata, L. J. Suva, J. Sakon, O. Matsushita and R. C. Gensure (2011). "Monthly administration of a novel PTH-collagen binding domain fusion protein is anabolic in mice." Calcif Tissue Int **88**(6): 511-520.

Ponnapakkam, T., R. Katikaneni, T. Nichols, G. Tobin, J. Sakon, O. Matsushita and R. C. Gensure (2011). "Prevention of chemotherapy-induced osteoporosis by cyclophosphamide with a long-acting form of parathyroid hormone." J Endocrinol Invest **34**(11): e392-397.

Ponnapakkam, T., R. Katikaneni, H. Suda, S. Miyata, O. Matsushita, J. Sakon and R. C. Gensure (2012). "A single injection of the anabolic bone agent, parathyroid hormone-collagen binding domain (PTH-CBD), results in sustained increases in bone mineral density for up to 12 months in normal female mice." Calcif Tissue Int **91**(3): 196-203.

Saito, W., K. Uchida, O. Matsushita, G. Inoue, H. Sekiguchi, J. Aikawa, H. Fujimaki and M. Takaso (2015). "Acceleration of callus formation during fracture healing using basic fibroblast growth factor-kidney disease domain-collagen-binding domain fusion protein combined with allogenic demineralized bone powder." J Orthop Surg Res **10**: 59.

Sides, C. R., R. Liyanage, J. O. Lay, S. T. L. Philominathan, O. Matsushita and J. Sakon (2012). "Probing the 3-D Structure, Dynamics, and Stability of Bacterial Collagenase Collagen Binding Domain (apo- versus holo-) by Limited Proteolysis MALDI-TOF MS." Journal of the American Society for Mass Spectrometry **23**(3): 505-519.

Spiriti, J. and A. van der Vaart (2010). "Mechanism of the calcium-induced trans-cis isomerization of a non-prolyl peptide bond in Clostridium histolyticum collagenase." Biochemistry **49**(25): 5314-5320.

Stratford, R., Jr., C. Vu, J. Sakon, R. Katikaneni, R. Gensure and T. Ponnapakkam (2014). "Pharmacokinetics in rats of a long-acting human parathyroid hormone-collagen binding domain peptide construct." J Pharm Sci **103**(2): 768-775.

Toyoshima, T., O. Matsushita, J. Minami, N. Nishi, A. Okabe and T. Itano (2001). "Collagen-binding domain of a Clostridium histolyticum collagenase exhibits a broad substrate spectrum both in vitro and in vivo." Connect Tissue Res **42**(4): 281-290.

Uchida, K., O. Matsushita, N. Nishi, G. Inoue, K. Horikawa and M. Takaso (2015). "Enhancement of periosteal bone formation by basic fibroblast-derived growth factor containing polycystic kidney disease and collagen-binding domains from Clostridium histolyticum collagenase." J Tissue Eng Regen Med.

Watanabe-Nakayama, T., M. Itami, N. Kodera, T. Ando and H. Konno (2016). "High-speed atomic force microscopy reveals strongly polarized movement of clostridial collagenase along collagen fibrils." Sci Rep **6**: 28975.

Wilson, J. J., O. Matsushita, A. Okabe and J. Sakon (2003). "A bacterial collagen-binding domain with novel calcium-binding motif controls domain orientation." Embo Journal **22**(8): 1743-1752.

Example 2 - Acceleration of bone formation during fracture healing by basic fibroblast growth factor fused with tandem collagen-binding domains from *Clostridium histolyticum* class I collagenase

The increased retention of recombinant basic fibroblast growth factor (bFGF) at fracture sites accelerates bone formation during fracture healing. We previously constructed a fusion protein consisting of bFGF and a polycystic kidney disease domain (PKD; s2b) and a collagen-binding domain (CBD; s3) derived from the *Clostridium histolyticum* class II collagenase (ColH); and reported the combination of the fusion protein and a collagen-like peptide, poly(Pro–Hyp–Gly)$_{10}$ (SEQ ID NO: 59), induced mesenchymal cell proliferation and callus formation at fracture sites. It is known that *C. histolyticum* also produces class I collagenase (ColG), which possesses tandem CBDs (s3a and s3b) at the C-terminus. First, we examined binding affinity of the four collagen anchors derived from two clostridial collagenases to a collagenous peptide, H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 (SEQ ID NO: 60), by surface plasmon resonance, which revealed that the tandem CBDs (s3a-s3b) shows the highest affinity to a collagenous peptide. Then, we constructed fusion proteins consisting of bFGF and single CBD (bFGF-s3b) or tandem CBDs (bFGF-s3a-s3b) to compare their biological abilities to those of the previous fusion construct (bFGF-s2b-s3). A fracture-model study showed that bFGF-s3a-s3b exhibit the highest abilities to induce mesenchimal cell proliferation and bone formation. Taken together, collangen anchors with higher collagen-binding affinity exert bFGF to show higher biological activities. The poly(Pro–Hyp–Gly)$_{10}$/bFGF-(CBD)$_2$ composite therefore appears to have the potential to promote bone fracture healing in the clinical setting.

Basic fibroblast growth factor (bFGF) is a mitogenic protein with angiogenic properties and is involved in bone remodeling during early bone repair [1;2]. Recombinant human bFGF has demonstrated efficacy in animal models of osteoporosis for regenerating bone fractures and defects [3;4]. In two recent clinical trials, bFGF treatment accelerated bone union at osteotomy and tibial fracture sites [5;6]. Although the findings from these studies strongly indicate that bFGF promotes bone remodeling and regeneration, exogenously added bFGF is rapidly diffused from bone defect sites.

Clostridium histolyticum, a pathogenic bacterium of gas gangrene, secretes two classes of collagenase, (class I, ColG and class II, ColH). These enzymes commonly contain a catalytic domain (s1), polycystic kidney disease domain (PKD, s2; SEQ ID NO: 61), and collagen-binding domain (CBD, s3). However copy numbers of PKD and CBD in the C-terminal collagen-anchors are different in ColG and ColH, being s2-s3a-s3b and s2a-s2b-s3 respectively [7;8]. We previously demonstrated that fusion proteins consisting of bFGF and either CBD (bFGF-s3) or PKD-CBD (bFGF-s2b-s3) derived from ColH accelerated bone formation in rat femurs when loaded onto collagen sheets compared to native bFGF [9]. When combined with high-density collagen sheets, bFGF-s2b-s3 promoted greater bone formation than bFGF-s3[10]. The combination of bFGF-s2b-s3 with the collagen-like peptide poly(Pro-Hyp-Gly)$_{10}$ (SEQ ID NO: 59) also induced greater bone formation compared to bFGF alone in mice bone fracture models[11]. In a more recent study, a fusion protein consisting of galectin-9 and tandem CBDs (s3a and s3b) derived from ColG displayed higher collagen-binding activity than the corresponding protein fused with PKD and CBD (s2b and s3) derived from ColH [12]. Based on these findings, we speculated that a bFGF fusion protein containing tandem CBDs from ColG would increase the retention of bFGF at the fracture site through the enhancement of collagen-binding activity, leading to improved bone formation and fracture repair.

Here, we evaluated dissociation constants between various collangen-anchors and mini-collagen *in vitro*. Following the results, we constructed fusion proteins consisting of bFGF and either single (bFGF-s3b) or tandem CBD(s) (bFGF-s3a-s3b) derived from ColG, and examined the bone formation ability of these fusion proteins with previously constructed two fusion proteins consisting of bFGF and collagen-anchors derived from ColH.

Materials and Methods

A mini-collagen peptide and a collagen-like polypeptide

A mini-collagen peptide, H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 (SEQ ID NO: 60), was synthesized by a *N*-(9-fluorenyl) methoxycarbonyl (Fmoc)-based strategy on Rink-amide resins (Novabiochem, San Diego, CA). In each cycle, Fmoc-amino acids (5 equivalents; Novabiochem) were reacted in the presence of *N,N'*-diisopropylcarbodiimide (5 equivalents; Wako Pure Chemical, Osaka, Japan) and 1-hydroxybenzotriazole (5 equivalents; Wako Pure Chemical) in *N,N*-dimethylformamide for 90 min. Fmoc deprotection was performed by 20% (v/v) piperidine in DMF for 20 min. Peptide cleavage and deprotection steps were performed by treatment with a standard

trifluoroacetic acid (TFA) scavenger cocktail (TFA: *m*-cresol: thioanisole: water: ethanedithiol: 82.5: 5: 5: 5: 2.5, v/v) for 4 hours at room temperature. The peptides were purified by HPLC on a Cosmosil 5C18-AR-II column (20 × 250 mm, Nacalai Tesque, Kyoto, Japan) with CH_3CN in water, both containing 0.05% (v/v) TFA. Purity of the product was confirmed by RP-HPLC on a Cosmosil 5C18-AR-II (4.6 × 250 mm, Nacalai Tesque) with a linear gradient of CH_3CN in water, both containing 0.05% (v/v) TFA. Mass spectrometric analysis was performed with a Bruker Autoflex III MALDI-TOF MS (Bruker Daltonics, Leipzig, Germany). H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 (SEQ ID NO: 60): MS (MALDI-TOF) *m/z* calcd. for $C_{159}H_{232}N_{44}O_{52}$ ($[M+H]^+$): 3590.7 found 3590.6. A HPLC profile of H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 (SEQ ID NO: 60) is shown in **Fig. 11**. A collagen-like polypeptide, poly(Pro-Hyp-Gly)$_{10}$ (SEQ ID NO: 59) was obtained from PHG Co., Ltd. (Hyogo, Japan) [13].

Collagen-anchors derived from Clostridial collangenases, ColG and ColH

CBD (s3) and PKD-CBD (s2b-s3) derived from the *C. histolyticum* class II collagenase ColH were purified as described previously[14]. CBD (s3b) and CBD-CBD (s3a-s3b) derived from the *C. histolyticum* class I collagenase ColG were purified as described previously[17].

Quantitative analysis of collagen anchor binding to a collagenous peptide

Binding of collagen-anchors to the mini-collagen peptide was measured by surface plasmon resonance using a BIACORE apparatus (Biacore, Uppsala, Sweden) in the same manner as reported previously [15]. Briefly, the peptide was dissolved in 10 mM sodium acetate (pH 6.0) at a concentration of 0.1 mg/ml, and was covalently immobilized on a CM5 sensor chip (Biacore) using the standard amine coupling procedure recommended by the manufacturer. Resonance was measured in 10 mM sodium HEPES (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, and 0.005% Tween-20 at a flow rate of 20 µl/min at 25°C. After each binding step, the chip was regenerated with a 180-s pulse of 0.1 M HCl. Values for the apparent dissociation constant, K_D(app), were calculated from equilibrium binding data for eight protein concentrations (100 nM – 300 µM) by direct fitting to the following equation by the least squares method,

$$cRU = cRU_{max} \times [protein]/(K_D + [protein]) \quad \text{(Eq. 1)}$$

where cRU is the response at equilibrium corrected for bulk refractive index errors using a sham-coupled flow cell blocked with ethanolamine, [protein] is the analyte concentration, and K_D is the dissociation constant.

Collagen-binding bFGF

Four collagen-binding bFGF fusion proteins (CB-bFGFs) were used in this study (**Fig. 6**). Two fusion proteins, bFGF-s3 and bFGF-s2b-s3, consiting of human bFGF, and CBD or PKD-CBD derived from ColH were prepared as previously described [10]. In order to prepare bFGF-s3b (ColG), an expression plasmid, pCHG115 DNA encoding a fusion protein between GST and a C-terminal collagen-binding domain (s3b, ColG) was digested with *Bam*HI and *Eco*RI at the linker region, and ligated with a hbFGF-encoding DNA fragment pretreated with *Bgl*II and *Eco*RI. *Escherichia coli* DH5α was transformed with the ligation mixture.to confirm the nucleotide sequence of resultant plasmid (pCHG115-hbFGF) by Sanger sequencing. *E. coli* BL21 CodonPlus RIL (Agilent Technologies, Santa Clara, CA) was transformed with the plasmid to express the GST-bFGF-s3b fusion protein. The fusion protein was purified, and the GST moiety was cleaved off as described previously [10]. Another fusion protein consisting of bFGF and tandem CBDs derived from ColG (bFGF-s3a-s3b) was produced in the same manner using another expression plasmid, pCHG112.

Proliferation Assay

Periosteum was collected from the distal femurs of 10-week-old Wistar rats as previously described [9], and was then digested with 0.2% type I collagenase (Wako Pure Chemical Industries, Ltd., Tokyo, Japan) for 2 h at 37°C. The digested sample was passed through a 40-μm filter to obtain single-cell suspensions of nucleated cells, which were then seeded at 1×10^4 cells/cm^2 in 6-well culture plates containing α-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The plates were incubated at 37°C for 7 days in a 5% CO_2 atmosphere and the passage 0 (P0) cells were then detached from the plate surface by treatment with 0.25% trypsin and 1 mM EDTA for 5 min. The cells were collected and seeded at 1.25×10^3 cells/well in 96-well plates. α-MEM containing either bFGF, bFGF-s3 (ColH), bFGF-s2b-s3 (ColH), bFGF-s3b (ColG), or bFGF-s3a-s3b (ColG) was then added to the culture supernatant at concentrations of 0 (control), 0.1, 1, and 10 pM. Cell proliferation was evaluated after 2 days of treatment using a water-soluble tetrazolium (WST) assay kit (Cell Count Reagent SF; Nacalai Tesque, Kyoto, Japan) following the manufacturer's protocol and a previously described procedure [9].

Fracture model

A femur fracture model was generated using 9-week-old C57BL/6J mice. The mice were fed standard rodent chow (CRF-1; Oriental Yeast, Tokyo, Japan), and were housed under

controlled environmental conditions (temperature, 23±2°C; humidity, 55±10%; lighting, 12-h light/dark cycle) in a semi-barrier system at Nippon Charles River Laboratories (Kanagawa, Japan). Femur fractures were generated by first making a 4-mm medial parapatellar incision in the left knee under sterile conditions. After drilling a 0.5-mm hole in the intracondylar notch, a 0.2-mm tungsten guide wire was inserted retrograde into the intramedullary canal, and a section of the femur was removed using a wire saw (0.22 mm in diameter) with a lateral approach. To stabilize the fracture, a stainless steel screw (0.5 mm in diameter) was inserted into the intramedullary canal after removing the guide wire. After generating the fracture, PBS (control), poly(Pro-Hyp-Gly)$_{10}$ (SEQ ID NO: 59) gel containing 0.058 nmoles of bFGF, bFGF-s3, bFGF-s2b-S3, bFGF-s3b, or bFGF-s3a-s3b was immediately injected into the fracture site (n=8, each treatment). The dose of bFGF was determined based on the results of a previous study [11]. All animal procedures followed the guidelines of the Animal Ethics Committee of Kitasato University.

Quantification of new bone volume and bone mineral content

To quantify the new bone volume and bone mineral content in the control and treated region, femurs were removed from sacrificed mice four weeks after the fracture treatment, and stored in 4% paraformaldehyde for 48 h at 4°C. The femurs were transferred to PBS and imaged using an inspeXio SMX-90CT microfocus X-ray CT system (Shimadzu, Tokyo, Japan) with the following settings: acceleration voltage, 90 kV; current, 110 mA; voxel size, 20 lm/pixel; and matrix size, 1024×1024. New bone volume and bone mineral content were quantified in the micro-CT images of whole femurs using Tri-3D-Bon three-dimensional (3D) image analysis software (Ratoc System Engineering, Tokyo, Japan) at 10-mm regions of interest (500 slices) of the midfemur, as previously described [14;16]. Bone mineral content was estimated by comparing the measured densities of each femur sample in the micro-CT images to those in a hydroxyapatite (HA) calibration curve, which was constructed by plotting the data generated from phantom images prepared with 200, 300, 400, 500, 600, 700, and 800 mg HA/cm^3. New bone was defined as having a threshold density value of ≥300 mg/cm^3.

Histological evaluation

To examine the effects of poly(Pro-Hyp-Gly)$_{10}$/bFGF-s3a-s3b (ColG) treatment on bone formation, femurs were excised from control and treated animals 14 days after the creation of fractures. The femur samples were demineralized in a 20% EDTA solution for 28 days, and the remaining tissue was then embedded in paraffin. The femurs were sectioned (3 μm) through the

long axis in the coronal plane, and the obtained sections were stained with hematoxylin and eosin (HE) for morphological analysis.

Statistical analysis

Differences among the PBS (control), bFGF, bFGF-s3, bFGF-s2b-s3, bFGF-s3b and bFGF-s3a-s3b groups were examined using one-way ANOVA with Fisher's least significant difference (LSD) test. The level of significance was set at $p < 0.05$. All statistical analyses were conducted using SPSS software (Version 19.0; SPSS Inc., Chicago, IL).

Results

Binding affinity of collagen-anchors

Dissociation constants of four collagen-anchors to the mini-collagen peptide, H-Gly-Pro-Arg-Gly-$(Pro\text{-}Hyp\text{-}Gly)_{12}$-$NH_2$ (SEQ ID NO: 60), were measured by surface plasmon resonance (Table 2). Although s2b-s3 (PKD-CBD, ColH) showed a lower Kd value compared to the s3 alone (CBD, ColH), the values of s3b (CBD, ColG) and s3a-s3b (CBD-CBD, ColG) were spprox. 10-fold lower than those of the ColH anchors. It can be expected that the ColG-derived anchors bind more tightly to collagenous peptides than the ColH-derived anchors.

Table 2: Binding affinity of various collagen anchors

Collagen anchor	K_D (x 10^{-5} M)	Ratio
ColGS3aS3b	4.46 ± 0.45	0.100
ColGS3b	4.54 ± 0.15	0.102
ColHS2bS3	44.5 ± 0.55	1.00
ColHS3	75.2 ± 0.41	1.69

Regarding Table 2, anchor proteins were dissolved in HBS-Ca buffer at concentrations ranging from 1 x 10^{-7} M to 3 x 10^{-4} M. Binding to the collagenous peptide, H-Gly-Pro-Arg-Gly-$(Pro\text{-}Hyp\text{-}Gly)_{12}$-$NH_2$ (SEQ ID NO: 60), was measured by surface plasmon resonance. Data were directly fit to an equation described in Materials and Methods section by least squares method to calculate values for the apparent dissociation constant (K_D) and uncertainty.

***In-vitro* biological activities of fusion proteins**

The biological activities of the four CB-bFGF's were evaluated by measuring the proliferation of rat periosteal mesenchymal cells *in vitro* (**Fig. 7**). Two days after treatment with 0.1 pM bFGF-s3a-s3b (ColG), the number of cultured periosteal mesenchymal cells had significantly

increased compared to the control (α-MEM) treatment group. In contrast, no significant increase was detected in the bFGF, bFGF-s3 (ColH), bFGF–s2b-s3 (ColH), or bFGF–s3b (ColG)-treated cells. However, when the concentration of the bFGF or CB-bFGF's was increased to 1 or 10 pM, the cell number had increased significantly in all forms of the growth factors compared to α-MEM-treated cells.

***In-vivo* callus formation induced by CB-bFGF/poly(Pro-Hyp-Gly)$_{10}$ composite material**

Poly(Pro-Hyp-Gly)$_{10}$ gel was mixed with either PBS, bFGF (controls) or one the four prepared CB-bFGF's, and applied at fractured site in mice femurs. After 4 weeks of recovery, callus formation at the fracture sites was evaluated by micro-CT image analysis (**Figs. 8A-8F**). Compared with PBS-injected fracture sites, callus volume and bone mineral content was significantly higher at sites treated with poly(Pro-Hyp-Gly)$_{10}$ in combination with bFGF or the CB-bFGF fusion proteins (**Fig. 9,** $P<0.05$). Notably, however, bFGF-s2b-s3 (ColH), bFGF-s3b (ColG) and bFGF-s3a-s3b (ColG) resulted in higher callus volume and bone mineral content compared to bFGF (**Fig. 9**, $P<0.05$). Among the three fusion proteins, bFGF- s3a-s3b (ColG) exhibited the highest efficacy for bone repair, as the callus volume and bone mineral content were significantly higher than those of all the other groups (**Fig. 9**, $P<0.05$).

Histomorphometric findings

To investigate the mechanism by which poly(Pro-Hyp-Gly)$_{10}$/bFGF-s3a-s3b (ColG) accelerated new bone formation, histological evaluation of the treated fracture sites was performed after 2 weeks when soft callus formation was first detected in the mouse femur fracture model. Compared to the control group, large calluses were observed at the fracture sites treated with either bFGF-s2b-s3 (ColH), bFGF-s3b (ColG) or bFGF-s3a-s3b (**Figs. 10A-10F**). Notably, the calluses formed by the bFGF-s3a-s3b-treatment were clearly larger than those observed in the other treatment groups (**Figs. 10A-10F**). This finding indicated that poly(Pro-Hyp-Gly)$_{10}$/bFGF-s3a-s3b (ColG) accelerated periosteal cell proliferation in the early stages of fracture healing.

Discussion

Clostridial collagenases possess collagen-anchors at their C-termini. The anchors bind to collagen fibrils and collagenous peptides with triple-helical conformation, but not to denatured collagen (gelatin) [17]. The anchors are made of two types of domains, PKD and CBD, where former enhances the binding of the latter. The enzymes possess collagen anchors made of various copy numbers of PKD(s) and CBD(s). Previously we have shown the callus-inducing potential of

composite materials made of collagen-carrier (high density collagen sheet/powder or demineralized bone matrix) and bFGF fused with an anchor made of a single copy each of PKD and CBD (bFGF-PKD-CBD) derived from *Clostridium histolyticum* class II collagenase, ColH [14;16]. Recently we tried to use collagenous-peptide gel instead of collagen-carrier since the former is more easily applicable by injection. In aged mice fracture models, a novel composite material made of poly(Pro-Hyp-Gly)$_{10}$ and bFGF-PKD-CBD induced greater bone formation than one made of poly(Pro-Hyp-Gly)$_{10}$ and bFGF [11]. Hence, we could speculate that efficacies of this composite material can be optimized by switching anchors with various binding affinity to the collagenous peptide carrier.

In order to estimate the binding affinity of various anchors to the peptide carrier, poly(Pro-Hyp-Gly)$_{10}$, we synthesized a longer collagenous peptide, H-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_{12}$-NH_2 as a ligand for surface plasmon resonance assay, where the first triplet, Pro-Arg-Gly, was introduced to keep its water-solubility and triple-helical conformation. Since the peptide was immobilized to the sensor chip at the N-terminus, it could be safely expected that the alteration near the N-terminus does not affect the binding significantly. A single CBD (ColG s3b) showed a K_D value ($4.54 \pm 0.15 \times 10^{-5}$ M) to this peptide similar to that ($5.72 \pm 0.473 \times 10^{-5}$ M) reported previously to a shorter collagenous peptide, (G(POG)$_8$) [15], indicating that the quantitative analysis performed here is reproducible. Collagen anchors (s3a-s3b and s3b) derived from class I enzyme (ColG) showed significantly higher affinity to this peptide than ones (s2b-s3 and s3) derived from class II enzyme (ColH), suggesting that the formers are more appropriate anchors toward the peptide carrier than the latters. Presence of an additional CBD (s3a) did not significantly enhance binding of ColG s3b to the synthetic peptide, suggesting that the peptide used for this assay is still too short to allow simultaneous binding of the two CBDs. An alternative possibility is that the binding between S3a and the collagen peptide is sufficiently weak to be reflected in the apparent K_D value. Binding assay and/or small-angle X-ray diffraction study using a longer collagenous peptide would be necessary to solve this question.

Provided that the efficacy correlates with the binding affinity, we could expect more callus formation using bFGF fused with the ColG anchors when combined with the collangenous peptide. Hence, we prepared four CB-bFGF's with one of the anchors described above. In order to confirm that bFGF moiety in each CB-bFGF construct is intact, cell proliferation assay was performed *in vitro*. Four CB-bFGF and bFGF promoted cell proliferation in dose dependent manner, which indicates that bFGF moiety is active regardless to the various anchor moiety. Specific activity of the

bFGF-CBD-CBD (s3a-s3b) seems to be slightly higher than the other CB-bFGF's at the lower concentrations (0.1 - 1.0 pM), which might be due to the binding of this CB-bFGF to collagen produced by the mesenchymal cells.

Then, osteogenic potential of the composite materials made of poly(Pro-Hyp-Gly)$_{10}$ and either of the four CB-bFGF's was compared using a mouse femur fracture model. When combined with the gel-like carrier made of poly(Pro-Hyp-Gly)$_{10}$, bFGF-CBD (ColG) and bFGF-CBD-CBD (ColG) induced large soft callus formation at 2 weeks and significantly more callus during fracture healing compared to bFGF, bFGF-PKD-CBD (ColH), or bFGF-CBD (ColH). Among the examined, bFGF-CBD-CBD (ColG) resulted in the highest callus volume and bone mineral content.

The binding affinity of collagen anchors may affect the osteogenic activity of the corresponding fusion proteins, as the rapid diffusion of target molecules from defect sites would limit their osteogenic potential [10]. We previously demonstrated that bFGF–PKD–CBD (ColH) has higher binding affinity to collagen-carrier and induces greater bone formation compared to bFGF-CBD (ColH) [10]. Among the collagen anchors used in the present study, ColG CBD(s) showed approximately 10-times higher affinity than ColH anchors to a collagenous peptide. This finding is consistent with the *in-vivo* results, where the treatment with bFGF fused with ColG anchors accelerated osteogenesis more efficiently compared to bFGF fused with ColH anchors when combined with a collagenous peptide carrier.

Taken together, osteogenic potentials seem to correlate with the binding affinity of collagen-anchors at least in the range we examined. It also seems likely that the tandem CBDs (ColG) increase the retention time of bFGF most at fracture sites when introduced together with collagen-like polypeptide poly(Pro-Hyp-Gly)$_{10}$ and thereby accelerate bone formation. These results suggest that bFGF-CBD-CBD (ColG) combined with poly(Pro-Hyp-Gly)$_{10}$ is a promising therapeutic material for stimulating bone repair in the clinical setting.

Previous studies have demonstrated that fusion proteins between growth factors and CBDs have superior biological activity compared to native growth factor [18;19]. For example, Han et al. [18] reported that bone morphogenetic protein containing a CBD derived from von Willebrand factor increased the *in-vitro* alkaline phosphatase activity of the mouse osteoblastic cell line MC3T3-E1. As bFGF stimulates periosteal mesenchymal cells [9;10;16], here, we examined the proliferation ability of bFGF and several CB-bFGF fusion proteins using rat periosteal

mesenchymal cells and demonstrated that bFGF-CBD-CBD stimulated the highest cell proliferation activity of the examined proteins at a concentration as low as 1 pM. Taken together, the findings from this study indicate that bFGF-CBD-CBD possesss both high collagen-binding affinity and biological activity, including the ability to stimulate callus formation during fracture healing.

A recombinant collagen-binding bFGF fusion protein containing tandem CBDs from *C. histolyticum* class I collagenase ColG strongly induced bone formation when injected in combination with the collagen-like peptide poly(Pro-Hyp-Gly)$_{10}$ into mouse femur fracture sites. The high osteogenic properties of bFGF-CBD-CBD/poly(Pro-Hyp-Gly)$_{10}$ suggest that this composite material has the potential to promote fracture healing in the clinical setting.

References for Example 2

[1] Khan SN, Bostrom MP, Lane JM: Bone growth factors. Orthop Clin North Am 2000;31:375-388.

[2] Ueno M, Urabe K, Naruse K, Uchida K, Minehara H, Yamamoto T, Steck R, Gregory L, Wullschleger ME, Schuetz MA, Itoman M: Influence of internal fixator stiffness on murine fracture healing: two types of fracture healing lead to two distinct cellular events and FGF-2 expressions. Exp Anim 2011;60:79-87.

[3] Kato T, Kawaguchi H, Hanada K, Aoyama I, Hiyama Y, Nakamura T, Kuzutani K, Tamura M, Kurokawa T, Nakamura K: Single local injection of recombinant fibroblast growth factor-2 stimulates healing of segmental bone defects in rabbits. J Orthop Res 1998;16:654-659.

[4] Kawaguchi H, Kurokawa T, Hanada K, Hiyama Y, Tamura M, Ogata E, Matsumoto T: Stimulation of fracture repair by recombinant human basic fibroblast growth factor in normal and streptozotocin-diabetic rats. Endocrinology 1994;135:774-781.

[5] Kawaguchi H, Jingushi S, Izumi T, Fukunaga M, Matsushita T, Nakamura T, Mizuno K, Nakamura T, Nakamura K: Local application of recombinant human fibroblast growth factor-2 on bone repair: a dose-escalation prospective trial on patients with osteotomy. J Orthop Res 2007;25:480-487.

[6] Kawaguchi H, Oka H, Jingushi S, Izumi T, Fukunaga M, Sato K, Matsushita T, Nakamura K: A local application of recombinant human fibroblast growth factor 2 for tibial shaft fractures: A randomized, placebo-controlled trial. J Bone Miner Res 2010;25:2735-2743.

[7] Bauer R, Wilson JJ, Philominathan ST, Davis D, Matsushita O, Sakon J: Structural comparison of ColH and ColG collagen-binding domains from Clostridium histolyticum. J Bacteriol 2013;195:318-327.

[8] Bauer R, Janowska K, Taylor K, Jordan B, Gann S, Janowski T, Latimer EC, Matsushita O, Sakon J: Structures of three polycystic kidney disease-like domains from Clostridium histolyticum collagenases ColG and ColH. Acta Crystallogr D Biol Crystallogr 2015;71:565-577.

[9] Uchida K, Matsushita O, Naruse K, Mima T, Nishi N, Hattori S, Ogura T, Inoue G, Tanaka K, Takaso M: Acceleration of periosteal bone formation by human basic fibroblast growth factor containing a collagen-binding domain from Clostridium histolyticum collagenase. J Biomed Mater Res A 2014;102:1737-1743.

[10] Uchida K, Matsushita O, Nishi N, Inoue G, Horikawa K, Takaso M: Enhancement of periosteal bone formation by basic fibroblast-derived growth factor containing polycystic kidney disease and collagen-binding domains from Clostridium histolyticum collagenase. J Tissue Eng Regen Med 3-18-2015.

[11] Sekiguchi H, Uchida K, Inoue G, Matsushita O, Saito W, Aikawa J, Tanaka K, Fujimaki H, Miyagi M, Takaso M: Acceleration of bone formation during fracture healing by poly(pro-hyp-gly)10 and basic fibroblast growth factor containing polycystic kidney disease and collagen-binding domains from Clostridium histolyticum collagenase. J Biomed Mater Res A 2016;104:1372-1378.

[12] Fukata Y, Itoh A, Nonaka Y, Ogawa T, Nakamura T, Matsushita O, Nishi N: Direct cytocidal effect of galectin-9 localized on collagen matrices on human immune cell lines. Biochim Biophys Acta 2014;1840:1892-1901.

[13] Kishimoto T, Morihara Y, Osanai M, Ogata S, Kamitakahara M, Ohtsuki C, Tanihara M: Synthesis of poly(Pro-Hyp-Gly)(n) by direct poly-condensation of (Pro-Hyp-Gly)(n), where n=1, 5, and 10, and stability of the triple-helical structure. Biopolymers 10-15-2005;79:163-172.

[14] Saito W, Uchida K, Matsushita O, Inoue G, Sekiguchi H, Aikawa J, Fujimaki H, Takaso M: Acceleration of callus formation during fracture healing using basic fibroblast growth factor-kidney disease domain-collagen-binding domain fusion protein combined with allogenic demineralized bone powder. J Orthop Surg Res 2015;10:59.

[15] Wilson JJ, Matsushita O, Okabe A, Sakon J: A bacterial collagen-binding domain with novel calcium-binding motif controls domain orientation. EMBO J 4-15-2003;22:1743-1752.

[16] Saito W, Uchida K, Ueno M, Matsushita O, Inoue G, Nishi N, Ogura T, Hattori S, Fujimaki H, Tanaka K, Takaso M: Acceleration of bone formation during fracture healing by injectable collagen powder and human basic fibroblast growth factor containing a collagen-binding domain from Clostridium histolyticum collagenase. J Biomed Mater Res A 2014;102:3049-3055.

[17] Matsushita O, Koide T, Kobayashi R, Nagata K, Okabe A: Substrate recognition by the collagen-binding domain of Clostridium histolyticum class I collagenase. J Biol Chem 3-23-2001;276:8761-8770.

[18] Han X, Zhang W, Gu J, Zhao H, Ni L, Han J, Zhou Y, Gu Y, Zhu X, Sun J, Hou X, Yang H, Dai J, Shi Q: Accelerated postero-lateral spinal fusion by collagen scaffolds modified with engineered collagen-binding human bone morphogenetic protein-2 in rats. PLoS One 2014;9:e98480.

[19] Shiozaki Y, Kitajima T, Mazaki T, Yoshida A, Tanaka M, Umezawa A, Nakamura M, Yoshida Y, Ito Y, Ozaki T, Matsukawa A: Enhanced in vivo osteogenesis by nanocarrier-fused bone morphogenetic protein-4. Int J Nanomedicine 2013;8:1349-1360.

CLAIMS

We claim:

1. A collagen-binding therapeutic agent comprising two collagen-binding domains linked by a domain linker and a therapeutic agent linked to at least one of the collagen-binding domains by a therapeutic agent linker, wherein the collagen-binding agent lacks collagenase activity, and wherein each of the two collagen-binding domains are selected from the group consisting of any one of the polypeptides of SEQ ID NOs: 1-39, a polypeptide having at least 90% sequence identity to any one of SEQ ID NOs: 1-39, and a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 1-39,

2. The collagen-binding agent of claim 1, wherein the therapeutic agent is selected from the group consisting of FGF, parathyroid hormone (PTH), PTH/PTHrP receptor agonist, PTH/PTHrP receptor antagonist, bone morphogenic protein (BMP), G-CSF, BMP-2, BMP-3, anti-sclerostin antibody, growth hormone, IGF-1, VEGF, TGF-β, KGF, TGF-α, TGF-β1, TGF-β receptor, CT, GH, GM-CSF, EGF, PDGF, celiprolol, activins and connective tissue growth factors.

3. The collagen-binding agent of claim 1 or 2, wherein one of the collagen-binding domains is selected from the group consisting of the polypeptides of any one of SEQ ID NOs: 15-30, a polypeptide having at least 90% sequence identity to any one of SEQ ID NOs: 15-30, and a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 15-30 and, wherein the other collagen-binding domain is selected from the group consisting of the polypeptides any one of SEQ ID NOs: 1-14 and 31-39, a polypeptide having at least 90% sequence identity to any one of SEQ ID NOs: 1-14 and 31-39, or a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 1-14 and 31-39.

4. The collagen-binding agent of any one of the preceding claims, wherein the two collagen-binding domains are selected from the group consisting of any one of SEQ ID NOs: 15-30, a polypeptide having at least 90% sequence identity to any one of SEQ ID NOs: 15-30, and a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 15-30.

5. The collagen-binding agent of any one of the preceding claims, wherein the two collagen-binding domains are selected from the group consisting of the polypeptides of any one of SEQ ID NOs: 1-14 and 31-39, a polypeptide having at least 90% sequence identity to any

one of SEQ ID NOs: 1-14 and 31-39, or a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 1-14 and 31-39.

6. The collagen-binding agent of any one of the preceding claims, wherein the collagen-binding agent comprises a polypeptide selected from the group consisting of any one of the polypeptides of SEQ ID NOs: 40-47, a polypeptide having at least 90% sequence identity to any one of SEQ ID NOs: 40-47, and a fragment of at least 8 consecutive amino acids of any one of SEQ ID NOs: 40-47.

7. The collagen-binding agent of any one of the preceding claims, wherein the domain linker comprises a polypeptide.

8. The collagen-binding agent of any one of the preceding claims, wherein the domain linker comprises a polypeptide selected from the group consisting of any one of the polypeptides of SEQ ID NOs: 48-55 or a polypeptide having at least 80% sequence identity to any one of the polypeptides of SEQ ID NOs: 48-55.

9. The collagen-binding agent of any one of the preceding claims, wherein the therapeutic agent is selected from the group consisting of a polypeptide, a hormone, a growth factor, a cytokine, a small molecule, a polynucleotide, a carbohydrate, and a lipid.

10. The collagen-binding agent of any one of the preceding claims, wherein the therapeutic agent comprises a polypeptide.

11. The collagen-binding agent of any one of the preceding claims, wherein the therapeutic agent is selected from the group consisting of FGF, parathyroid hormone (PTH), PTH/PTHrP receptor agonist, and PTH/PTHrP receptor antagonist.

12. The collagen-binding agent of any one of the preceding claims, wherein the therapeutic agent linker comprises a polypeptide.

13. The collagen-binding agent of any one of claims 10-12, wherein the C terminus of the therapeutic agent is linked to the N-terminus of the collagen-binding agent by the therapeutic agent linker.

14. A pharmaceutical composition comprising any one of the collagen-binding agents of claims 1-13 and a pharmaceutical carrier.

15. A method of treating a condition comprising administering any one of the collagen-binding agents of claims 1-13 or the pharmaceutical composition of claim 14 to a subject in an amount effective to treat the condition.

16. The method of claim 15, wherein the subject is a mammal.

17. The method of claim 16, wherein the mammal is a human.

18. The method of any one of claims 15-17, wherein the condition is selected from the group consisting of hyperparathyroidism, a hair condition (either excessive hair growth or hair loss), a collagenopathy, a wound, a bone condition, spinal fusion, ischemic heart diseases, peripheral nerve disorder and spinal cord injury.

19. The method of any one of claims 15-17, wherein the condition comprises a collagenopathy selected from the group consisting of osteogenesis imperfecta, Stickler's syndrome, Ehlers-Danlos syndrome, Alport's syndrome, Caffey's disease, and localized collagen or cartilage damage.

20. Use of the composition of any one of claims 1-14 in the manufacture of a medicament for treating a condition.

Fig. 1A

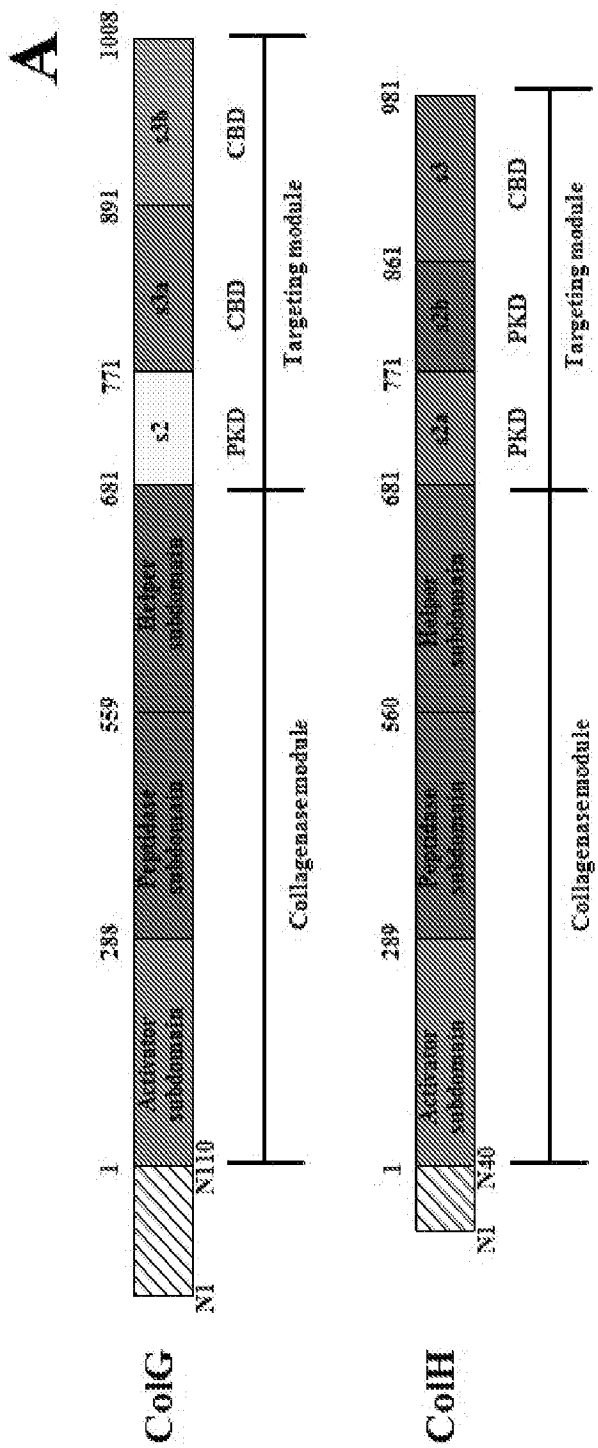

A
ColG
1
288
559
681
771
891
1088
N1
N110
Peptidase subdomain
Helical subdomain
S2
S3a
S3b
PKD
CBD
CBD
Collagenase module
Targeting module
ColH
1
289
560
681
771
861
981
N1
N40
Peptidase subdomain
Helical subdomain
S4
S3
PKD
PKD
CBD
Collagenase module
Targeting module

Fig. 1B

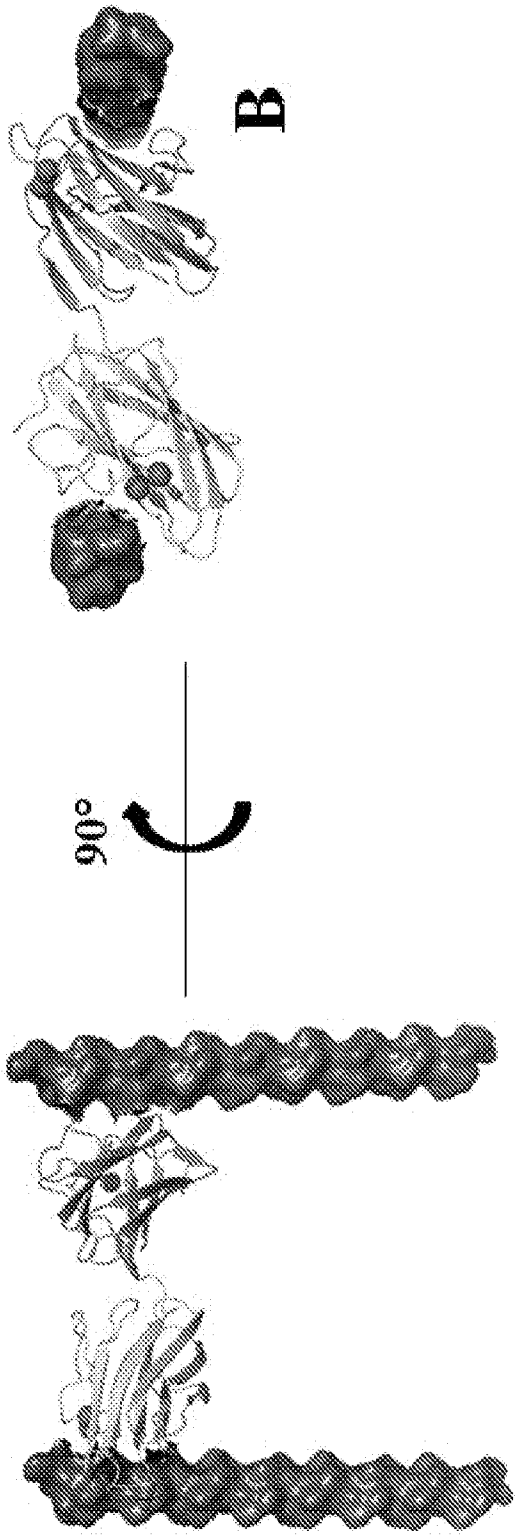

B
90°

Fig. 1C

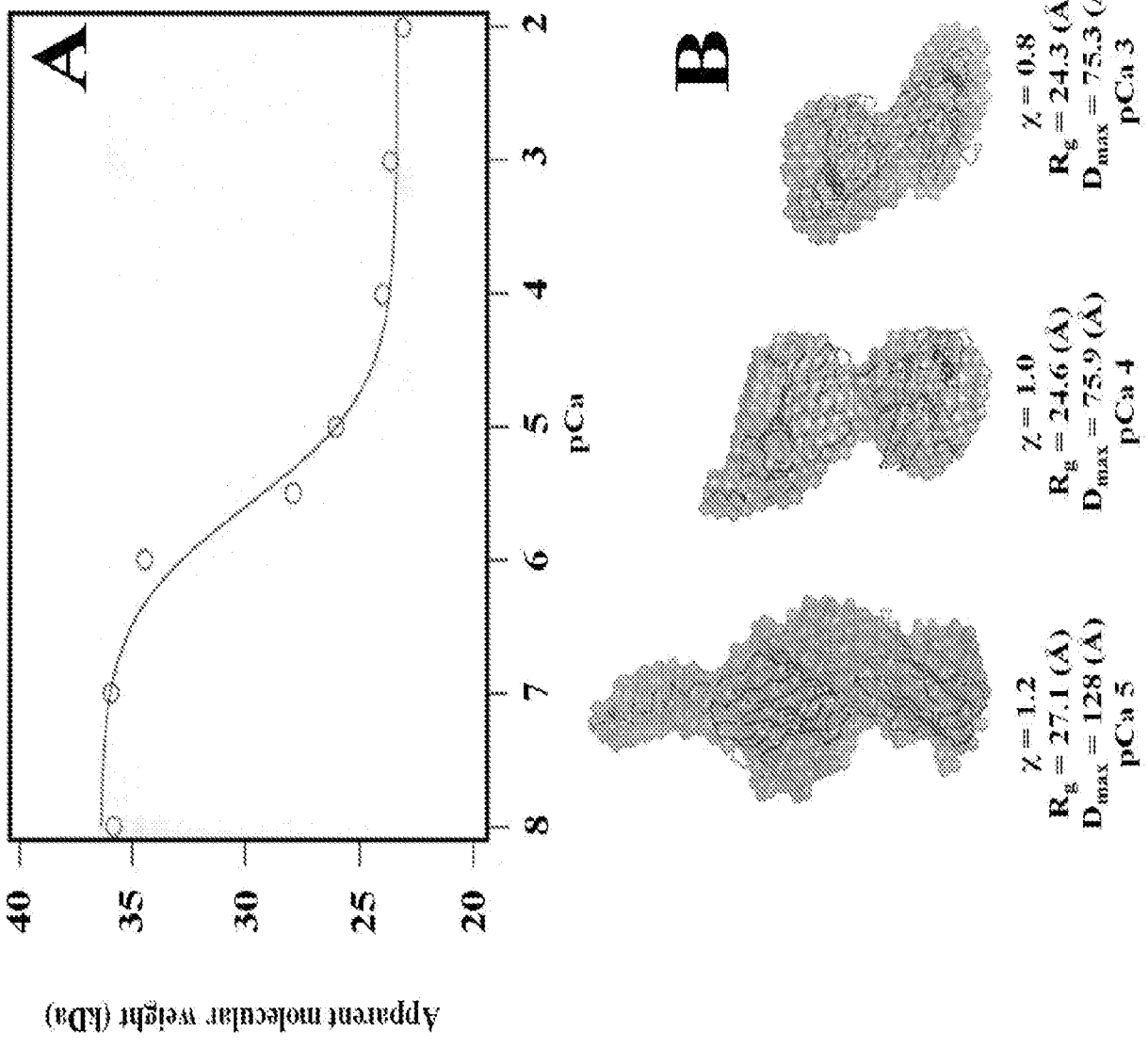

A
Apparent molecular weight (kDa)
40
35
30
25
20
8
7
6
5
4
3
2
pCa
B
χ = 1.2
R_g = 27.1 (Å)
D_max = 128 (Å)
pCa 5
χ = 1.0
R_g = 24.6 (Å)
D_max = 75.9 (Å)
pCa 4
χ = 0.8
R_g = 24.3 (Å)
D_max = 75.3 (Å)
pCa 3

Fig. 2A

Fig. 2B

Fig. 3

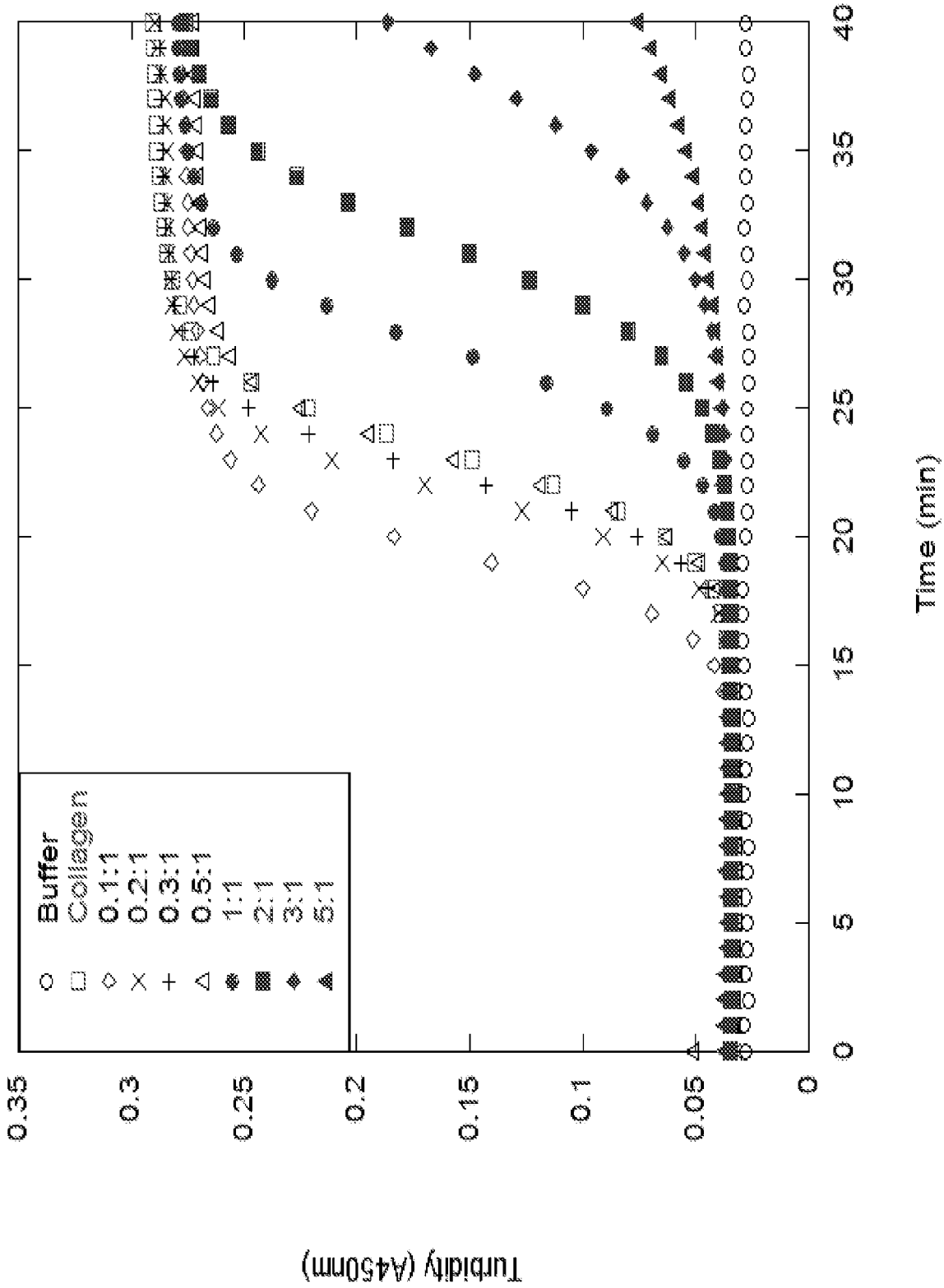

Collagen fibril formation in the presence of s3a-s3b
Buffer
Collagen
0.1:1
0.2:1
0.3:1
0.5:1
1:1
2:1
3:1
5:1
Turbidity (A450nm)
Time (min)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0
5
10
15
20
25
30
35
40

Fig. 4C

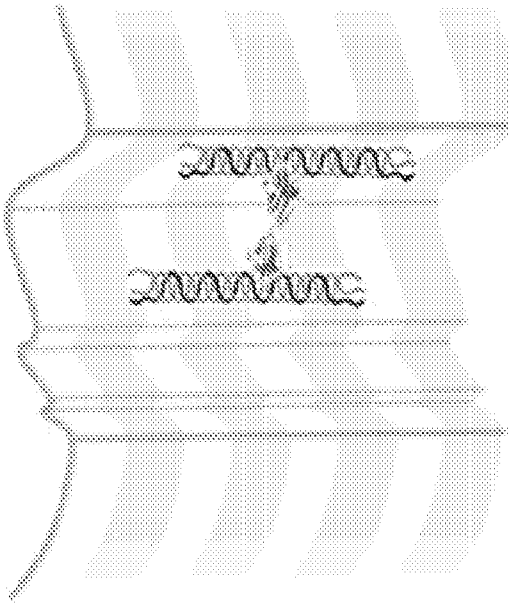


Fig. 4B

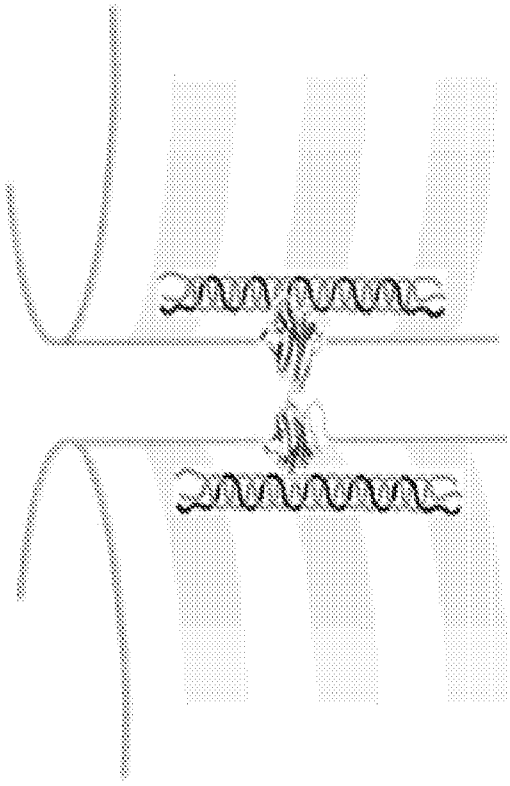


Fig. 4A

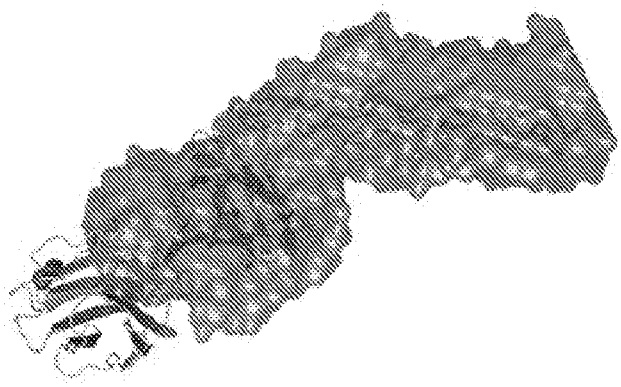


Fig. 5

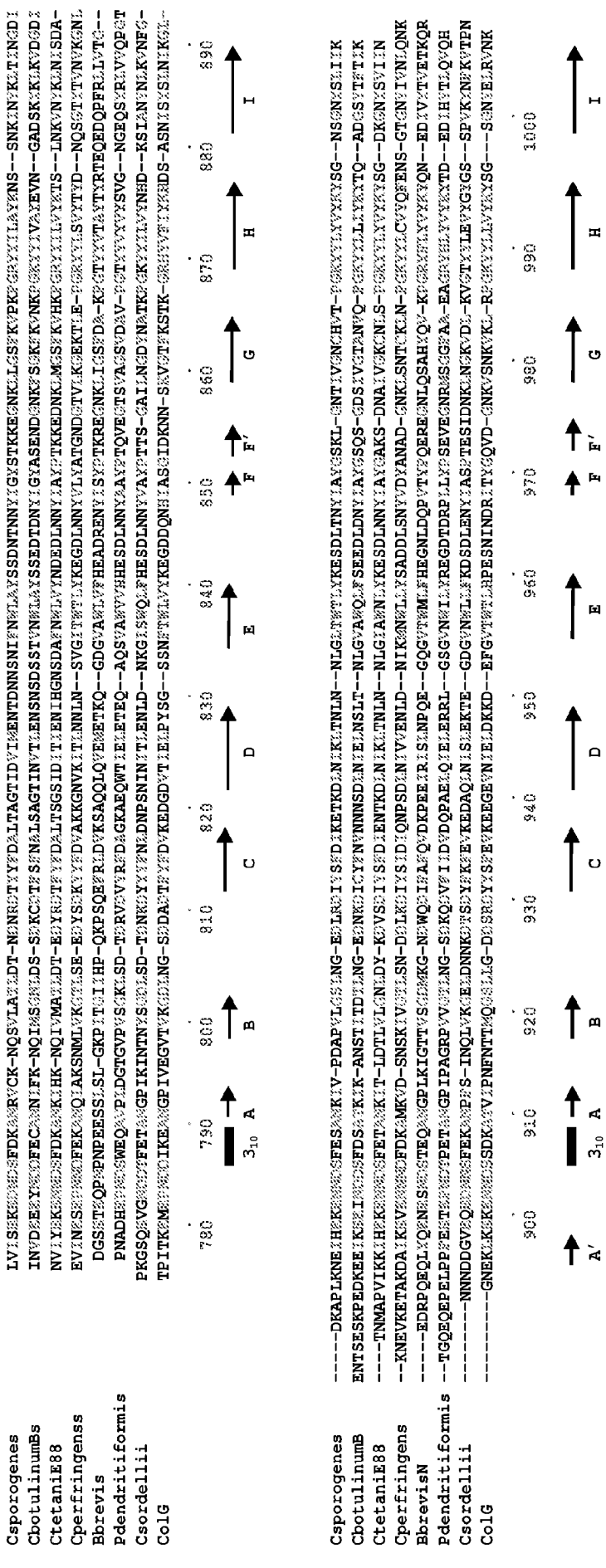

Csporogenes
CbotulinumBs
CtetanieE88
Cperfringenss
Bbrevis
Pdendritiformis
Csordellii
ColG
780
790
800
810
820
830
840
850
860
870
880
890
A
B
C
D
E
F
F′
G
H
I
3_{10}
900
910
920
930
940
950
960
970
980
990
1000
A′

Fig. 6

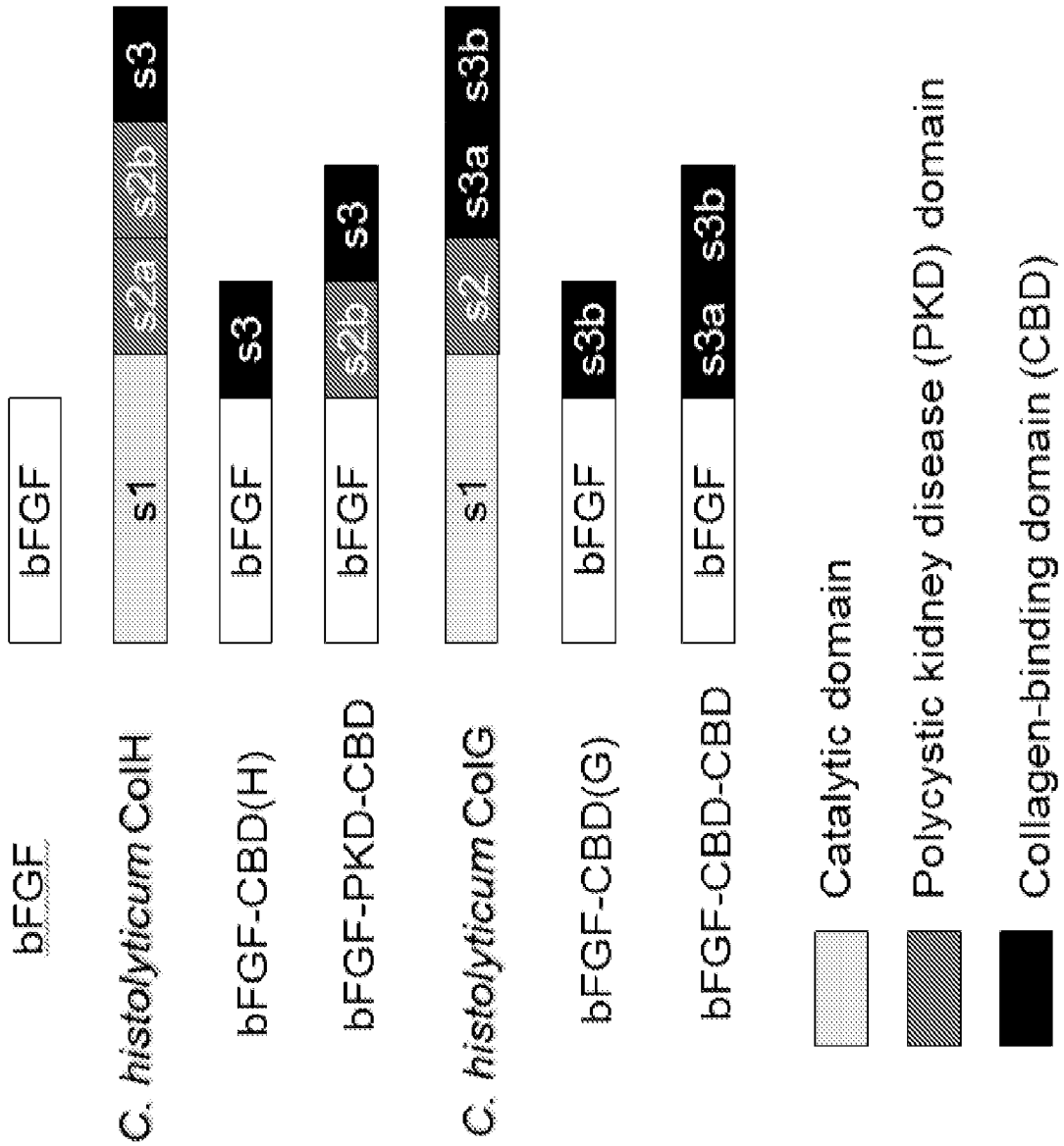
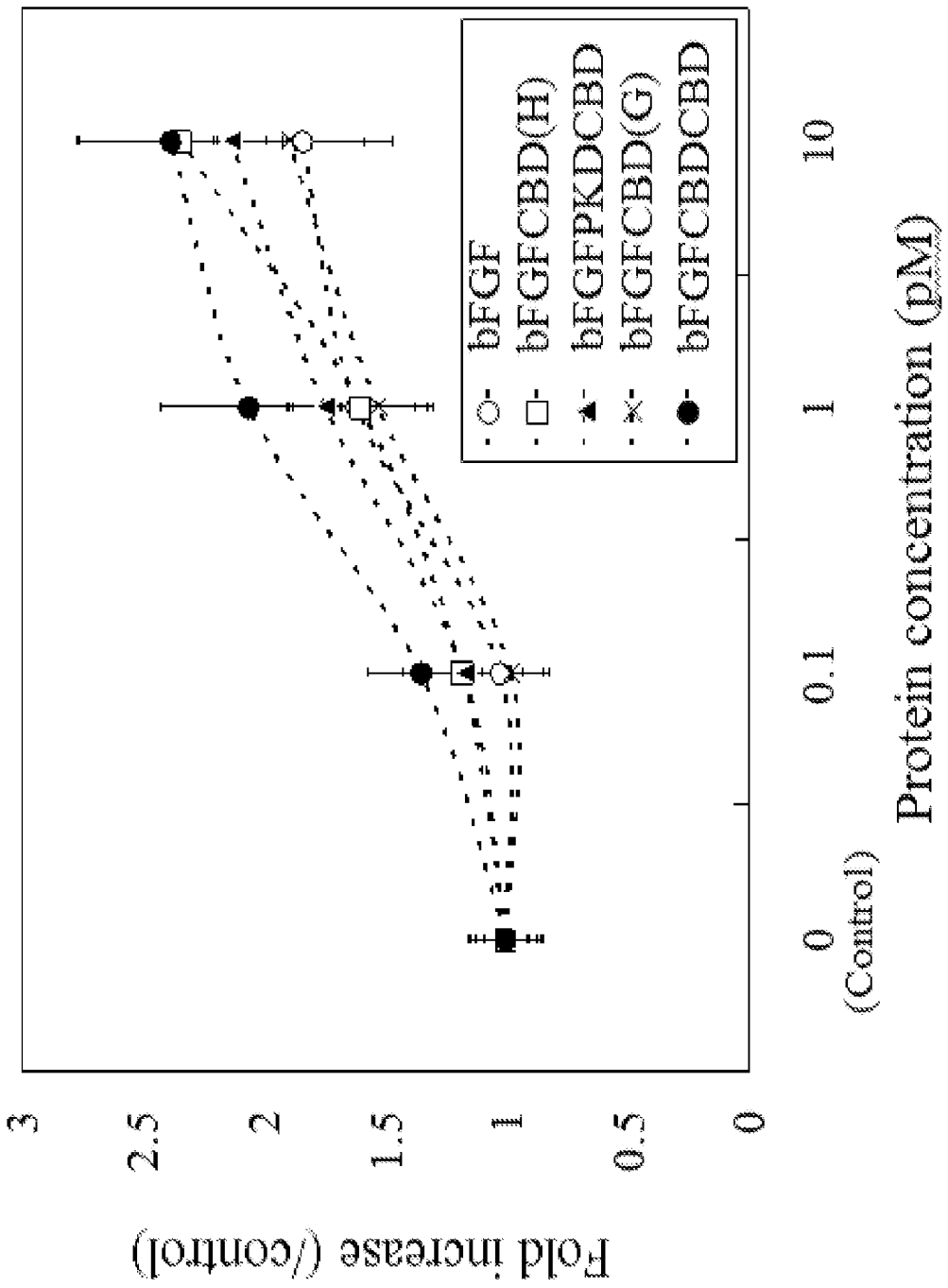

bFGF
bFGF
C. histolyticum ColH
s1
s2a
s2b
s3
bFGF-CBD(H)
bFGF
s3
bFGF-PKD-CBD
bFGF
s2b
s3
C. histolyticum ColG
s1
s2
s3a
s3b
bFGF-CBD(G)
bFGF
s3b
bFGF-CBD-CBD
bFGF
s3a
s3b
Catalytic domain
Polycystic kidney disease (PKD) domain
Collagen-binding domain (CBD)

Fig. 7


Fold increase (/control)
3
2.5
2
1.5
1
0.5
0
0
(Control)
0.1
1
10
Protein concentration (pM)
bFGF
bFGFCBD(H)
bFGFPKDCBD
bFGFCBD(G)
bFGFCBDCBD

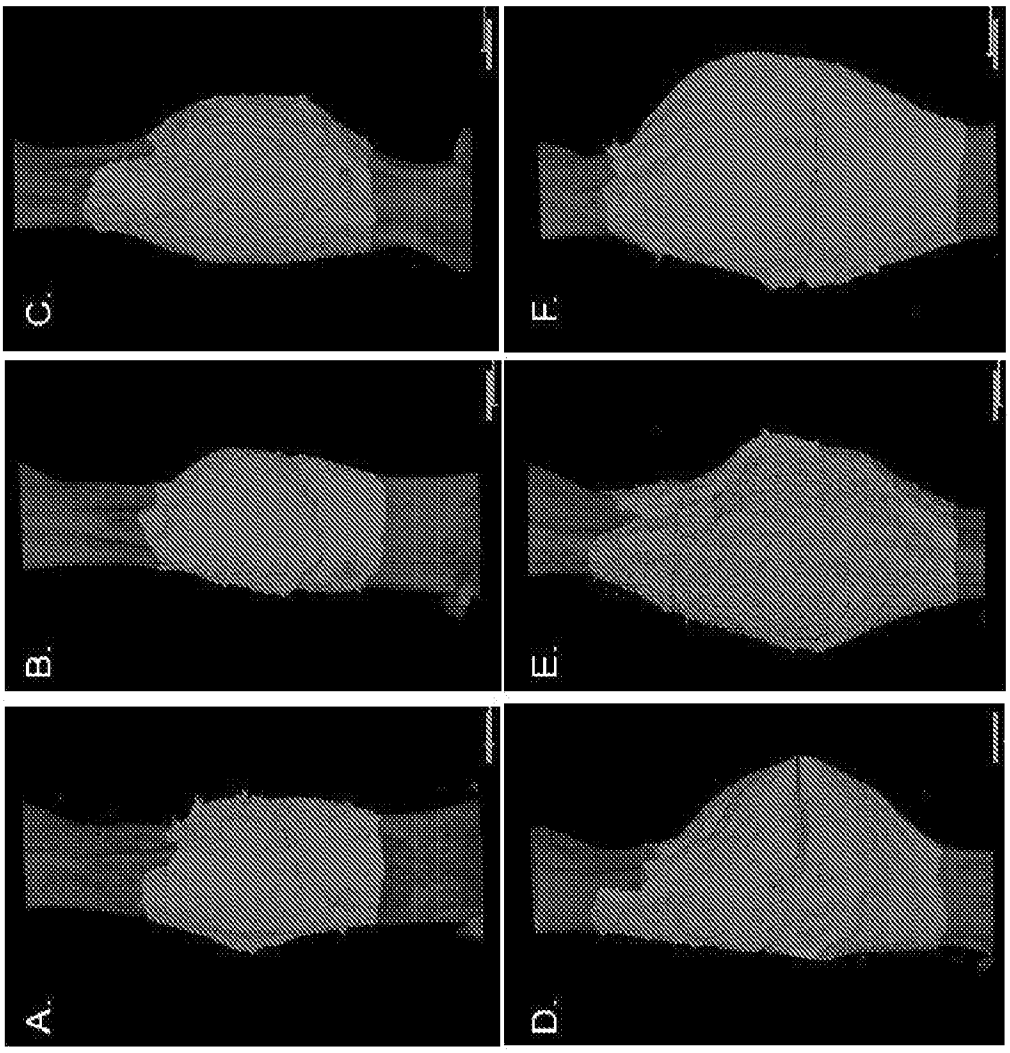


Fig. 8A Fig. 8B Fig. 8C

Fig. 8D Fig. 8E Fig. 8F

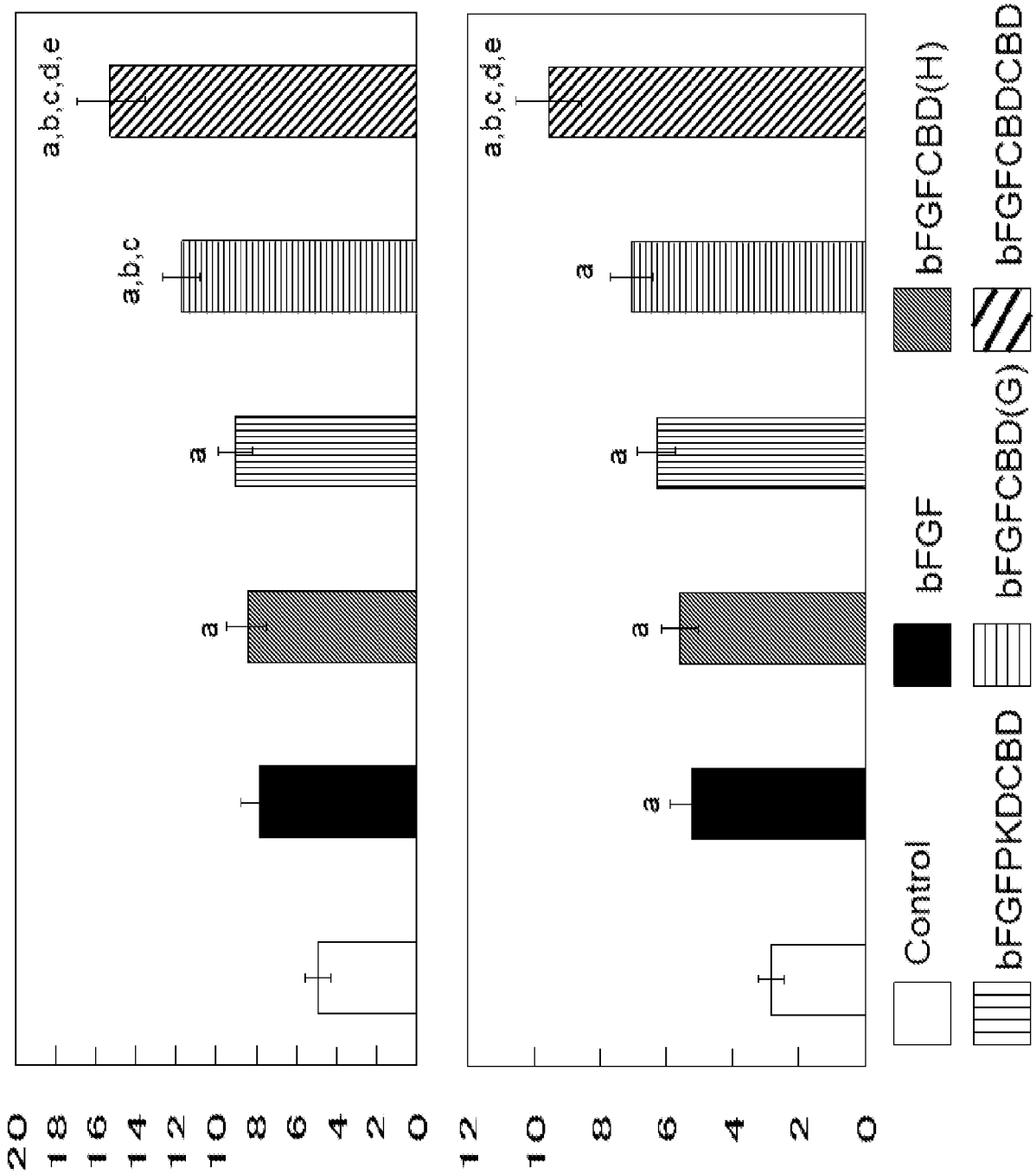

Callus volume (mm³)
20
18
16
14
12
10
8
6
4
2
0
a,b,c,d,e
a,b,c
a
a
a
Bone mineral content (mg)
12
10
8
6
4
2
0
a,b,c,d,e
a
a
a
a
Control
bFGF
bFGFCBD(H)
bFGFPKDCBD
bFGFCBD(G)
bFGFCBDCBD

Fig. 9A

Fig. 9B

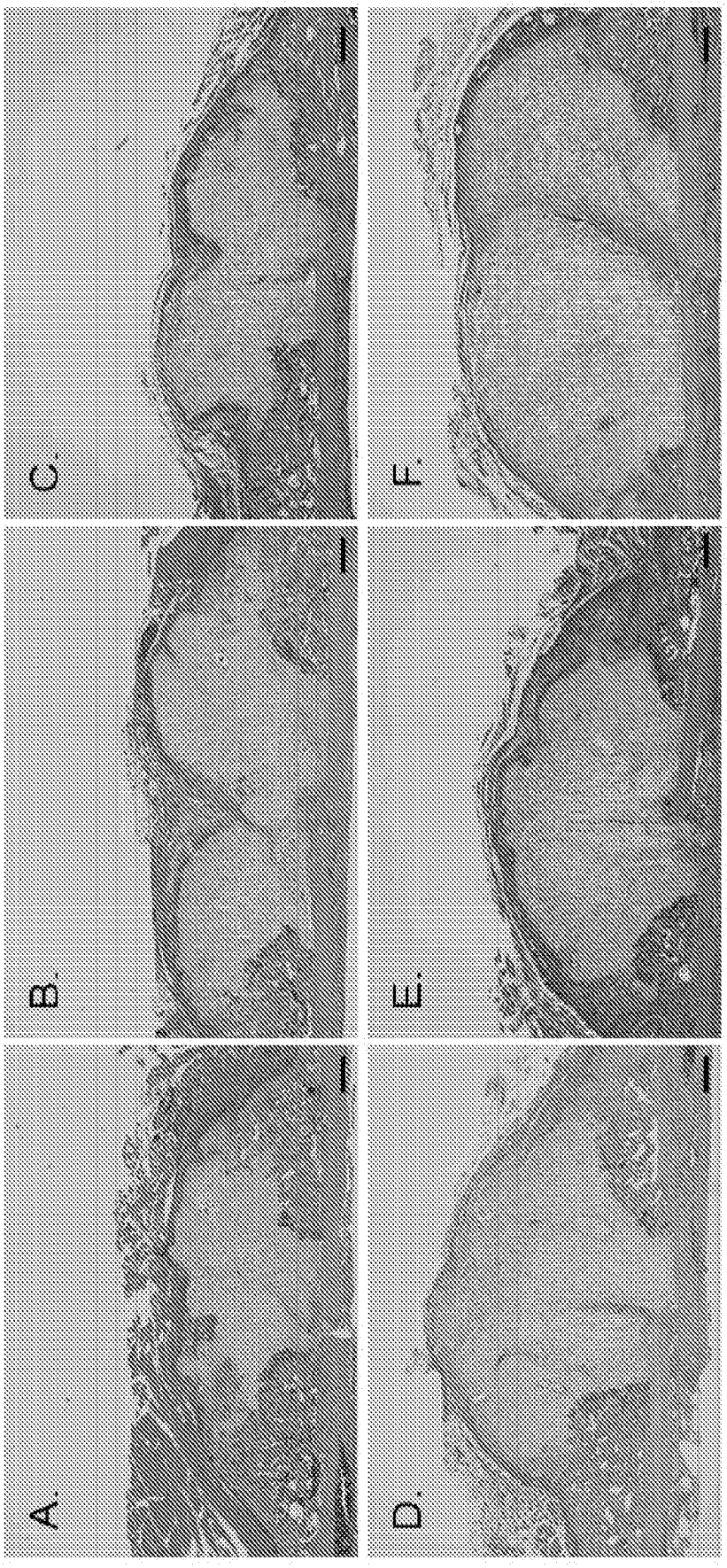


Fig. 10A Fig. 10B Fig. 10C

Fig. 10D Fig. 10E Fig. 10F

Fig. 11

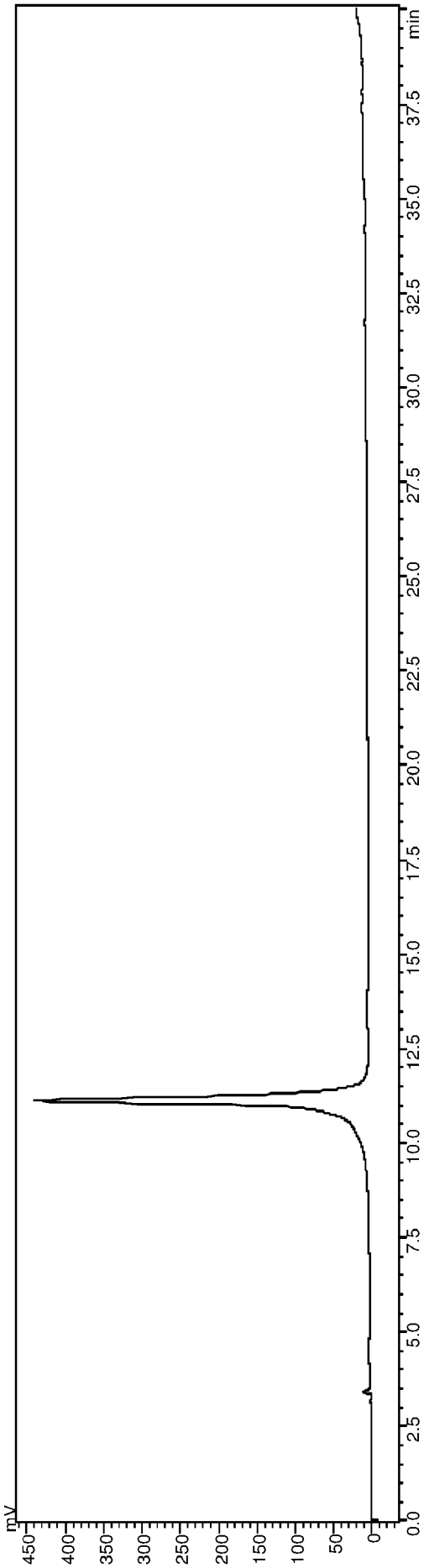

mV
450
400
350
300
250
200
150
100
50
0
min
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
25.0
27.5
30.0
32.5
35.0
37.5

Fig. 12

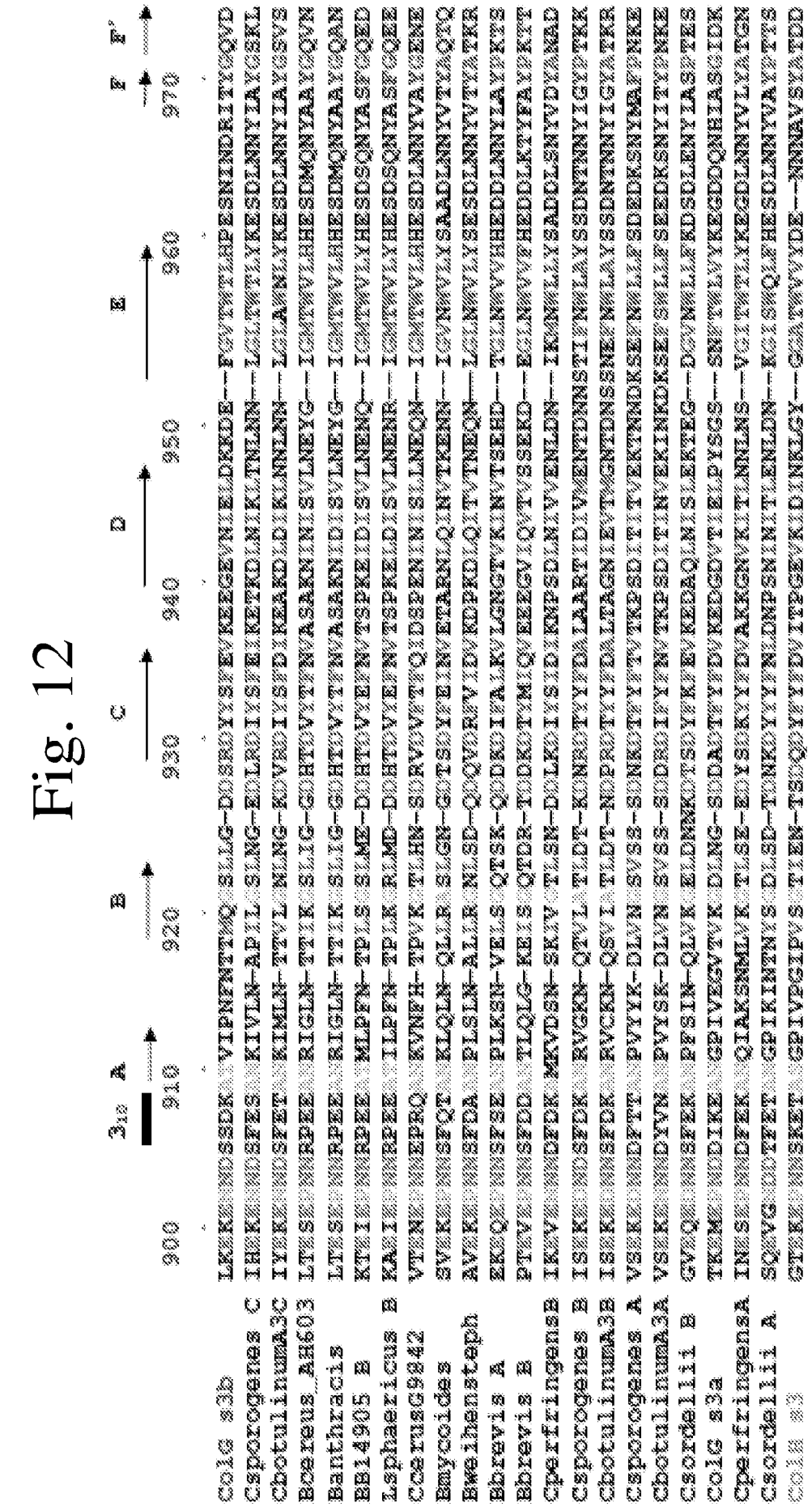


Fig. 12 (continued)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/017665

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 8/65; A61K 38/00; A61K 38/10; A61K 38/16; A61K 38/48; C12N 9/52 (2018.01)
CPC - A61K 8/64; A61K 8/65; C07K 2319/00; C07K 2319/70; C12N 9/52 (2018.02)

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)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/94.67; 424/94.6; 424/192.1; 514/1.1; 514/17.2; 530/356 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2015/0038423 A1 (THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS et al) 05 February 2015 (05.02.2015) entire document	1-3
A	BAUER et al. "Structural comparison of ColH and ColG collagen-binding domains from Clostridium histolyticum," J Bacteriol, 09 November 2012 (09.11.2012), Vol. 195, Pgs. 318-327. entire document	1-3
A	MATSUSHITA et al. "Substrate recognition by the collagen-binding domain of Clostridium histolyticum class I collagenase," J Biol Chem, 09 December 2000 (09.12.2000), Vol. 276, Pgs. 8761-8770. entire document	1-3
A	US 2014/0335146 A1 (UCHIDA et al) 13 November 2014 (13.11.2014) entire document	1-3
A	US 2015/0284701 A1 (THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS et al) 08 October 2015 (08.10.2015) entire document	1-3

☐ Further documents are listed in the continuation of Box C. ☐ 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
18 April 2018	07 MAY 2018
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/017665

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-20
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/017665

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, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

SEQ ID NOs.1-40 were searched.