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Characterization by high-resolution crystal structure analysis of a triple-helix region of human collagen type III with potent cell adhesion activity

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ABSTRACT

Collagen is one of the most abundant and important proteins in the human body. Human collagen type III (hCOL3A1) belongs to the fibril-forming collagens and is widely distributed in extensible connective tissue like skin, internal organs, or the vascular system. It plays key roles in wound healing, collagen fibrillogenesis, and normal cardiovascular development in human. The charged residues are considered to be an important characteristic of hCOL3A1, especially for collagen binding and recognition. Here we found that a triple helix fragment of hCOL3A1, Gly489-Gly510, contained multiple charged residues, as well as representative Glu-Lys-Gly and Glu-Arg-Gly charged triplets. We solved the crystal structure of this new fragment to a high-resolution of 1.50 Å and identified some important conformations of this new triple-helix region, including strong hydrogen bonds in interchain and interhelical interactions in addition to obvious flexible bending for the triple helix. We also found that the synthetic collagen peptides around this region exhibited potent activities through integrin-mediated peptide-membrane interaction. We then developed a method to produce a recombinant protein consisting of 16 tandem repeats of the triple-helix fragment of hCOL3A1 with strong activity without cytotoxicity. These results provide a strong base for further functional studies of human collagen type III and the method developed in this study can be applied to produce hCOL3A1-derived proteins or other tandem-repeat proteins with membrane adhesion activity.

1. Introduction

The most abundant protein in the animal body is collagen, mainly located extracellular matrix (ECM). It participates in the formation of many tissues, such as tendons, ligaments, skin, cornea, and bone. In human, at least 28 different types of collagen can be classified as fibril-forming collagens (Type I, II, III, V, and XI) and non-fibrillar collagens [1]. Collagens self-associate to form supramolecular arrangements for various functions in different tissues, such as cell adhesion, cell membrane repair, signaling, migration, and maturation [2]. Collagen is also involved with host-defense proteins, including C1q, collectins, and macrophage scavenger receptors [3]. In fibril-forming collagens, the triple-helix conformation of collagen is formed by three polypeptide chains, with glycine repeated at every third position.

Type III collagen is the second most abundant collagen, and it is widely distributed in extensible connective tissue, including skin,

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internal organs, and the vascular system. It was found to be important in wound healing, collagen I fibrillogenesis, and normal cardiovascular development in human [4]. Besides the common proline and its post-translational modified 4-hydroxyproline (4-Hyp, "O" for short), multiple charged residues occurred at the X and Y positions of hCOL3A1, forming Glu-Lys-Gly, Glu-Arg-Gly or other charged triplets. These charged residues are considered to be an important characteristic of hCOL3A1, especially for collagen binding and recognition [5]. Several peptidic structures in the triple-helix region of hCOL3A1 have been solved, using host-guest peptides (host peptide stands for POG or Pro-hydroxyproline-Gly) [6] or native peptides [7]. Here we noticed that a triple-helix fragment of human type III collagen, Gly489-Gly510, contains multiple charged residues and both Glu-Lys-Gly and Glu-Arg-Gly triplets. Then we solved the crystal structure of this new region to a resolution of 1.50 Å. Our findings reveal some important conformations of triple-helix region in hCOL3A1 at high resolution. The synthetic collagen peptides around this region and it's derived recombinant protein all exhibited potent cell adhesion activities through integrin-mediated peptide-membrane interaction. These results provide a strong base for further functional studies of human collagen type III and the method developed in this study can be used for producing hCOL3A1-derived proteins or other tandem-repeat proteins with membrane adhesion activity.

2. Materials and methods

2.1. Crystallization

Host-guest peptides C3Pa (Pro-Hyp-Gly)$_3$-FRGPAGPNGIPG-(Pro-Hyp-Gly)$_3$ and C3Pb (Pro-Hyp-Gly)$_3$-IPGEKGPAGERG-(Pro-Hyp-Gly)$_3$ were both synthesized of 95% pure with a standard solid-phase FMOC method [8] by Taihe Biotechnology Co., LTD (Beijing, China). Before crystallization screening, the peptide powder was dissolved in double-distilled water to a concentration of 15 mg/ml. Crystal screening was performed at 16 °C using hanging-drop vapor diffusion. The drops were set on a siliconized cover clip by equilibrating a mixture containing 1 μl protein solution (15 mg/ml C3Pa in water) and 1 μl reservoir solution (30% (w/v) PEG 400, 0.1 M Na Acetate pH 4.6, 0.1 M Cadmium Chloride) against a 1000 μl reservoir solution. After one week, single crystals formed and were flash frozen by liquid nitrogen for future data collection. C3Pb peptide was crystallized in a similar way with a different reservoir solution (0.2 M NaCl, 0.1 M Bis-Tris pH 6.5, 25% (w/v) PEG 3350).

2.2. Data collection, structure determination, and refinement

The datasets of C3Pa and C3Pb were all collected at beamline BL-18U1 of Shanghai Synchrotron Radiation Facility with wavelength of 0.97930 Å. The crystals were kept at 100 K during X-ray diffraction data collection. Data were indexed and scaled with HKL2000 [9]. Five percent of the data were randomly selected for the calculation of R_{free}. Both crystals belong to the $P2_1$ space group, but with different cell parameters. Phases of C3Pa and C3Pb were solved by BALBES from the CCP4 program suite [10], and their initial models were built by ARP/wARP [11]. All refinement procedures were carried out with PHENIX.refine [12] and COOT [13]. Table S1 shows the detailed statistics of data collection and refinement.

2.3. Accession numbers

Coordinates and structure factors have been deposited in the Protein Data Bank with accession number 6A0C for crystal structure of C3Pa and accession number 6A0A for C3Pb.

2.4. Development of a method to express tandem-repeat protein T16

Recombinant protein T16 consists of 16 tandem repeats of the triple-helix fragment of hCOL3A1, Gly483-Pro512. Briefly, the DNA fragment of T16 was synthesized (Genewiz, China) and inserted into the commercially available expression plasmid pET32a(+). The resulting recombinant expression vectors were transformed into BL21 (DE3). After induced by IPTG (isopropylthio-β-galactoside), the expressed protein was purified with Nickel-chelate chromatography.

2.5. Cell adhesion assay

According to previous research, 100 μl protein solution (200 μg/ml) and blank solution (PBS) were added to a 96-well plate at 4 °C overnight. The nonspecific binding site was blocked with 100 μl of 1% heat-denatured bovine serum albumin (BSA) and then washed with PBS two times. 3T3 cells (10^5/well) were incubated 60 min at 37 °C, followed by washing 4 times in PBS. LDH was tested by Roche's Cytotoxicity Detection Kit. Relative cell adhesion rates were compared with standard products.

2.6. Competition inhibition assay

Plates were coated with 100 μl of 200 μg/ml T16 protein or a test peptide. 3T3 cells (1×10^6 cells/ml) were incubated with 10 μM RGD peptides in PBS to compete for the cell surface receptors for 30 min before seeding [14]. The 3T3 cells without RGD peptides were used as the control. The assay was undertaken in triplicate, and the data were presented as mean ± SD. The attached cells were measured by the LDH method. The Type I collagen adhesion of cells without RGD peptide was used as a reference.

2.7. Cytotoxicity assay

The cytotoxicity of T16 protein and the peptides to 3T3 cells were measured using Cell Counting Kit-8 (CCK-8) following instructions in the manual provided by the manufacturer (Dojindo Molecular Technologies, Japan).

3. Results

3.1. Overall structure

To unravel the structural characteristics of Gly489-Gly510 in hCOL3A1, a series of peptides were designed and synthesized for high-throughput crystal screening. With most crystals of poor quality, we finally determined two crystal structures using host–guest peptide with hCOL3A1 peptide in the middle (Fig. 1A), including (POG)$_3$-$_{490}$FRGPAGPNGIPG$_{501}$-(POG)$_3$ (C3Pa for short) and (POG)$_3$-$_{499}$IPGEKGPAGERG$_{510}$-(POG)$_3$ (C3Pb for short), to a resolution of 1.50 Å. Both structures adopt a standard triple helical conformation (Fig. 1B). The three chains are typically designated as leading, middle, and trailing chains, with one residue interval. The asymmetric unit of both C3Pa and C3Pb contains one triple-helix molecule. Since four amino acids ($_{498}$GIPG$_{501}$) are overlapped in C3Pa and C3Pb peptides, we could build the structure from Gly 489 (involving one Gly residue in host peptide) to Gly510 of hCOL3A1 (Fig. 1B and C).

Both C3Pa and C3Pb share a similar structure in shape, but the two peptides are not identical. Structural alignment for the whole molecule shows that the root-mean-square (r.m.s.) deviations of Cα between the two structures is 1.22 Å (Fig. 2A). The host (POG)$_3$ residues at both ends and guest residues in the middle all showed

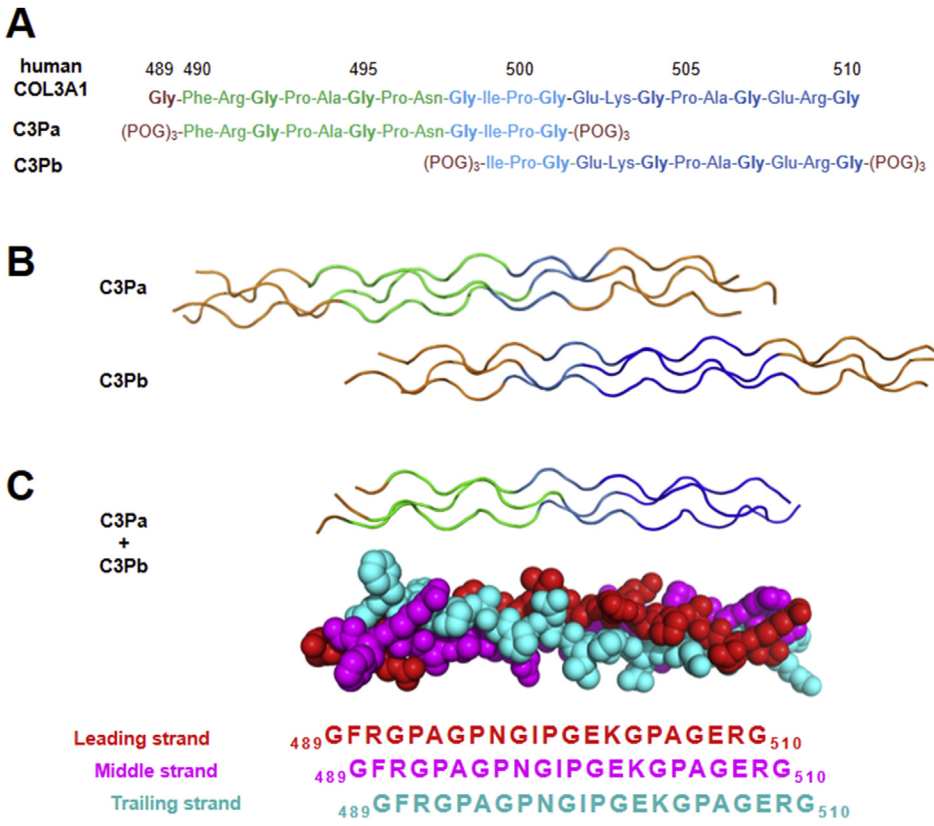


Fig. 1. Overall structures of C3Pa and C3Pb. (A) The amino acid sequence of human COL3A1 (Gly489 to Gly510), C3Pa and C3Pb. The host peptide of $(POG)_3$ is colored in orange, with the guest peptide of C3Pa and C3Pb in green and blue, respectively. The overlapped guest peptide of C3Pa and C3Pb is colored in sky blue. (B) Side view (N-terminus at the left) of the overall structure of C3Pa and C3Pb. Their colors are consistent with their amino acid sequence. (C) Based on the overlapped region ($_{498}$GIPG$_{501}$), the continuous structure of human COL3A1 (Gly489 to Gly510) was built by superposed C3Pa and C3Pb structures. Besides cartoon representation, this structure is also shown in spheres, with three different colors to distinguish the leading, middle, and trailing strands. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

main chain differences. We also compared the two structures with several reported host-guest peptide structures (Fig. 2B and C). It is obvious that the structure of C3Pb looks very similar to other 3-peptide structures (PDB entry: 2DRT, 1QSU, and 1BKV) when compared to C3Pa.The r.m.s. deviations between C3Pb and the other 3 structures are around 1 Å (0.99 Å ~ 1.19 Å), but those of C3Pa are much larger with 1.29 Å ~ 1.90 Å (Fig. 2D). The guest peptide from Phe490 to Pro500 in C3Pa moves away from the central axis of the whole peptide (Fig. 2B). It seems that the C3Pa peptide bends with a larger angle, the importance of which will be discussed later.

3.2. Interchain and interhelical hydrogen bonds

Many polar residues with long side chains occur in Gly489-Gly510 of hCOL3A1, so various interchain hydrogen bonds were observed in the structures (Fig. S1, Supplementary material). It's interesting that Arg491 residues in three strands all formed strong hydrogen bonds with adjacent strands, which happened to form the shape of a triangle, or “arginine triangle” (Fig. S1Bd). This rare structure for collagen could help to stabilize the triple helix. In the C3Pa and C3Pb structures, the collagen molecules form regular staggered stacking with many interhelical hydrogen bonds in between (Fig. S2). These types of interactions may play crucial roles in collagen fibril formation in vivo.

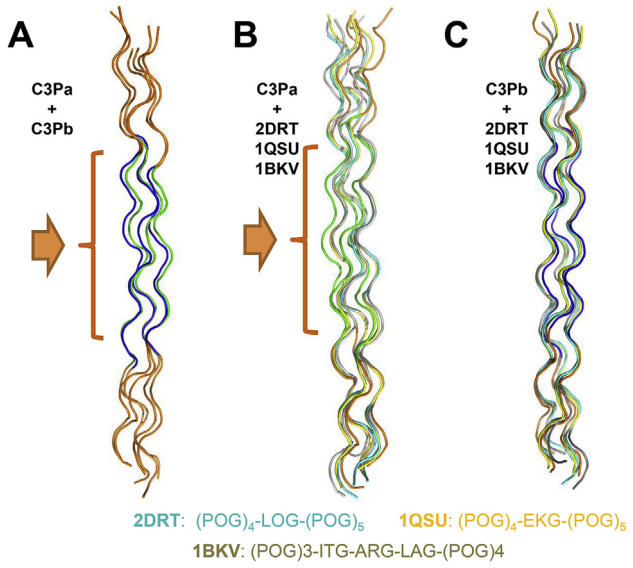
3.3. Flexibility of triple helix in C3Pa and C3Pb

Overall, the C3Pa structure exhibits a significant bending comparing with C3Pb (Fig. 3A and B). If we count the host peptide of $(POG)_3$, the full-length peptide of C3Pa triple helix exhibits a 169.43° angle, or 10.57° bend. The C3Pb full-length peptide exhibits a much smaller bend with 5.69°. The guest peptide of C3Pa, Gly489-Gly501 of hCOL3A1, exhibits a 164.88° angle, or 15.12° bend (Fig. 3A). In contrast, Gly498-Gly510 of hCol3A1 in C3Pb only exhibits a 9.36° bend, suggesting that this Gly489-Gly501 of hCOL3A1 has relatively strong flexibility. It can be speculated that this apparent bend may be related to the biological function of Gly489-Gly501 of hCOL3A1 because similar bending of collagen main chain has been proved to be critical in forming favorable packing interactions between integrin $\alpha 2\beta 1$ and its binding collagen peptide [15].

3.4. Cell adhesion activity of collagen peptides and its derived recombinant protein

Cell adhesion activity is an important biological function of collagen fibers, including human collagen type III. Using mouse fibroblast BALB 3T3 cells, we tested the adhesion activities of three native hCOL3A1 peptides, C1P, C2P and C3P, which overlap or partially overlap the Gly489-Gly510 helical region (Fig. 4A). The longest peptide, C1P (Gly483-Pro512 of hCOL3A1), showed the most potent cell adhesion activity (Fig. 4B).

It has been proven that the recombinant collagen-mimetic proteins with identical units of triple helix peptide arranged in a way of tandem repeats can self-assemble into mini-fibrils for biomedical and industrial application [16]. Therefore, we



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	r.m.s. derivation (Å)				
	C3Pa	C3Pb	2DRT	1QSU	1BKV
C3Pa	-	1.22	1.60	1.29	1.90
C3Pb	1.22	-	1.19	1.13	0.99

Fig. 2. Structural superposition between C3Pa, C3Pb and other similar host-guest peptides. (A) Structural alignment of all Cα atoms between C3Pa and C3Pb. The host peptide of $(POG)_3$ is colored in orange, with the guest peptide of C3Pa and C3Pb in green and blue, respectively. (B) Structural alignment of all Cα atoms between C3Pa and three other structures: PDB entry 2DRT in cyan, PDB entry 1QSU in yellow, and PDB entry 1BKV in gray. (C) Structural alignment of all Cα atoms between C3Pb and three other structures. (D) The r.m.s. derivations between the superposed structures. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

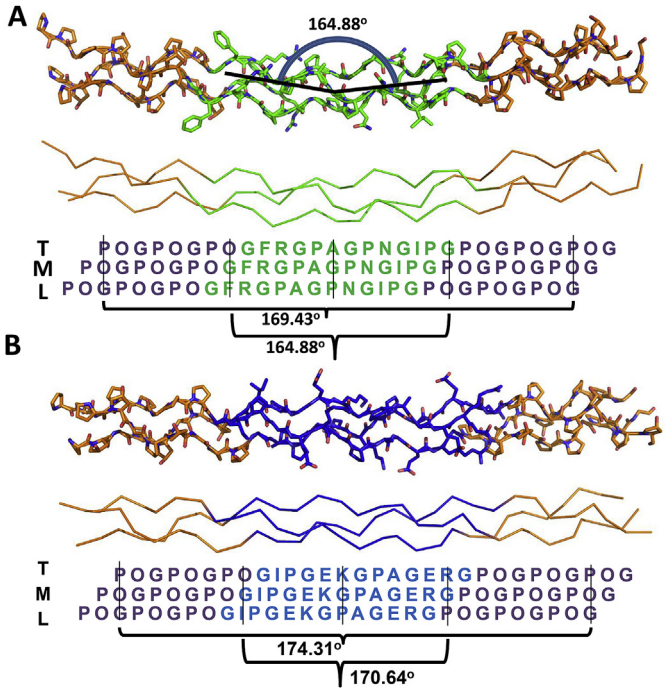

Fig. 3. The flexibility of the triple helix in C3Pa (A) and C3Pb (B). Structures are all shown in stick representation. The bending angles are calculated based on the central point of the Cα atoms of the residues in the same position in each of the three chains (164.88° for C3Pa and 170.64° for C3Pb).

constructed a recombinant collagen protein containing 16 tandem repeats of the C1P peptide, designated T16 (Fig. 4A). We found that this 480aa recombinant protein T16, could be easily expressed with thioredoxin tag in *E.*coli (Fig. S3A), and remain stable after removing the tag (Fig. S3B). We found that this T16 protein has very strong cell adhesion activity, much higher than single C1P peptide (same quantity) or Type I collagen control (Fig. 4B).

To further confirm the activity of C1P and T16, two peptides with randomly selected sequences in the triple-helix region of hCOL3A1 (P1 and P2 in Fig. 4A) were synthesized and tested using the cell adhesion assay. Besides, an unrelated peptide HR2P-M2 which is derived from the heptad repeat (HR) 2 domain in MERS-CoV spike (S) protein S2 subunit [17] and an unrelated protein BSA were included as controls. Like HR2P-M2 and BSA, P1 and P2 peptides showed no significant adhesion activity. As expected, T16 and C1P exhibited much stronger cell adhesion activity than that of Type I collagen (Fig. 4B), suggesting that the high cell adhesion activity is resulted from the specific sequences of C1P and the crystal structural properties that we have determined.

3.5. Cell adhesion of C1P and T16 are mediated by integrins

Integrins are the principal family of cell surface proteins that interact with the extracellular matrix, like collagen. Here we used a RGD peptide, the common inhibitor of integrin-ligand interactions, in the cell adhesion competition assay to compete with C1P or T16. As shown in Fig. 4C, addition of the RGD peptide resulted in a significant decrease of the cell adhesion activities of both C1P peptide and T16 protein, suggesting that the C1P peptide and T16 protein may share a same binding site in integrins with the RGD peptide, although neither C1P peptide nor T16 protein containing RGD sequence. Further identification of the motif in C1P and T16 for binding to integrins is a subject of future studies.

To determine the cytotoxicity of T16 and peptides, CCK-8 assay was performed. No obvious cytotoxicity was found for T16 protein and the peptides tested (Fig. 4D). No CC_{50} value could be determined for them because of the lack of cytotoxicity at the concentration as high as 1000 μg/ml. Therefore, the T16 protein with remarkable collagen properties is expected to be safe for application as a collagen-related biomaterial in clinics.

4. Discussion

Here, we determined the crystal structure of Gly489-Gly510 in hCOL3A1 at high resolution. We found some important conformations in this triple helical region of hCOL3A1. On one hand, many polar residues are involved in Gly489-Gly510 of hCOL3A1; and they provide many strong direct hydrogen bonds in interchain or interhelical interactions. On the other hand, Gly489-Gly501 of hCOL3A1 exhibits an obvious bend of 15.12°. Such relatively large flexible bending is uncommon among collagen solo structures. These features suggest that this triple helical region may have important biological functions, since both the charged residues and flexible triple helix backbone have been proven to be essential in collagen recognition by its receptor, like integrin α2β1 [15].

The collagen triple helix is an important binding motif for many different kinds of molecules. In particular, the charged residues of Lys, Arg, Glu or Asp in the triple-helix region of collagen have been reported to be key residues in collagen recognition [18]. Charged residues are asymmetrically distributed along the collagen triple helix in human type III collagen, forming Glu-Lys-Gly or Glu-Arg-Gly triplets. Glu-Lys-Gly triplet has been reported to be the

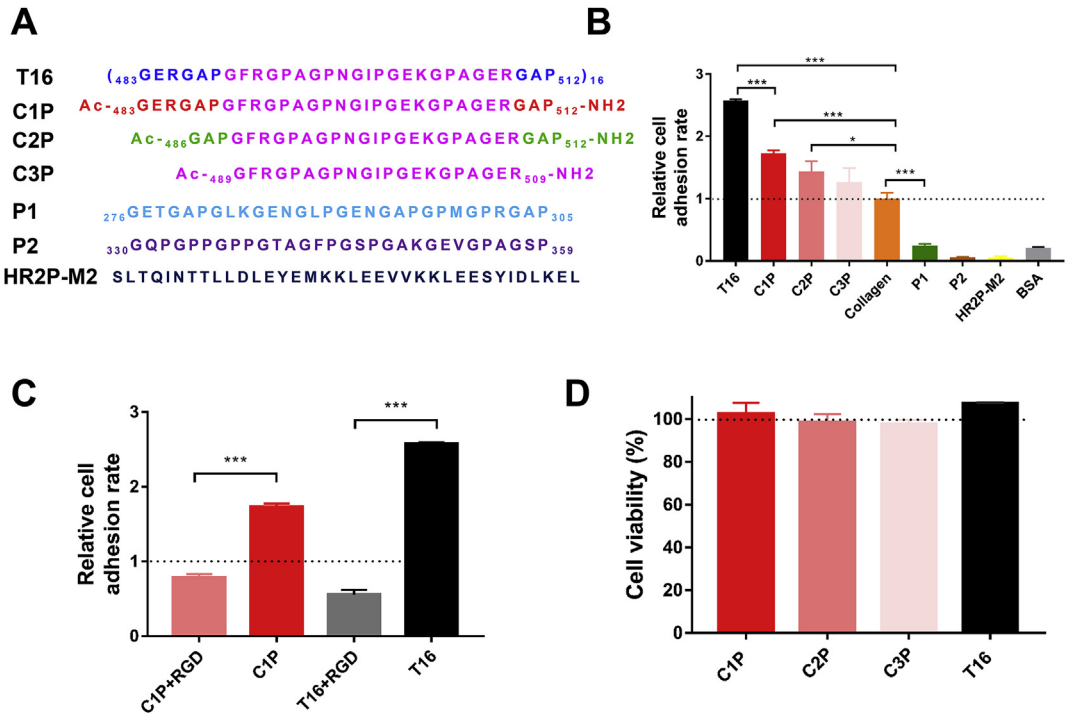

Fig. 4. The cell adhesion activity of hCOL3A1-derived peptides and protein. (A) The amino acid sequences of hCOL3A1-derived native peptides (C1P, C2P, C3P), tandem-repeat protein (T16), randomly selected from the triple-helix region of hCOL3A1 (P1, P2) and irrelevant peptide (HR2P-M2). (B) Adhesion of the peptides (C1P, C2P, C3P, P1, P2, and HR2P-M2) and the proteins (T16, type I collagen, and 1% heat-denatured BSA) to 3T3 cells, respectively. Substrate concentrations are 0.2 mg/ml for peptides and proteins. The type I collagen was included as a reference to calculate relative cell adhesion rate. (C) Competition cell adhesion assay. The 3T3 cell -adhesion activity of T16 and C1P (200 μg/ml) was significantly decreased after RGD peptide (10 μM) binding with integrin. All peptides and proteins were compared with type I collagen without RGD. (D) Peptides and T16 did not show cytotoxicity at concentration of 1 mg/ml. Data are shown as mean ± s.d. The asterisks represent statistical differences. *P < 0.05; **P < 0.01; ***P < 0.001, ns P > 0.05. Student's two-tailed *t*-test was used for the comparisons.

ligand-binding site of the macrophage scavenger receptor, and Glu-Arg-Gly triplet was found to be the integrin-binding site on collagen [5]. The Gly489-Gly510 region, containing both two kinds of triplets, may be an active triple helical site in hCOL3A1 and play an important role in cell adhesion and ligand reorganization. We selected two peptides from other triple-helix region of hCOL3A1 (Fig. 4A), both lacking Glu-Lys-Gly and Glu-Arg-Gly triplets, then found that they exhibited much weaker cell adhesion activities than C1P peptides containing Gly489-Gly510 region (Fig. 4C). So the potent cell adhesion activity of C1P peptide is closely related to its sequence composition. Juming et al. has reported that the recombinant collagen-like proteins containing tandem repeats of the type I collagen peptide shows high cell adhesion activities [19]. Using similar approach, we constructed a recombinant collagen protein T16, which consists of 16 tandem repeats of the triple-helix fragment of hCOL3A1, C1P peptide. As expected, T16 showed stronger cell adhesion activity than C1P peptide under same mass concentration (Fig. 4B). It suggested that the recombinant protein containing multiple tandem repeats of the triple-helix fragment may have more stable helical conformation or more favorable ligand binding configuration to facilitate the cell membrane attachment and adhesion.

The model peptide Pro-Hyp-Gly (POG) is the most stabilizing sequence found in collagen, and it has been studied extensively for structure, stability and dynamic changes [20]. The crystal structure of $(POG)_n$ repeating peptides showed a linear structure of the triple helix [21,22], seems to indicate that the triple helix of collagen is a rigid rod-like conformation with little flexibility. However, a recent study using analytical ultracentrifugation and small-angle X-ray scattering to analyze a series of $(POG)_n$ repeating peptides found that a small degree of nonlinearity exists in these triple helices with the degree of bending approximated as 4–17° from linearity [23]. Therefore, in addition to the interruptions in the Gly-X-Y repeat, the regular Gly-X-Y repeated collagen could also have a certain degree of flexibility. This flexible bending of the collagen triple helix is hard to find in crystal owing to the packing pressure of crystallization. However, we observed such a certain degree of flexibility in our two structures, especially for C3Pa with a 15.12° bend. The only difference between C3Pa peptide and $(POG)_{10}$ model peptide is the guest peptide of hCOL3A1, Phe490~Gly501. Consequently, we reason that the bending which occurs in the crystal structure results from the intrinsic property of this hCOL3A1 peptide. Interestingly, the integrin-binding peptide GFOGER also showed an 8°~9° angle between host POG peptide and guest peptide, termed as "junctional kinking", and this angle was increased to 16° when this peptide bound to the integrin I domain [15]. Similar bending has also been reported in some high resolution crystal structures for other imino acid poor peptide bound to its target proteins, such as $(GPO)_3$ GPQGLAGQRGIVGLOGQRGER$(GPO)_3$ with matrix metalloproteinase 1 [24], and $(GPO)_4$GPRGRT$(GPO)_4$ with a collagen binding protein CAN [25]. It suggests that the bending of collagen peptide can help its binding with target proteins. Therefore, the 15.12° bend of hCOL3A1 Phe490~Gly501 suggests that this triple helix region is highly flexible and may be involved in ligand reorganization, cell adhesion or other biological activities. Integrins are cell surface glycoproteins that serve as receptors for the extracellular matrix (ECM). It mediated the interaction of extracellular matrix with cell membrane. RGD peptide is a specific inhibitor of human PS2 group α subunit integrins [26]. In this study, we have shown that RGD peptide competitively inhibits the adhesion activity of C1P peptide and its derived recombinant protein T16. This suggests that the cell adhesion activity of the Gly489-Gly510 in

hCOL3A1 is mediated by human PS2 group α subunit integrin on the cell surface. However, further study on their specific interaction pattern and binding site is warranted.

In summary, our structures present detail at the atomic level for molecular flexibility and stability of Gly489-Gly510 of hCOL3A1. Using tandem repeat strategy, we expressed a recombinant protein containing 16 tandem repeats of this region, and we found that it has no cytotoxicity but much stronger cell adhesion activity than that of the commercially available human collagen type I. Our findings provide a base for further functional studies of the human collagen type III and a method for producing hCOL3A1-derived proteins and other tandem-repeat proteins with membrane adhesion activity.

Author contributions

S.J., L.L., and R.Z. conceived the idea and supervised the project; C.H., Y.Z., W.X., and S.Y. performed the experiments and analyzed the results; C.H. and Y.Z. wrote the original draft; R.Z., L.L., and S.J. revised the manuscript.

Disclosure

The authors indicate no potential conflict of interests regarding the publication of this paper.

Conflicts of interest

All authors agree with the submission and have no conflicting financial interests.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.018.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2018.12.018.

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