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Specific inhibition of procollagen C-endopeptidase activity by synthetic peptide with conservative sequence found in chordin

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Procollagen C-endopeptidase (BMP-1) and N-endopeptidase (ADAMTS-2) are key enzymes for correct and efficient conversion of fibrillar procollagens to their self assembling monomers. Thus, they have an essential role in building and controlling the quality of extracellular matrices (ECMs). Here, we tested inhibition of activity of the largest variant of BMP-1, a recombinant mammalian tolloid (mTld), *in vitro* by three synthetic peptides with conservative amino-acid sequences found in chordin using procollagen type I as a substrate. We also verified the specific action of best inhibitory 16 amino-acid peptide in the procollagen type I cleavage assay with the use of ADAMTS-2 (procollagen N-endopeptidase). Subsequently, we determined the critical residues and minimal sequence of six amino acids in the original 16 amino-acid peptide required to maintain the inhibitory potential. Studies on the interactions of 6 and 16 amino acid long peptides with the enzyme revealed their binding to non-catalytic, regulatory domains of mTld; the inhibitory activity was not due to the competition of peptides with the substrate for the enzyme active center, because mTld did not cleave the peptides. However, in the presence of mTld both peptides underwent cyclization by disulfide bond formation. Concluding, we have shown that procollagen C-endopeptidase may be specifically blocked *via* its non-catalytic domains by synthetic peptide consisting of 6 amino acids in the sequence found in highly conservative region of chordin. Thus, we hypothesize that the 6 amino-acid peptide could be a good candidate for antifibrotic drug development.

Keywords: procollagen C-endopeptidase, N-endopeptidase, chordin

INTRODUCTION

Fibrillar collagens are the most abundant proteins in the ECM. They are synthesized and secreted as highly soluble precursors, procollagens. To self-assemble into collagen monomers, the precursors require conversion (Lapiere *et al.*, 1971; Leung *et al.*, 1979; Prockop *et al.*, 1979; Prockop & Kivirikko, 1984; Hojima *et al.*, 1989; 1994; Kessler *et al.*, 1996; Li *et al.*, 1996; Arnold *et al.*, 1997; Colige *et al.*, 1997). This processing is performed by two zinc metalloproteases, procollagen N-endopeptidase (ADAMTS-2) cleaving off the N-propeptides, and procollagen C-endopeptidase (BMP-1) removing the C-propeptides (Hojima *et al.*, 1989; 1994).

BMP-1 also acts further as an upstream regulator of the ECM by activating pro-lysyl oxidase (Cronshaw *et al.*, 1995; Panchenko *et al.*, 1996). It activates also other ECM components, such as monomers of laminin-5 (Amano *et al.*, 2000), whose activation is critical for building basement membranes, and procollagen V (Imamura *et al.*, 1998),

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Abbreviations: ADAMTS-2, procollagen N-endopeptidase; BMP-1, procollagen C-endopeptidase; ECM, extracellular matrix; MRP, horseradish peroxidase; IPTG, iospropyl β-D-thiogalactoside.

which is one of the first collagens synthesized in the walls of micro-vessels. BMP-1 has also been identified in signaling pathways in embryogenesis and tissue development, including dorsal-ventral patterning of developing embryos (Piccolo *et al.*, 1997; Blader *et al.*, 1997; Marques *et al.*, 1997; Scott *et al.*, 2000; Rattenhol *et al.*, 2002; Wolfman *et al.*, 2003; Ge *et al.*, 2004; Steiglitz *et al.*, 2004; Zhang *et al.*, 2004; Ge & Greenspan, 2006). These processes involve proteolytic activation of multifunctional morphogenetic factors, such as BMP-2 and -4, and GDF-8 and -11. The signaling molecule pro-biglycan (a small proteoglycan), which promotes bone formation is also activated by BMP-1 (Scott *et al.*, 2000). Other roles of BMP-1 have been linked to its ability to process procollagen VII (Rattenholl *et al.*, 2002), myostatin (Wolfman *et al.*, 2003), dentin matrix protein 1 (Steiglitz *et al.*, 2004), and osteoglycin (Ge *et al.*, 2004). Recently, a link between BMP-1 and TGF-βs has been reported in that the metalloprotease is able to process latent TGFβ-binding proteins (LTBPs) (Ge & Greenspan, 2006). An association of procollagen C-endopeptidase with the cytoplasmic domain of adrenoergic receptor α1A has been shown in a yeast two-hybrid system and indicates some unidentified function of this metalloproteinase inside the cells (Zhang *et al.*, 2004).

Previous studies have shown that functional variants of BMP-1 are critical for survival during embryo development. Transgenic mice homozygous for inactive alleles of the BMP-1 gene lacked all active variants of BMP-1, and as a result, the neonates died at birth due to persistent hernia of the gut in the umbilical region (Suzuki *et al.*, 1996). Some of these neonates or even fetuses also suffered internal hemorrhages possibly due to weakness of blood vessel walls. In addition to reduced ossification of certain skull bones, electron microscopy analysis of collagenous tissues revealed abnormal barbered wire-like collagen fibrils that indicated a lack of efficient removal of procollagen C-propeptides. Thus those results strongly suggested that BMP-1 variants play a critical role in the formation of healthy blood vessels and skeletal components during tissue development.

At least three variants of BMP-1 arising from alternative splicing of the RNA product of the BMP-1 gene have been identified (Takahara *et al.*, 1994). One of these variants, known as mammalian Tolloid (mTld), is a homologue of the *Drosophila* developmental protein tolloid (Wozney *et al.*, 1988; Kessler *et al.*, 1996; Li *et al.*, 1996; Marques *et al.*, 1997). Tolloid is a metalloprotease, which liberates decapentaplegic (DPP), a morphogen, from its inhibitory protein, short gastrulation (SOG). Xolloid is another homologue of mTld and is directly involved in the regulatory pathway of BMP-2 in *Xenopus* (Piccolo *et al.*, 1997) and BMP-4 in zebra fish (Blader *et al.*, 1997). Xolloid liberates BMP-2 and BMP-4 from inhibitory complexes with the non-collagenous inhibitor chordin.

Analysis of cDNAs encoding BMP-1 (Takahara *et al.*, 1994) has revealed sequences encoding: (a) a 70 kDa polypeptide identical to BMP-1 (Kessler *et al.*, 1996); (b) an 80 kDa polypeptide similar to the 70 kDa protein except that it contains an additional His-rich sequence (Takahara *et al.*, 1994); and (c) a polypeptide of about 100 kDa that is homologous to *Drosophila* tolloid (Marques *et al.*, 1997).

The structure of these three variants includes an N-terminal domain, which ends with sequences that are likely cleaved by a furin-like enzyme. The zinc-binding protease domain is identical in all splicing variants of procollagen C-endopeptidase. They also share more than 90% homology with the protease domain of two recently identified human and mouse metalloproteases whose domain structures are identical to mTld (Wozney *et al.*, 1988).

The C-terminal regions of the three variants differ by the number of CUB domains (complement, sea urchin EGF, bone morphogenetic protein-1). The 70 kDa polypeptide contains three CUB domains. The last two CUB domains in this enzyme are separated by an EGF-like domain (epidermal growth factor). The 80 kDa polypeptide is identical to the 70 kDa polypeptide except for the insertion of a 121 amino-acid region rich in histidine, serine and threonine, after the last CUB domain (Takahara *et al.*, 1994). The 100 kDa variant contains an additional EGF domain followed by two additional CUB domains. The domain structure of the enzyme has been determined previously (Sieron *et al.*, 2000) indicating that the CUB domains and EGF motifs are involved in governing substrate specificity of the variants (Hartigan *et al.*, 2003; Garrigue-Antar *et al.*, 2004).

The other zinc metalloproteinase acting in the conversion of fibrillar procollagens to collagens is procollagen N-endopeptidase (ADAMTS-2), which specifically cleaves the N-propeptides in the pro-α chains of procollagen types I and II (Hojima *et al.*, 1989; 1994). Mutations in the gene encoding procollagen N-endopeptidase have been detected in patients with human Ehlers-Danlos syndrome type VII (currently, dermatosparaxis) and in dermatosparacic calfs and sheep (Lapiere *et al.*, 1971; Colige *et al.*, 1997).

A process strongly relaying on the structure of ECM is angiogenesis, which is the outgrowth of new blood vessels from existing vessels (Risau, 1997). It is a physiological process involving several steps and various cell types and growth factors (Senger, 1996; Sun *et al.*, 2005). The process requires cell migration and proliferation related to remodeling of the extracellular matrix (Dvorak *et al.*, 1995). The key role in

this process is played by matrix proteinases, both degrading and building ECM. Importantly, angiogenesis is also a hallmark in the pathology of many diseases, including cancer, ischemia, atherosclerosis, and inflammatory diseases (Montesano *et al.*, 1990; Gailit & Clark, 1994; Bugge *et al.*, 1996a; 1996b; Folkman & D'Amore, 1996).

Here we have tested the possibility of inhibition of one of the two critical enzymes controlling quality of ECM, namely an active procollagen C-endopeptidase variant mTld using rationally designed peptides with amino-acid sequences that are highly conservative in chordin, one of numerous substrates of mTld. We have also tested possible mechanism of inhibition by studying interactions between mTld and inhibitory peptide.

MATERIALS AND METHODS

Recombinant proteins. Recombinant bovine ADAMTS-2 was prepared in insect cells Sf9 (Invitrogen). The bovine cDNA encoding ADAMTS-2 was obtained as previously described (Colige *et al.*, 1997). The construct was inserted into the *Not*I/*Xba*I sites of pVL1392 vector and recombined with the baculovirus gold (Pharmingen) according to the manufacturer's protocol. The insect cells were infected with viral particles. Cells containing the virus were cultured in SF-900 II SFM medium (GIBCO BRL). Recombinant protein from the medium was identified after separation in 10% SDS/polyacrylamide gel.

Enzymatically active recombinant variant of procollagen C-endopeptidase mTld was prepared in HT-1080 cells according to the method published by Sieron *et al.* (2000). Active ADAMTS-2 and procollagen I were purified from chick embryo tendons using procedures described by Hojima *et al.* (1989).

For antibody production, recombinant human mTld was prepared in the *Escherichia coli* expression system. The complete cDNA was obtained as described previously (Li *et al.*, 1996). The coding sequence for the full-length protein was inserted into the *Sph*I/*Sal*I restriction sites of the pQE32 plasmid. *E. coli* at log phase of growth in one liter of culture medium was induced with 1 mM IPTG for 4 h at 30°C. The cells were collected by centrifugation and the recombinant protein was recovered from inclusion bodies. mTld was identified under denaturing conditions based on its migration in 12% SDS/polyacrylamide gel. Proteins isolated from inclusion bodies of bacteria transformed with empty vector were also separated in the gel and compared with the material isolated from transformed bacteria.

Polyclonal antibodies against recombinant proteins. Polyclonal antibodies against both metalloproteases were produced in New Zealand rabbits by Bio-Synthesis, Inc. (Lewisville, TX, USA). Briefly, the recombinant proteins were separated in polyacrylamide gels containing 0.1% SDS. The gels were stained with Coomassie Blue R250. Bands corresponding to mTld or ADAMTS-2 were excised and shipped to Bio-Synthesis, Inc. To characterize the antibodies' specificity, complete antisera from four consecutive bleedings were tested using either the antigens or purified recombinant enzymes synthesized in HT-1080 cells, as described previously (Sieron *et al.*, 2000). Secondary antibodies conjugated with horseradish peroxidase were used to visualize the antigens by the ECL method (Pharmacia-Amersham).

Preparation of synthetic peptides. The peptides used to inhibit mTld activity were prepared by Anova, formerly Cybersyn or by Sigma using state-of-the-art methods. Three peptides were prepared to test the residues critical for inhibitory potential. The sequences were designed based on conserved sequence found in chordin, which spans from residue 720 to 735 upstream from the mTld cleavage site between amino acids Ser853 and Asp854. The S1.16

Table 1. Sequence of peptides tested for inhibition of procollagen C-endopeptidase activity in mTld

Peptide name[a] (corresponding residues in chordin)	Amino acid sequence	M.W.	Soluble in	pH in water
S1.9 (737–745)	Ser-Gln-Pro-Val-His-Leu-Pro-Asp-Gln	1245	water	neutral
S1.16 (720–735)	Gln-Lys-Arg-Thr-Val-Ile-Cys-Asp-Pro-Ile-Val-Cys-Pro-Pro-Leu-Asn	2022	water	neutral
S1.16c (720–735)	Gln-Lys-Arg-Thr-Val-Ile-*Cys*-Asp-Pro-Ile-Val-*Cys*-Pro-Pro-Leu-Asn	2019	water	neutral
S1.19 (727–745)	Asp-Pro-Ile-Val-Cys-Pro-Pro-Leu-Asn-Cys-Ser-Gln-Pro-Val-His-Leu-Pro-Asp-Gln	2298	water	neutral
AS1.1 (720–735)	Gln-Lys-Arg-Thr-Val-Ile-*Ser*-Asp-Pro-Ile-Val-Cys-Pro-Pro-Leu-Asn	2005	water	neutral
AS2.1 (720–735)	Gln-Lys-Arg-Thr-Val-Ile-Cys-Asp-Pro-Ile-Val-*Ser*-Pro-Pro-Leu-Asn	2005	water	neutral
AS3.1 (720–735)	Gln-Lys-Arg-Thr-Val-Ile-*Ser*-Asp-Pro-Ile-Val-*Ser*-Pro-Pro-Leu-Asn	1989	water	neutral
AS1.11 (726–731)	Cys-Asp-Pro-Ile-Val-Cys	874	water	neutral
AS1.11c (726–731)	*Cys*-Asp-Pro-Ile-Val-*Cys*	871	water	neutral

[a]All peptides were conjugated with biotin on their amino-termini.

comprises of 16 amino acids identical in *Xenopus*, chick, and mammals chordin. The other two peptides partially overlapping S1.19 and S1.9 contained sequences, respectively from residue 727 to 745 and from 737 to 745 in chordin. Peptide variants were prepared as sequence modifications of the original peptide S1.16. The sequences of the peptides are listed in Table 1. Briefly, modifications have been made to peptide S1.16, which replaced Cys726 or Cys731 or both the cysteines at once with Ser (peptides AS1.1, AS2.1 and AS3.1, respectively, in Table 1). Finally, the smallest peptide AS1.11 tested in the mTld activity assays contained the six amino acids flanked by the two cysteines (Table 1). Also, variants of peptides S1.16 and AS1.11 (Table 1) were prepared by disulfide bridge formation upon peptide synthesis and assayed for inhibition of mTld procollagen C-endopeptidase activity.

Enzymatic activity assays. The enzyme inhibition assays of their activity were performed using previously described protocols (Sieron *et al.*, 1993; Fertala *et al.*, 1994; Hojima *et al.*, 1985; 1989; Arnold *et al.*, 1998). Briefly, two units of active mTld was incubated for 2 h at 35°C with about 0.3 μg of procollagen type I in a buffer containing 0.15 M NaCl, 7 mM $CaCl_2$, 0.015% Brij 30, 0.01% NaN_3, and 50 mM Tris/HCl, pH 7.5. To assay inhibition of the enzyme activity, the synthetic peptides dissolved in the assay buffer were added at concentrations indicated in Fig. 1B. The total volume of the reaction mixture was 20 μl. The cleavage was terminated by adding 0.25 volumes of 5-times concentrated protein sample buffer (10% glycerol, 0.5% SDS, 25 mM EDTA, and 0.05% bromophenol blue in 0.3 M Tris/HCl, pH 6.8) and boiling the sample for 5 min. Reaction products were separated in 7.5% SDS/polyacrylamide gels and assayed following staining and documenting with a digital camera using ImageQuant software (Molecular Dynamics). The comparison of relative enzymatic activities was based on the amount of free C-propeptide trimer measured with the help of the ImageQuant program (Molecular Dynamics, CA). The program counts pixels corresponding to the amount of stain in the measured spot in the gel, which subsequently is converted by the "reader" to an image. The results presented as a picture of SDS gel are from one representative experiment out of at least six reproducible assays.

Similarly, two units of chick ADAMTS-2 was incubated for 2 h at 35°C with 0.3 μg of procollagen type I in a final volume of 20 μl of buffer containing 0.1 M NaCl, 7 mM $CaCl_2$, 0.015% Brij 30, 0.02% NaN_3, and 25 mM Tris/HCl, pH 7.5. Products of the proteinase cleavage were separated in SDS/polyacrylamide gels and analyzed for liberation of N-propetides using the same procedure as for analysis of procollagen C-endopeptidase activity. Peptides were premixed at 25°C with the enzymes 30 min prior to incubation with the substrate at concentrations indicated in Fig. 1B. The picture of the gel represents one of at least six reproducible assays.

Assay of interactions of inhibiting peptides with the enzyme. Synthetic biotinylated peptides that blocked procollagen C-endopeptidase activity were characterized for their interactions with the enzyme or its fragments. The peptide was incubated with procollagen C-endopeptidase or its α-chymotrypsin-derived fragments under conditions previously described (Sieron *et al.*, 2000). The reaction products were analyzed after cross-linking by SDS/PAGE followed by Western blot and detection of biotin with specific anti-biotin antibody conjugated with HRP. Results of interactions of peptides with enzyme without subsequent cross-linking were analyzed by standard reverse phase high pressure liquid chromatography using Hi-Pore Reverse Phase Column RP-318 250 mm × 4.6 mm (BioRad) and standard buffers. The results presented in Fig. 3 are typical examples of one out of at least six reproducible repeated reverse phase HPLC column chromatography profiles.

RESULTS

Enzymatic activity of human mTld is specifically inhibited by synthetic peptides

A series of synthetic peptides were tested for their ability to block recombinant human mTld activity on procollagen I. Two peptides, S1.16 and S1.19 inhibited this activity (Fig. 1A) but not the activity of ADAMTS-2 on procollagen I (Fig. 1B). The blocking activity of peptide S1.16 was mapped by evaluating the activity of shorter peptides and peptides with Cys residues substituted with serines. Neither the cyclic version of peptide S1.16 nor peptide AS3.1, which did not contain any cysteines, blocked the enzyme activity (Fig. 1C). Peptides AS1.1 and AS2.1 that contained a single cysteine retained their inhibitory potential at a level similar to the original peptide S1.16. The assay of inhibition of the enzymatic activity with a short version of the peptide containing two flanking Cys residues (AS1.11) revealed the minimal 6-amino-acid sequence critical for blocking the procollagen C-endopeptidase activity on procollagen I (Fig. 1D).

Peptide S1.16 binds to non-protease domains of mTld

Biotinylated peptide S1.16 was incubated with intact human recombinant mTld or its frag-

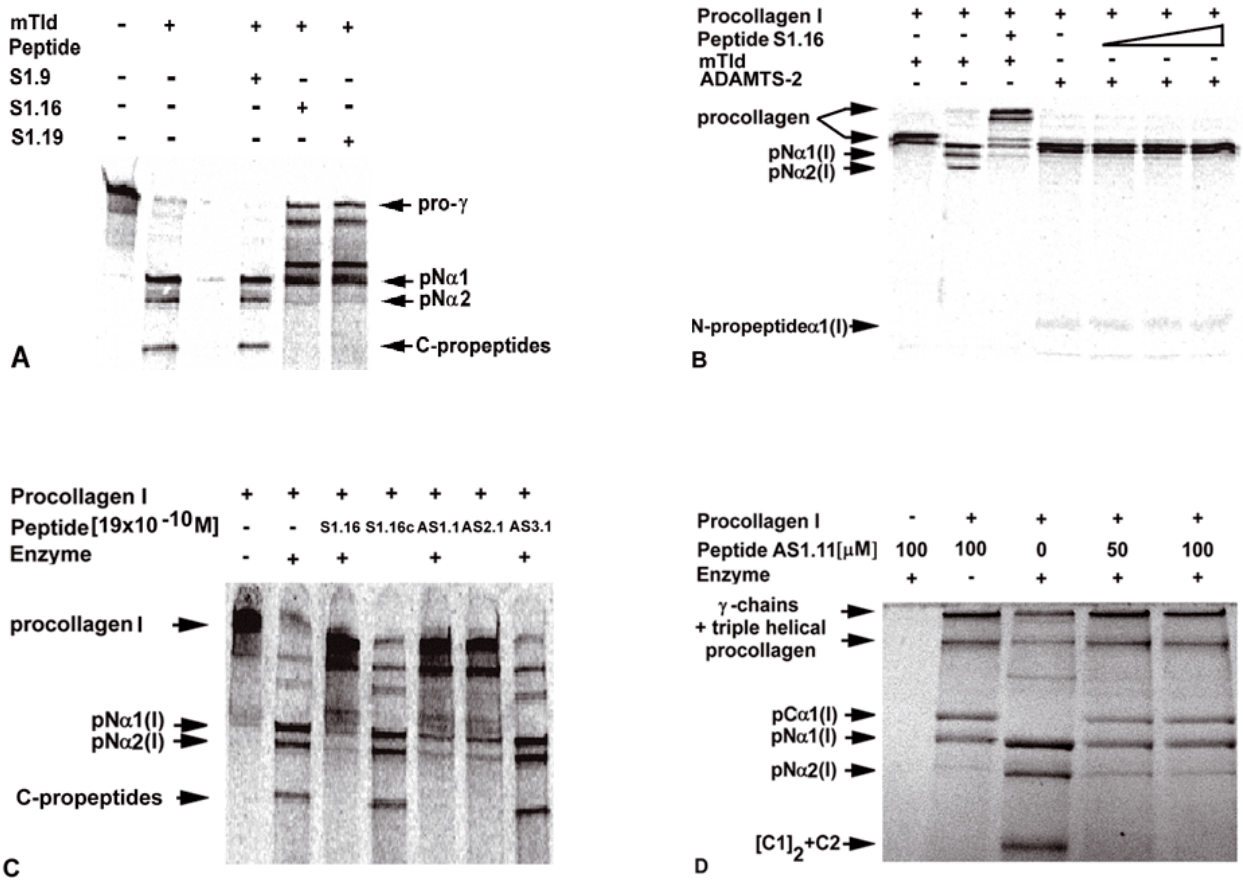

Figure 1. Assay of specific inhibitory activity of synthetic peptide inhibitors on procollagen C- and N-endopeptidases.

A. Assay of procollagen C-endopeptidase enzymatic activity on procollagen type I in the presence of peptides with amino-acid composition described in Methods section. The peptides were at a concentration of 19×10^{-10} M. B. Assay of inhibitory effect of peptide S1.16 on procollagen N-endopeptidase. The peptide concentrations were 19×10^{-10}, 38×10^{-10}, and 76×10^{-10} M. As a positive control for peptide inhibitory activity procollagen C-endopeptidase inhibition assay was conducted (first three lanes from left); C. Variations of peptide S1.16 as described in Methods assayed for inhibition of enzymatic activity of procollagen C-endopeptidase; D. Determination of the shortest variant of peptide S1.16 inhibiting enzymatic activity of procollagen C-endopeptidase.

ments obtained following cleavage with α-chymotrypsin as previously described by Sieron *et al.* (2000). Mixtures of purified products of mTld digestion with α-chymotrypsin were separated on 12% SDS/polyacrylamide gels and transferred to PVDF membranes. The membranes were probed with biotinylated S1.16 peptide. The results were examined using HRP-conjugated anti-biotin antibody and the ECL method for visualization. The biotinylated S1.16 peptide was bound to the full-length mTld and to its digestion products of about 90, 60 and 40 kDa (Fig. 2), which did not contain the catalytic zinc-binding protease domain (see also Sieron *et al.*, 2000). The peptides that were obtained by modifications of peptide S1.16 by disulfide bond formation between the two Cys residues (S1.16c) or AS1.11 by replacement of the second (AS2.1) or both cysteines (AS3.1) with serines showed just some interaction with the procollagen C-endopeptidase fragments obtained following α-chymotrypsin digestion, however, the pattern was different in that the non-blocking peptides did not bind to the 60 kDa fragment as the active peptides did (not shown).

Further analysis of the interaction between the S1.16 peptide and mTld revealed that the peptide changed its conformation in the presence of the enzyme by forming an internal disulfide bridge (Fig. 3). A gel filtration reverse-phase analysis of the incubation products with mTld under reducing conditions revealed retention time the same as for the peptide incubated without mTld (compare curve 5 with curve 2 in Fig. 3). The cyclic peptide did not block mTld activity but the linear peptide did inhibit its enzymatic activity, however, in the presence of the enzyme it formed a disulfide bond in a time-dependent manner (Fig. 3). A mixture of the peptide and enzyme pre-incubated for 2 h lacked the activ-

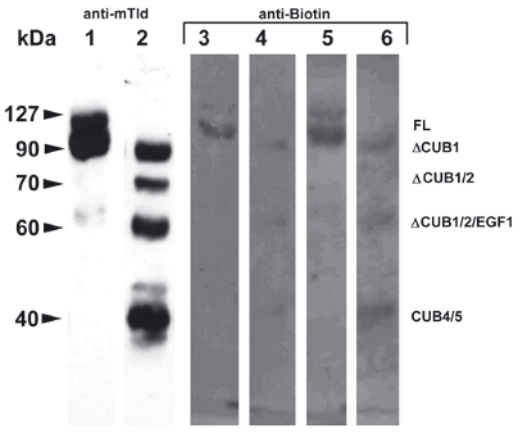

Figure 2. Interactions of synthetic peptide inhibitors with domains of procollagen C-endopeptidase.
Odd lanes contained intact procollagen C-endopeptidase and even lanes contained products of procollagen C-endopeptidase treated for 15 h with α-chymotrypsin at a concentration of 10 μg/ml as described previously by Sieron *et al.* (2000). The enzyme activity was stopped by addition of soybean inhibitor at a concentration of 50 μg/ml. Lanes 1 and 2 were probed with polyclonal anti-procollagen C-endopeptidase antibodies. Lanes 3 and 4 are reprobed lanes 1 and 2 for detection of biotinylated peptide S1.16 bound to full-length (lane 3) or α-chymotrypsin-generated fragments (lane 4) of procollagen C-endopeptidase using anti-biotin antibodies conjugated with horseradish peroxidase. Lanes 5 and 6 were lanes 1 and 2 reprobed second time for detection of biotinylated peptide AS1.11 bound to full length (lane 5) α-chymotrypsin-generated fragments (lane 6) of procollagen C-endopeptidase with anti-biotin antibodies conjugated with horseradish peroxidase.

ity on procollagen I, whereas pre-incubation of the enzyme with the cyclic peptide S1.16c did not abolish the activity on procollagen I (Fig. 1C).

DISCUSSION

The ability to control collagen fibril formation and their quality seems to be of general importance in such pathologies as fibrotic diseases. Inhibition of angiogenesis is important for fighting tumors and un-controlled cell proliferation in growing tumors (Byth *et al.*, 2001; Zeng *et al.*, 2005). Numerous attempts have been undertaken to develop specific inhibitors of biologically active molecules. Some investigators used peptides derived from natural proteins (Wu *et al.*, 1997; Huh *et al.*, 2005; Zhang *et al.*, 2006) others tested synthetic molecules directed against the action of particular factors such as VEGF or PDGF preventing binding to their receptors (Celik *et al.*, 2005; Sun *et al.*, 2005).

In our work we aimed to interrupt correct ECM formation by blocking the action of one of the metalloproteinase crucial for embryonic development. The key role of BMP-1 variants was found critical in transgenic mice which died at birth due to numerous organ abnormalities resulting from abnormal structure of collagen fibrils in their ECM (Suzuki *et al.*, 1996).

In the formation of collagen fibrils, one of the basic structural components of the ECM, procollagen N- and C-endopeptidases are key players (Lapiere *et al.*, 1971; Leung *et al.*, 1979; Prockop *et al.*, 1979).

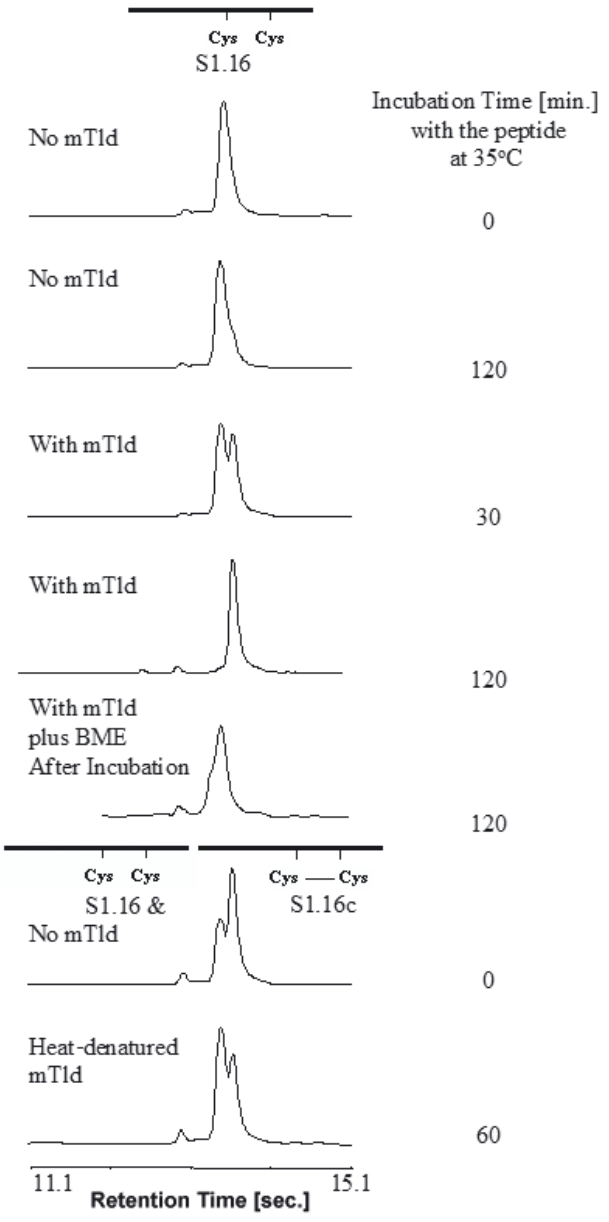

Figure 3. Structural changes of peptide inhibitor S1.16 following incubation with procollagen C-endopeptidase.
Positions of cysteines in the schematics of S1.16 and S1.16c are indicated. Horizontal line linking Cys in peptide S1.16c indicates disulfide bridge. With mTld – means incubation of the peptide in the presence of mTld. No mTld – means incubation of the peptide alone. For heat denaturation the mTld prior to the assay was incubated at 90°C for 10 min.

Knockout mice for either metalloproteinase were severely affected. Mice lacking active procollagen C-endopeptidase died at birth, those without active procollagen N-endopeptidase developed symptoms of dermatosparaxis (EDS VIIC) (Lapiere *et al.*, 1971; Li *et al.*, 2001). Based on observations that collagen monomers retaining C-propeptides do not form fibrils at all, whereas, monomers with N-propeptides form abnormal ribbon-like structures of weak fibrils, we proposed that in pathological conditions characterized by high content of collagen fibrils their formation could be inhibited by, knocking down the procollagen C-endopeptidase activity.

How and where the inhibitor acts can be determined based on crystals obtained from mixtures of the inhibitor and its target (Grams *et al.*, 1996). A different way to achieve the same information is to analyze the interaction of the partners in solution directly as Sieron and co-workers reported elsewhere (Sieron *et al.*, 2000). Here, we used the latter approach to show that the interaction of the inhibiting peptides occurred through the regulatory part of the enzyme consisting of five CUB domains and two EGF-like motifs. Previously, we identified that CUB1 to CUB3 and both EGF motifs but not CUB4 and CUB5 bound procollagen type I (Sieron *et al.*, 2000). Subsequently, others identified that CUB1 is required for proper folding and secretion of the enzyme to the extracellular compartment and both CUB1 and CUB2 together with the protease domain form the minimal structure required for procollagen C-endopeptidase activity (Garrigue-Antar *et al.*, 2004), whereas both EGF-like motifs hold CUB4/5 domains of mTld in locations that exclude substrates cleavable by BMP-1 (Hartigan *et al.*, 2003). The amino-acid residue in CUB2 essential for the C-endopeptidase activity seemed to be E483 in the β4-β5 loop of this domain (Grams *et al.*, 1996). The interaction of both peptides observed in our assays was with four α-chymotrypsin-generated peptides, containing all non-catalytic C-terminal domains, a fragment lacking the first or two CUB domains, one lacking the first two CUBs and the EGF domain, and one consisting of just the last two CUB domains. However, the interaction of the inhibitory peptide with the polypeptide lacking the first two CUB domains was the weakest, probably as a result of its lowest recovery following α-chymotrypsin cleavage. Altogether, the minimal fragment interacting with the inhibitors in our tests seemed to be CUB4/5 since it was present in all analyzed fragments obtained by digestion with α-chymotrypsin. This result also explains the specificity of the inhibitor to just mTld but not ADAMTS-2 or astacin and different MMPs (not shown). The inhibitory peptide could interfere with the action of the second EGF-like motif possibly by binding to the CUB4/5 domain and in this way changing its orientation in the entire molecule. Therefore, our results support the hypothesis of Hartigan and co-workers (2003) that the CUB4/5 domains may be responsible for mTld substrate specificity.

Somewhat surprising is our observation that the circular peptides were not active in contrast to their linear forms. It is commonly understood that cyclic peptides usually exhibit better activities then their linear forms. Here, we propose that the site for binding the peptide in the enzyme is hidden somewhere inside the CUB4/5 structure or in just one of these two domains and only after reaching it the inhibitor changes its conformation and a disulfide bond can be formed between two Cys residues stabilizing its interaction with the enzyme. The inhibitory peptide showed strong affinity for the enzyme as observed in assays with denatured mTld. Although mTld was heat-denatured, part of the inhibitory peptide pool was converted to the cyclic form. This also explains binding of the peptide to the α-chymotrypsin-generated fragmentes electro-blotted following heat denaturation and SDS/PAGE. The inhibitory action of peptides AS1.1 and AS2.1 lacking either of the two cysteines indicates that cysteine is critical for peptide activity at least in assays conducted *in vitro*. The absence of co-elution of the peptide with the enzyme indicates that the interaction is disrupted under the assay conditions applied here.

Our results indicate that the activity of procollagen C-endopeptidase could be specifically and efficiently blocked by a synthetic peptide with the amino-acid sequence found to be conserved in some of the enzyme substrates, namely chordin. Thus further studies on its inhibitory activity in biological systems is needed to evaluate if it could be a good candidate for the development of a drug inhibiting fibrosis and angiogenesis.

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REFERENCES

Amano S, Scott IC, Takahara K, Koch M, Champliaud MF, Gerecke DR, Keene DR, Hudson DL, Nishiyama T, Lee S, Greenspan DS, Burgeson RE (2000) Bone morphogenetic protein 1 is an extracellular processing enzyme of the laminin 5 γ2 chain. *J Biol Chem* **275**: 22728–22735.

Arnold WV, Sieron AL, Fertala A, Bachinger HP, Mechling D, Prockop DJ (1997) A cDNA cassette system for the synthesis of recombinant procollagens. Variants of procollagen II lacking a D-period are secreted as triple-helical monomers. *Matrix Biol* **16**: 105–116.

Arnold WV, Fertala A, Sieron AL, Hattori H, Mechling D, Bachinger HP, Prockop DJ (1998) Recombinant procollagen II: Deletion of D period segments identifies sequences that are required for helix stabilization and generates a temperature-sensitive N-proteinase cleavage site. *J Biol Chem* **273**: 31822–31828.

Blader P, Rastegar S, Fisher N, Strähle U (1997) Cleavage of the BMP-4 antagonist Chordin by zebrafish Tolloid. *Science* **278**: 1937–1940.

Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, Carmeliet P, Collen D, Degen JL (1996a) Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. *Proc Natl Acad Sci USA* **93**: 5899–5904.

Bugge TH, Kombrinck KW, Flick MJ, Daugherty CC, Danton MJ, Degen JL (1996b) Loss of fibrinogen rescues mice from the pleiotropic effects of plasminogen deficiency. *Cell* **87**: 709–719.

Byth HA, Mchunu BI, Dubery IA, Bornman L (2001) Assessment of a simple, non-toxic Alamar blue cell survival assay to monitor tomato cell viability. *Phytochem Anal* **12**: 340–346.

Celik I, Surucu O, Dietz C, Heymach JV, Force J, Hoschele I, Becker CM, Folkman J, Kisker O (2005) Therapeutic efficacy of endostatin exhibits a biphasic dose-response curve. *Cancer Res* **65**: 11044–11050.

Colige A, Li SW, Sieron AL, Nusgens BV,Prockop DJ, Lapiere CM (1997) cDNA cloning and expression of bovine procollagen I N-proteinase: a new member of the superfamily of zinc-metalloproteinases with binding sites for cells and other matrix components. *Proc Natl Acad Sci USA* **94**: 2374–2379.

Cronshaw AD, Fothergill-Gilmore LA, Hulmes DJS (1995) The proteolytic processing site of the precursor of lysyl oxidase. *Biochem J* **308**: 279–284.

Dvorak HF, Brown LF, Detmar M, Dvorak AM (1995) Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* **146**: 1029–1039.

Fertala A, Sieron AL, Ganguly A, Li SW, Ala-Kokko L, Anumula KR, Prockop DJ (1994) Synthesis of recombinant human procollagen II in a stably transfected tumour cell line (HT1080). *Biochem J* **298**: 31–37.

Folkman J, D'Amore PA (1996) Blood vessel formation: what is its molecular basis? *Cell* **87**: 1153–1155.

Gailit J, Clark RA (1994) Wound repair in the context of extracellular matrix. *Curr Opin Cell Biol* **6**: 717–725.

Garrigue-Antar L, Francois V, Kalder KE (2004) Deletion of epidermal growth factor-like domains converts mammalian tolloid into a chordinase and effective procollagen C-proteinase. *J Biol Chem* **279**: 49835–49841.

Ge G, Greenspan DS (2006) BMP1 controls TGFβ1 activation *via* cleavage of latent TGFβ-binding protein. *J Cell Biol* **175**: 111–120

Ge G, Seo NS, Liang X, Hopkins DR, Höök M, Greenspan DS (2004) Bone morphogenetic protein-1/Tolloid-related metalloproteinases process osteoglycin and enhance its ability to regulate collagen fibrillogenesis. *J Biol Chem* **279**: 41626–41633.

Grams F, Dive V, Yiotakis A, Yiallouros I, Vassiliou S, Zwilling R, Bode W, Stocker W (1996) Structure of astacin with a transition-state analogue inhibitor. *Nat Struct Biol* **3**: 671–675.

Hartigan N, Garrigue-Antar L, Kadler KE (2003) Bone morphogenetic protein (BMP)-1: identification of the minimal domain structure for C-proteinase activity. *J Biol Chem* **278**: 18045–18049.

Hojima Y, van der Rest M, Prockop DJ (1985) Type I procollagen carboxylterminal proteinase from chick embryo tendons. *J Biol Chem* **260**: 15996–16003.

Hojima Y, McKenzie JA, van der Rest M, Prockop DJ (1989) Type I procollagen N-proteinase from chick embryo tendons. Purification of a new 500-kDa form of the enzyme and identification of the catalytically active polypeptides. *J Biol Chem* **264**: 11336–11345.

Hojima Y, Behta B, Romanic AM, Prockop DJ (1994) Cleavage of type I procollagen by C- and N-proteinases is more rapid if the substrate is aggregated with dextran sulfate or polyethylene glycol. *Anal Biochem* **223**: 173–180.

Huh JI, Calvo A, Stafford J, Cheung M, Kumar R, Philp D, Kleinman HK, Green JE (2005) Inhibition of VEGF receptors significantly impairs mammary cancer growth in C3(1)/Tag transgenic mice through antiangiogenic and nonantiangiogenic mechanisms. *Oncogene* **24**: 790–800.

Imamura Y, Steiglitz BM, Greenspan DS (1998) Bone morphogenetic protein-1 processes the NH_2–terminal propeptide, and a furin-like proprotein convertase processes the COOH-terminal propeptide of pro-α1(V) collagen. *J Biol Chem* **273**: 27511–27517.

Kessler E, Takahara K, Biniaminov L, Brusel M, Greenspan DS (1996) Bone morphogenetic protein-1: the type I procollagen C-proteinase [see comments]. *Science* **271**: 360–362.

Lapiere CM, Lenaers A, Kohn LD (1971) Procollagen peptidase: an enzyme excising the coordination peptides of procollagen. *Proc Natl Acad Sci USA* **68**: 3054–3058.

Leung MK, Fessler LI, Greenberg DB, Fessler JH (1979) Separate amino and carboxyl procollagen peptidases in chick embryo tendon. *J Biol Chem* **254**: 224–232.

Li SW, Sieron AL, Fertala A, Hojima Y, Arnold WV, Prockop DJ (1996) The C-proteinase that processes procollagens to collagens is identical to the protein previously identified as bone morphogenic protein-1. *Proc Natl Acad Sci USA* **93**: 4127–5130.

Li SW, Arita M, Fertala A, Bao Y, Kopen GC, Langsjo TK, Hyttinen MM, Helminen HJ, Prockop DJ (2001) Transgenic mice with inactive alleles for procollagen N-proteinase (ADAMTS-2) develop fragile skin and male sterility. *Biochem J* **355**: 271–278.

Marques G, Musacchio M, Simell MJ, Wunnenberg-Stapleton K, Cho KWY, O'Connor M (1997) Production of a DPP activity gradient in the early *Drosophila* embryo through the opposing actions of the SOG and TLD proteins. *Cell* **91**: 417–426.

Montesano R, Pepper MS, Mohle-Steinlein U, Risau W, Wagner EF, Orci L (1990) Increased proteolytic activity is responsible for the aberrant morphogenetic behavior of endothelial cells expressing the middle T oncogene. *Cell* **62**: 435–445.

Panchenko MV, Stelter-Stevenson WG, Trupetskoy OV, Gacheru SN, Kagan HM (1996) Metalloproteinase activity secreted by fibrogenic cells in the processing of prolysyl oxidase. Potential role of procollagen C-proteinase. *J Biol Chem* **271**: 7113–7119.

Piccolo S, Agius E, Lu B, Goodman S, Dale L, De Robertis EM (1997) Cleavage of Chordin by Xolloid metalloprotease suggests a role for proteolytic processing in the regulation of Spemann organizer activity. *Cell* **91**: 407–416.

Prockop DJ, Kivirikko KI (1984) Heritable diseases of collagen. *N Engl J Med* **311**: 376–386.

Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA (1979) Biosynthesis of collagen. *N Engl J Med* **301**: 12–23, 75–85.

Rattenholl A, Pappano WN, Koch M, Keene DR, Kadler KE, Sasaki T, Timpl R, Burgeson RE, Greenspan DS, Bruckner-Tuderman L (2002) Proteinases of the bone morphogenetic protein-1 family convert procollagen VII to mature anchoring fibril collagen. *J Biol Chem* **277**: 26372–26378.

Risau W (1997) Mechanisms of angiogenesis. *Nature* **386**: 671–674.

Scott IC, Imamura Y, Pappano WN, Troedel JM, Recklies AD, Roughley PJ, Greenspan DS (2000) Bone morphogenetic protein-1 processes probiglycan. *J Biol Chem* **275**: 30504–30511.

Senger DR (1996) Molecular framework for angiogenesis: a complex web of interactions between extravasated plasma proteins and endothelial cell proteins induced by angiogenic cytokines. *Am J Pathol* **149**: 1–7.

Sieron AL, Fertala A, Ala-Kokko L, Prockop DJ (1993) Deletion of a large domain in recombinant human procollagen II does not alter the thermal stability of the triple helix. *J Biol Chem* **268**: 21232–21237.

Sieron AL, Tretiakova A, Jameson BA, Segall ML, Lund-Katz S, Khan MT, Li SW, Stöcker W (2000) Structure and function of procollagen C-proteinase (mTolloid) domains determined by protease digestion, circular dichroism, binding to procollagen type I, and computer modeling. *Biochemistry* **39**: 3231–3239.

Steiglitz BM, Ayala M, Narayanan K, George A, Greenspan DS (2004) Bone morphogenetic protein-1/tolloid-like proteinases process dentin matrix protein-1. *J Biol Chem* **279**: 980–986.

Sun J, Wang DA, Jain RK, Carie A, Paquette S, Ennis E, Blaskovich MA, Baldini L, Coppola D, Hamilton AD, Sebti SM (2005) Inhibiting angiogenesis and tumorigenesis by a synthetic molecule that blocks binding of both VEGF and PDGF to their receptors. *Oncogene* **24**: 4701–4709.

Suzuki N, Labosky PA, Furuta Y, Hargett L, Dunn R, Fogo AB, Takahara K, Peters DM, Greenspan DS, Hogan BL (1996) Failure of ventral body wall closure in mouse embryos lacking a procollagen C-proteinase encoded by BMP-1, a mammalian gene related to *Drosophila* tolloid. *Development* **122**: 3587–3595.

Takahara K, Lyons GE, Greenspan DS (1994) Bone morphogenetic protein-1 and a mammalian toloid homologue (mTld) are encoded by alternatively spliced transcripts which are differentially expressed in some tissues. *J Biol Chem* **269**: 32572–32578.

Wolfman NM, McPherron AC, Pappano WN, Davies MV, Song K, Tomkinson KN, Wright JF, Zhao L, Sebald SM, Greenspan DS, Lee SJ (2003) Activation of latent myostatin by the BMP-1/tolloid family of metalloproteinases. *Proc Natl Acad Sci USA* **100**: 15842–15846.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988) Novel regulators of bone formation: Molecular clones and activities. *Science* **242**: 1528–1534.

Wu Z, O'Reilly MS, Folkman J, Shing Y (1997) Suppression of tumor growth with recombinant murine angiostatin. *Biochem Biophys Res Commun* **236**: 651–654.

Zhang T, Xu Q, Chen FR, Han QD, Zhang YY (2004) Yeast two-hybrid screening for proteins that interact with α1-adrenergic receptors. *Acta Pharmacol Sin* **25**: 1471–1478.

Zhang Y, Ge G, Greenspan DS (2006) Inhibition of bone morphogenetic protein 1 by native and altered forms of α_2-macroglobulin. *J Biol Chem* **281**: 39096–39104.

Zeng Q, Li S, Chepeha DB, Giordano TJ, Li J, Zhang H, Polverini PJ, Nor J, Kitajewski J, Wang CY (2005) Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling. *Cancer Cell* **8**: 13–23.