Identification of binding partners interacting with the α1-N-propeptide of type V collagen

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INTRODUCTION

The ECM (extracellular matrix) is composed of a complex combination of macromolecules organized into tissue-specific networks. The major structural ECM components are the fibrillar collagens, which aggregate into highly organized banded collagen fibrils. The low-abundant fibrillar type V collagen plays an important role in the control of type I/V collagen fibrillogenesis, as illustrated by studies in genetically modified mouse models, which demonstrated that type V collagen is involved in fibril nucleation and regulation of collagen fibril diameter [1–3]. Furthermore, although embryonic fibroblasts of col5a1−/− mice synthesize and secrete normal amounts of type I collagen, collagen fibrils are virtually absent and mice die at the onset of organogenesis [3]. These findings indicate that a certain threshold of type V collagen should be maintained for normal collagen fibrillogenesis.

The most broadly distributed isoform of type V collagen is the [α1(V)]α2(V) heterotramer [1]. During fibril formation, this heterotrimer is incorporated into type I collagen fibrils, thereby regulating the diameter of these fibrils through partial retention of the α1(V)-N-propeptide [1,3]. The current working model predicts that the entire type V collagen triple helix is buried within the fibril, whereas type I collagen is present along the fibril surface. The α1(V)-N-propeptide extends outwards from the gap zones of the heterotypic fibrils and is thus the only part of the type V collagen molecule that emerges at the surface of these fibrils [1].

The α1(V)-N-propeptide exhibits a unique multidomain structure (Figure 1A): a TSPN-1 (thrombospondin-1 N-terminal domain-like) region, also known as the PARP (proline/arginine-rich) protein domain, followed by a VAR (variable region), which together form the NC3 (non-collagenous 3) domain and a short interrupted triple helix (COL2). The COL2 region is adjacent to a short stretch, the NC2 domain. This flexible hinge region, which precedes the α1(V) major triple helix (COL1), allows the N-propeptide to project away from the major axis of the molecule. As such, the COL2 domain extends across the gap zone, placing the non-collagenous domains at the surface of heterotypic type I/V collagen fibrils [1]. The α2(V)-N-propeptide is shorter and less complex than its α1(V) counterpart. It is composed of a CRR (cysteine-rich repeat) domain, which precedes a small COL2

Abbreviations used: AD, activation domain; ADAMTS-2, a disintegrin and metalloproteinase with thrombospondin motifs 2; BMP-1, bone morphogenetic protein 1; CRR, cysteine-rich repeat; CUB, C1r/C1s, urchin embryonic growth factor and BMP-1 domain; ECM, extracellular matrix; EDC, N-ethyl-N-(3-dimethylaminopropyl)carbodi-imide; EDS, Ehlers–Danlos syndrome; FN, fibronectin; HEK, human embryonic kidney; MLLT1, myeloid/lymphoid or mixed-lineage leukaemia (trithorax homologue, Drosophila) translocated to 1; MMP-2, matrix metalloproteinase 2; NC2, flexible hinge region; NC3, non-collagenous 3; NHS, N-hydroxysuccinimide; PARP, proline/arginine-rich protein; PCGF2, polycomb group ring finger 2; PCPE-1, procollagen C-proteinase enhancer-1; QDO, quadruple dropout; RU, resonance unit; SPR, surface plasmon resonance; TGF-β1, transforming growth factor β1; TIMP-1, tissue inhibitor of metalloproteinases-1; TNC, tenascin C; TSPN-1, thrombospondin-1 N-terminal domain-like; UCMD, Ulrich congenital muscular dystrophy; VAR, variable region; vWF-A, von Willebrand factor type A; X-α-Gal, 5-bromo-4-chloroindol-3-yl α-d-galactopyranoside; Y2H, yeast two-hybrid; ZC3H4, zinc finger CCCH-type-containing 4.

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domain (Figure 1A) [4]. Processing of the \( \alpha 1(V) \)-N-propeptide is complex as it occurs in a tissue-specific manner at different sites and can be mediated by BMP-1 (bone morphogenetic protein 1)/procollagen C-proteinase [4,5] and/or ADAMTS-2 (a disintegrin and metalloproteinase with thrombospondin motifs 2) enzymes [6] (Figure 1), whereas the \( \alpha 2(V) \)-N-propeptide remains intact in the mature molecule [4]. Whereas BMP-1 cleavage of the \( \alpha 1(V) \)-N-propeptide, which represents the \textit{in vivo} dominant processing activity, releases the TSPN-1 domain and retains the VAR, COL2 and NC2 domains [4] (Figure 1), ADAMTS-2 cleavage releases the entire NC3 domain (TSPN-1 and VAR) (Figure 1A) [6].

Dysregulation of collagen fibrillogenesis is a hallmark of several subtypes of the EDS (Ehlers–Danlos syndrome). Classic EDS (MIM# 130 000 and MIM# 130 010) is characterized by fragile and hyperextensible skin, poor wound healing with atrophic scarring and joint hypermobility. As a result of impaired collagen fibrillogenesis, the dermis of classic EDS patients shows a decrease in fibril density and the presence of large irregular collagen fibrils (called collagen ‘cauliflowers’) [7]. Approx. 50% of the patients with classic EDS harbour a heterozygous mutation in COL5A1 or COL5A2, encoding the \( \alpha 1(V) \)- and the \( \alpha 2(V) \)-collagen chains respectively. Most commonly, mutations introduce a premature termination codon, thereby leading to a non-functional COL5A1 allele and a decreased amount of type V collagen protein (COL5A1 haploinsufficiency) [8,9]. A minority of mutations affects the type V collagen structure, including a number of glycine substitutions and exon-skipping mutations in the collagen triple helix, as well as C-propeptide mutations which interfere with the incorporation of the mutant chains into the type V collagen heterotrimer [8,9]. Recently, we have shown that mutations in the \( \alpha 1(V) \)-signal peptide result in decreased type V collagen secretion into the ECM [10]. Interestingly, until now, only two mutations in the highly conserved \( \alpha 1(V) \)-N-propeptide have been described allowing the expression of the mutant protein: a single homozygous amino acid substitution (p.G530S) [11] and a splice-acceptor mutation (IVS4-2A>G) [12] (Figure 1A). Whereas the p.G530S substitution is located in the COL2 domain and is suggested to interfere with the correct folding of this region, the IVS4-2A>G splice-acceptor mutation generates different mutant transcripts, affecting the VAR domain and deleting the BMP-1 cleavage site and two of the four cysteine residues. These latter transcripts are translated, and mutant proteins with abnormal \( \alpha 1(V) \)-N-propeptides are secreted and interfere with normal collagen fibrillogenesis.

Various binding partners to type V collagen have already been identified in previous studies, including thrombospondin, insulin, the small leucine-rich proteoglycan decorin, heparin (as reviewed in [13]), heparan sulfate [14], biglycan [15], osteonectin/SPARC (secreted protein acidic and rich in cysteine) [16], tenasin-X [17], integrins [18], DDRs (discoidin domain receptors) 1 and 2 [19] and uPARAP (urokinase plasminogen activator receptor-associated protein) [20]. However, since pepsinized type V collagen was used for all these studies, the ligands identified only bind to the type V collagen triple helix, as the N- and the C-propeptides are removed during pepsin digestion. In the present study, we intended to identify proteins that interact with the \( \alpha 1(V) \)-N-propeptide domain using the Y2H (yeast two-hybrid) approach. We selected the \( \alpha 1(V) \)-N-propeptide because this is the only domain that can mediate type V collagen interactions in the ECM as it protrudes from the surface of heterotypic type I/V collagen fibrils. Furthermore, we decided to generate a human dermal skin cDNA library as a prey library because the skin is the major affected tissue in classic EDS patients. Identifying new interaction partners of the \( \alpha 1(V) \)-N-propeptide will yield further insights into the process of collagen assembly and the pathogenesis of (classic) EDS. Moreover, these molecules may represent new candidate genes for hitherto unresolved EDS cases.
MATERIALS AND METHODS

Preparation of bait construct, library preparation and Y2H experiment

To screen for new interactions between the α1(V)-N-propeptide and proteins expressed in the ECM of human dermal fibroblasts, we used the Matchmaker Two-Hybrid System (Clontech Laboratories). All procedures were performed according to the manufacturer’s protocol. In order to generate the Y2H bait construct, pGBKTT7-pNα1(V), we started from the previously described plasmid pCEP4-pNα1(V) [5]. We performed a PCR on the pCEP4-pNα1(V) plasmid using primers (Supplementary Table S1 at http://www.BiochemJ.org/bj/433/bj4330371add.htm) that introduced unique restriction endonuclease sites for unidirectional cloning into the yeast DNA-binding domain vector pGBKTT7 (Clontech Laboratories). The pGBKTT7 vector carries the tryptophan reporter gene. To verify whether the pNα1(V) insert was cloned in-frame, the resulting pGBKTT7-pNα1(V) bait vector was sequenced.

To generate our prey cDNA library, poly(A)^+ RNA was isolated from human dermal fibroblasts and converted into cDNA with random primers (Clontech Laboratories). Competent yeast cells (strain AH109) were co-transformed with this dermal fibroblast library, the pGADT7-Rec prey vector [which contains the linearized AD (activation domain) and carries the leucine reporter gene (Clontech Laboratories)] and the pGBKTT7-pNα1(V) bait plasmid. Transformed yeast cells were spread on a series of agar-containing plates deficient in (i) tryptophan, (ii) leucine, (iii) trypthophan and leucine, (iv) tryptophan, leucine and histidine, or (v) tryptophan, leucine, histidine and adenine [QDO (quadruple dropout)] and incubated for 4–6 days at 30°C. Yeast colonies in which a bait–prey interaction occurred, survived stringent nutritional selection. Surviving colonies were plated on to QDO plates containing the chromogenic substrate X-α-Gal (5-bromo-4-chloroindol-3-yl α-d-galactopyranoside) (Clontech Laboratories). Yeast colonies turned blue when a bait–prey interaction occurred. Transformations were also carried out with positive control (pGADT7-RecT+pGBKTT7-53) and negative control (pGADT7-RecT+pGBKTT7-Lam) plasmids (Clontech Laboratories).

Selection and analysis of positive interactions

Blue colonies were transferred on to fresh QDO X-α-Gal plates and incubated for 4–6 days at 30°C. This process was repeated twice, and colonies that maintained their phenotype and survived stringent nutritional selection were considered to be positive clones. To screen the inserts (encoding possible interaction partners) of the pGADT7-Rec prey vector, we performed a PCR (touch-down programme 72.1°C–65.1°C) on the surviving colonies using the 5'AD-LD-Amplimer and 3'AD-LD-Amplimer primers (see Supplementary Table S1). The resulting PCR fragments were sequenced (ABI3730XL, Applied Biosystems), and a BLAST search was performed. We only retained sequences which contained an open reading frame and encoded proteins that were present in the GenBank® database. Sequences that were omitted included those that were out-of-frame, that corresponded to unknown genes, that started with a poly(A) tail, indicating that the gene was probably inverted and that contained multiple stop codons or open reading frames that could not be identified in the GenBank® database. In addition, genes which were on a list of known false positives, that are frequently found in Y2H screens independently of the target used, were omitted from further analysis [21]. Putative interacting proteins were retested by co-transformation in yeast AH109 cells and stringent nutritional selection.

Tissue extracts and recombinant proteins

To confirm the protein–protein interactions identified, tissue extracts and several native and recombinant proteins were used. Details are provided in the Supplementary Online Data and Supplementary Figure S1 at http://www.BiochemJ.org/bj/433/bj4330371add.htm. The purity of the proteins was analysed by SDS/PAGE (6% or 10% gels). Gels were stained with Coomassie Blue.

SPR (surface plasmon resonance) binding assays

Measurements were performed using a Biacore 3000 or a Biacore T100 instrument (GE Healthcare). PCPE-1 (procollagen C-proteinase enhancer-1) [22], and the pNα1(V)-protein were covalently immobilized to the dextran matrix of a CM5 sensorchip via their primary amine groups. The carboxymethylated dextran surface was activated by the injection of a mixture of 0.2 M EDC (N-ethyl-N’-(3-dimethylaminopropyl)carbodi-imide) and 0.05 M NHS (N-hydroxysuccinimide). PCPE-1 was diluted in 10 mM maleate buffer (pH 6.0), and the pNα1(V)-protein was diluted in acetic acid (pH 3.5) before injection over the activated surface of the sensorchip. Residual active groups were blocked by injection of 1 M ethanolamine (pH 8.5). Immobilization levels of 1438 RU (resonance units) for PCPE-1, and 7318 RU for the pNα1(V)-protein were obtained. A control flow cell was activated by the NHS/EDC mixture and deactivated by 1 M ethanolamine (pH 8.5) without any coupled protein. Control sensorgrams were subtracted online from the sensorgrams obtained on immobilized PCPE-1 and pNα1(V)-protein in order to yield specific binding responses. The α1(V)-N-propeptide (65 μg/ml) was injected at 20 μl/min for 4 min over immobilized PCPE-1 with 50 mM Tris/HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.005% P20 surfactant as running buffer in the presence or absence of 5 mM CaCl₂ [22]. TGF-β1 (transforming growth factor β1) (3.1 μg/ml) was injected over the immobilized pNα1(V)-protein at 30 μl/min for 2 min in 10 mM Hepes (pH 7.4) containing 0.15 M NaCl and 0.005% P20 surfactant. The sensorchip surface was regenerated either with a pulse of 2 M NaCl and/or a pulse of 1.5 M NaCl and 1.5 M guanidinium chloride.

SPR arrays were handled in a Biacore Flexchip system (GE Healthcare), a commercially available high-density array platform, and analysed as described previously [23]. The pNα1(V)-protein was printed directly in triplicate at concentrations varying from 50 to 200 μg/ml on to the gold surface of a Gold Affinity chip (GE Healthcare) using a non-contact PiezoArray spotter (PerkinElmer). Six drops of 330 pl each were delivered to the surface of the chip (total spotted volume: 1.9 nl, spot diameter: 250–300 μm, spotted amount: 100–400 pg/spot). The chip was blocked with blocking buffer containing mammalian proteins (Superblock, Pierce) for five 5 min periods. The blocked chip was then equilibrated with PBS containing 0.05% Tween 20 (Sigma) at 500 μl/min for 90 min. Human type I collagen (150 nM), type VI collagen (250 nM) and laminin 1 (used as a negative control) were diluted in PBS containing 0.05% Tween 20, and flowed at 500 μl/min over the chip surface for 25 min. The dissociation of the complexes was monitored in PBS containing 0.05% Tween 20 for 40 min. Data collected from reference spots (gold surface) and buffer spots were subtracted from those collected on spotted proteins to obtain specific binding curves.

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Co-immunoprecipitation assay

Protein–protein interactions between the pNα1(V)-protein and respectively FN (fibronectin), α1(VI)-collagen chain, TIMP-1 (tissue inhibitor of matrix metalloproteinasises-1), MMP-2 (matrix metalloproteinaise 2), TNC (tenascin C), TGF-β1 and the type I collagen C-propeptide were confirmed using the Protein A Dynabeads® system (Invitrogen). HEK (human embryonic kidney)-293T cells expressing the pNα1(V)-protein [5] were cultured in growth medium containing hygromycin (Invitrogen). The medium fraction was collected, concentrated using the Centriprep Centrifugal Filter Unit with Ultracel-30 membrane (Millipore) and stored until use at −20°C.

To facilitate the detection of the different interacting proteins by Western blot analysis, a FLAG tag was included at their N-terminus. Expression constructs encoding the interacting domains of TNC and MMP-2 were generated in the FLAG tag vector pMET7-fC-SH2, whereas expression constructs encoding the interacting domains of the α1(VI)-collagen chain, FN and TIMP-1 using Lipofectamine™ 2000 reagent (Invitrogen). HEK-293T cells were transfected by CaCl2 methodology. Subsequently, HEK-293T cells were transfected with the expression plasmids encoding the potential interacting domains of the α1(VI)-collagen chain, FN and TIMP-1 using Lipofoctamine™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol. The HEK-293T cells were transfected with the expression plasmids encoding the potential interacting domains of the α1(VI)-collagen chain, FN and TIMP-1 using Lipofoctamine™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol. The HEK-293T cells were transfected with the expression plasmids encoding the potential interacting domains of the α1(VI)-collagen chain, FN and TIMP-1 using Lipofoctamine™ 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

For the co-immunoprecipitation assay, the monoclonal anti-α1(V)-N-propeptide antibody [18G5, directed against the NC2 domain of the α1(V)-N-propeptide [5]] was bound to the Protein A Dynabeads®. To preclude non-specific binding of the concentrated pNα1(V)-medium to the Protein A Dynabeads®, we performed a titration experiment. Concentrated pNα1(V)-medium was incubated with Protein A Dynabeads® and the optimal protein concentration was determined by Western blotting after subtraction of non-specific binding. For each co-immunoprecipitation assay, a negative control, which consisted of Protein A Dynabeads® incubated with the concentrated pNα1(V)-medium, was included. Subsequently, concentrated pNα1(V)-medium was incubated for 4 h at 4°C with the Dynabeads®–18G5 complex with constant rotation. The Dynabeads®–18G5–pNα1(V)-protein complex was washed and incubated overnight at 4°C with constant rotation in the presence of cell lysates of HEK-293T cells, transfected with one of the expression plasmids encoding the different potential interacting domains, or in the presence of 10 ng of human recombinant TGF-β1 or 3 μg of affinity-purified type I collagen C-propeptide. The resulting complex was washed and eluted with non-reducing lane marker sample buffer (ThermoScientific) supplemented with DTT (dithiothreitol) (Sigma) (except for the type I collagen C-propeptide), boiled and loaded on NuPAGE® 4–12% Bis-Tris gels (Invitrogen) or on a self-poured SDS/10% PAGE gel for TNC. After SDS/PAGE, Western blotting was performed using the mouse 18G5 antibody [5,10], the mouse anti-FLAG M2 antibody (Sigma—Aldrich) for probing the interaction partner proteins and the mouse anti-TGF-β1 antibody (TB21, ab27969, Abcam), followed by incubation with the secondary antibody ECLplex goat anti-mouse IgG–Cy5 (indodiscarbocyanine) (GE Healthcare). For the co-immunoprecipitation assay with the pNα1(V)-protein and the type I collagen C-propeptide, rabbit antibodies against the COBUCUB2 (where COB is C1r/C1s, urchin embryonic growth factor and BMP-1 domain) fragment of mouse PCPE-1 and guinea-pig antibodies against the mouse type I C-propeptide were used [24]. In order to detect the pNα1(V)-protein, a rabbit polyclonal antibody directed against type V collagen (ab7046, Abcam) was used. Next, the blot was probed with the secondary antibodies Alexa Fluor® 488-conjugated goat anti-rabbit (A-11008, Molecular Probes) and Alexa Fluor® 594-conjugated goat anti-guinea pig (A-11076, Molecular Probes). Scanning of the membranes was performed on a Typhoon 9400 instrument (GE Healthcare). To investigate the specificity of the co-immunoprecipitation assay, negative control samples, containing proteins which do not interact with the pNα1(V)-protein (a gift from A. Dheedene, Center for Medical Genetics, Ghent, Belgium), were included. These samples consisted of cell lysates of HEK-293T cells expressing SON DNA-binding protein, MLT1 [myeloid/lymphoid or mixed-lineage leukaemia (trithorax homologue, Drosophila)2] and 3ZC5 (zinc finger CCHC-type-containing 4), each of which was generated in the FLAG tag vector pMG2-p85. In addition, as a negative control, the co-immunoprecipitation assays were carried out with the cell lysate of untransfected HEK-293T cells (Supplementary Figure S2 at http://www.BiochemJ.org/bj/433/bj4330371add.htm).

Solid-phase binding assay

An amount of 1 μg/well of purified peptidized fetal bovine type I collagen and acid-soluble and peptidized type V collagen were coated on to 96-well microtitre plates (Greiner) overnight at 4°C. The wells were blocked with 3% (w/v) dried non-fat milk in PBS for 2 h. Serial dilutions (0–40 μg/ml) of peptidized human type VI collagen, diluted in PBS containing 0.05% Tween 20 and 3% (w/v) dried non-fat milk, were added to the wells and incubated overnight at 4°C. After several washes, bound type VI collagen was revealed with a monoclonal antibody against type VI collagen (5C6, Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.) followed by incubation with a secondary mouse antibody conjugated to peroxidase (Dako). The signal was detected using 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) as the chromogenic substrate. Absorbance was measured at 405 nm with an ELISA microplate reader.

Enzyme digests

A 2 μg amount of the purified pNα1(V)-protein was incubated overnight at 37°C, without or with 100 ng of BMP-1 (1:20 enzyme/substrate ratio) in assay buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl and 5 mM CaCl). Where indicated, 1.5 μg of PCPE-1 was added. Digestion products were precipitated with 10% trichloroacetic acid after adding 1% Triton X-100. After centrifugation at 20000 g for 30 min, pellets were washed twice with ice-cold ethanol and acetone, resuspended in Laemmli buffer and analysed by SDS/PAGE (7.5% gels) under reducing conditions. Proteins were electrotransferred on to PVDF membranes (Immobilon-P, Millipore) overnight in 10 mM Caps [3-(cyclohexylamino)propan-1-sulfonic acid] (pH 11) and 5% methanol. Then, membranes were saturated for 2 h with 10% (w/v) dried non-fat milk in PBS (Sigma—Aldrich). Double immunolabelling using two different colorimetric detection kits was performed to discriminate BMP-1 from the pNα1(V)-protein and digestion product bands. The membrane was first probed with a primary polyclonal antibody against the TSPN-1 domain [5]...
followed by incubation with an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Bio-Rad Laboratories). Detection of the protein bands was performed using an alkaline phosphatase colorimetric kit (AP Color kit, Bio-Rad Laboratories). Subsequently, the membrane was incubated with a primary polyclonal antibody against BMP-1 followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Dako) and the signal was detected using a horseradish peroxidase colorimetric kit (Sigma–Aldrich).

The time course of the processing of the type V procollagen homotrimer by BMP-1 was followed as described previously [25]. Briefly, recombinant type V procollagen (3 μg) was incubated with 100 ng of BMP-1 at 37°C in 48 μl of 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM CaCl2 and 0.1% Brij-35, in the presence or absence of 200 ng of PCPE-1. Aliquots (6.4 μl) were removed at 0, 2, 4 and 6 h and the reaction was stopped by heating at 60°C for 15 min in Laemmli’s loading buffer containing 4% (w/v) 2-mercaptoethanol. Digestion products were analysed by SDS/PAGE (10% gels) and silver staining.

Relative band intensities were determined after scanning the original membranes (or the dried gel) on optimized non-edited images, using TINA image analysis software (version 2.07d, Raytest isotopenmessgeräte), after background subtraction.

RESULTS AND DISCUSSION

Since the Y2H system has been used successfully to identify interactions with extracellular proteins (e.g. type IV collagen, thrombospondin 1, laminin β3, the NC1 domain of type VII collagen, emilin and microfibril-associated glycoprotein-2 [26]) and to study protein assembly of type X collagen [27], we reasoned that the Y2H assay could be successful in identifying binding partners of the type V collagen N-propeptide. We thus designed a Y2H bait construct pGBK7-T7-pNα(I)-V, which encompassed the entire α(I)-V-N-propeptide domain and 11 triplets of the COL1 domain [5]. Taking into account that skin is the major affected tissue in classic EDS patients, we generated a human dermal fibroblast cDNA library.

Screening of this library with the pGBK7-T7-pNα(I)-V bait vector resulted in the growth of numerous yeast colonies (~4000 clones), even after stringent nutritional selection. Owing to this high number of positive yeast colonies, a PCR strategy was developed to screen all different bands. Approx. 32% of the positive yeast colonies (1128 colonies) yielded one specific PCR band, indicating that only one insert, encoding one interaction partner, was present. The remaining positive yeast colonies yielded multiple PCR bands, suggesting that multiple inserts were present; the absence of a PCR band indicates that the PCR conditions were not optimal. Direct sequencing of the 1128 specific PCR products yielded interpretable results for ~60% of the PCR fragments. Sequences that encoded nuclear and cytoskeleton proteins and proteins known as being false-positive Y2H interactions (e.g. ribosomal proteins and proteasome subunits; see Supplementary Table S2 at http://www.BiochemJ.org/bj/433/bj4330371add.htm) were omitted from further investigation. This resulted in the retention of nucleotide sequences encoding domains of 12 polypeptide chains/proteins putatively interacting with the α(I)-V-N-propeptide (Table 1). Each interacting domain was identified at least twice. These binding partners included ECM proteins that are involved in matrix assembly and cell-matrix interactions (the different α-chains of types I and VI collagen, FN and TNC), and ECM proteins that are involved in collagen biosynthesis and remodelling (PCPE-1, MMP-2, TIMP-1, TGF-β1 and N-acetylgalactosaminyltransferase 7). Moreover, through the Y2H assay, information about the interacting domains could be provided (Table 1). However, it should be noted that interactions between the α(I)-V-N-propeptide and the proteins identified might involve multiple regions and also regions that are not included in this Y2H screen since our fibroblast cDNA library does not contain all protein-coding full-length sequences. In addition, some interactions might depend on post-translational modifications and/or the structure of mature processed protein.

To validate the identified interactions, several protein–protein-binding assays were applied. The binding of the α(I)-V-N-propeptide to PCPE-1, TGF-β1, and types I and VI collagen was confirmed by SPR (Figure 2). As was shown previously for the binding of the procollagen C-propeptide to PCPE-1 [22], the binding of the α(I)-V-N-propeptide to PCPE-1 also increased in the presence of 5 mM CaCl2 (Figure 2A). The complexes formed between PCPE-1 and the α(I)-V-N-propeptide were stable as demonstrated by their very low spontaneous dissociation rate (Figure 2A). Soluble TGF-β1 bound to the α(I)-V-N-propeptide, but dissociated rapidly (Figure 2B). In addition, SPR arrays showed the formation of very stable complexes of the α(I)-V-N-propeptide with pepsinized types I

Table 1 Proteins putatively interacting with the α(I)-V-N-propeptide

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The interacting domain was deduced by direct sequencing of the inserts of the Y2H pGADT7-Rec prey vector. FN type-III, FN type-III repeat; NTR, netrin-1 domain; AA, amino acid.
and VI collagen (Figure 2C). Additionally, we confirmed by co-immunoprecipitation that the α1(V)-N-propeptide binds to the identified domains of some of the binding partners [FN, α1(VI)-collagen chain, TIMP-1, MMP-2 and TNC] (Figure 3A), to the full-length TGF-β1 and to the C-propeptide of type I collagen (Figure 3B). No interaction was detected with four negative controls (SON, MMLT1, PCGF2 and ZC3H4, results not shown). Hence, from the 12 proteins identified that interact with the α1(V)-N-propeptide, 11 were confirmed by SPR and/or co-immunoprecipitation. Unfortunately, the interaction of the α1(V)-N-propeptide with the N-acetylgalactosaminyltransferase 7 enzyme could not be confirmed since the protein is not commercially available for SPR studies and, for some reason, our different attempts to produce the recombinant protein failed. This enzyme belongs to the family of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferases (EC 2.4.1.41) [28]. Since this family of enzymes is poorly characterized, the putative physiological significance of this interaction remains to be determined.

Some proteins identified from our Y2H screen were reported previously as type V collagen ligands. This is the case for the α1- and α2-chains of type I collagen [1,29] and FN [30]. However, our findings add information to the previously reported data. First, we show that the binding site for FN in type V collagen was located within the α1(V)-N-propeptide domain. Second, our Y2H results suggest that the α1(V)-N-propeptide interacts with the C-propeptide domains of the α1(I)- and α2(I)-collagen chains. These interactions may represent an important factor in the growth control of the heterotypic type I/V collagen fibrils. The binding of the α1(V)-N-propeptide with the type I collagen C-propeptide domains can regulate directly the rate of type I procollagen and thus the accretion of heterotypic fibrils by limiting the processing collagen type I molecule deposit. Moreover, although the triple-helix domains of the α1(I)- and α2(I)-collagen chains were not identified in our Y2H screen, we showed, using SPR assays and solid-phase binding assays, that the type I collagen triple-helix domain may also be involved in the type I-type V collagen interaction (Figures 2C and 3A). The fact that this interacting protein domain is absent from our Y2H screen suggests that the triple-helix conformation is likely to be a prerequisite for this interaction to occur. Another possibility is that the sequence encoding this domain was not withheld due to our selection procedure.

New binding partners were also identified. We demonstrated for the first time a direct interaction between the α1(V)-N-propeptide and type VI collagen (Figure 2C). The most common type VI collagen form is the heterotrimer α1(VI)α2(VI)α3(VI) (encoded by COL6A1, COL6A2 and COL6A3 genes respectively) [31]. Each α1-chain consists of a central short collagenous domain flanked by several vWF-A (von Willebrand factor type A) modules at the N- and C-termini [31]. Our Y2H data revealed that different regions of the type VI collagen molecule are involved in the α1(V)-N-propeptide interaction. Whereas the α1(VI)- and α3(VI)-chains interact with the α1(V)-N-propeptide through their globular domains (Table 1), the α2(VI)-chain interacts with the α1(V)-N-propeptide through its triple helix (Table 1). We showed that the α1(V)-N-propeptide-binding sites are retained in the pepsinized form of type VI collagen (Figure 2C). Treatment with pepsin usually removes the globular domains of collagens. However, it has been shown that pepsinized type VI collagen consists of a mixture of dimers and tetramers which retain small globular domains at the extremities of the molecules [32], suggesting that the binding sites located within the globular domains are likely to be present in the pepsinized molecule. Moreover, using a solid-phase binding assay, we investigated whether pepsinized type VI collagen can bind to the intact form of type V collagen extracted from tissues with acetic acid (Figure 4). Mature type V collagen retains the VAR, COL2 and NC2 domains, while the TSPN-1 domain is removed by BMP-1 during processing (Figure 1). When pepsinized type VI collagen was used as soluble ligand, a strong and saturable binding was obtained with acid-soluble type V collagen (Figure 4A). A weaker binding of pepsinized type VI collagen resulted with type I collagen. In contrast, no binding to pepsinized type V collagen was detected (Figure 4A). These results underscore the Y2H data and show that type VI collagen binds to mature type V collagen found in tissues (Figure 4C).
Figure 3  Co-immunoprecipitation assay

(A) The 18G5 antibody was bound on Protein A Dynabeads®. The pNa1(V)-protein was immobilized on the Protein A Dynabeads®—18G5 antibody complex. The Protein A Dynabeads®—18G5—pNa1(V)-protein complex was incubated with cell lysates of HEK-293T cells, which expressed FLAG-tagged domains of different identified interacting proteins [α1(VI)-collagen chain, FN, TIMP-1, MMP-2 and TNC]. The interaction complexes were eluted and evaluated by SDS/PAGE and Western blotting. The blot was developed using the 18G5 antibody (probed to the pNa1(V)-protein) and the anti-FLAG M2 antibody (probed against the FLAG tag epitope). For these specific interacting domains, an interaction with the pNa1(V)-protein was shown. In the control sample, no non-specific interaction of the pNa1(V)-protein with the Protein A Dynabeads® was detected. 

(B) The pNa1(V)-protein was immobilized on Protein A Dynabeads® by the 18G5 antibody. The Protein A Dynabeads®—18G5—pNa1(V)-protein complex was incubated with either recombinant human TGF-β1 or affinity-purified type I collagen C-propeptide. These interaction complexes were eluted and evaluated by SDS/PAGE and Western blotting. The pNa1(V)-protein was detected by a rabbit antibody against type V collagen, whereas a mouse antibody against TGF-β1 and a guinea-pig antibody against mouse type I procollagen C-propeptide were used to reveal TGF-β1 and the type I collagen C-propeptide. Both proteins (domains) could be revealed, thereby confirming the interaction between the pNa1(V)-protein and TGF-β1 and the type I collagen C-propeptide respectively. We also used a rabbit antibody to the CUB1CUB2 domains of PCPE-1, which revealed a band corresponding to PCPE-1. An explanation for this could be that this band originates from the culture medium of the HEK-293-EBNA (Epstein–Barr nuclear antigen-expressing HEK-293) cells used to express the pNa1(V)-protein, known to express PCPE-1 (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/433/bj4330371add.htm). Fraction 1 = supernatant of the Protein A Dynabeads® after incubation with pNa1(V)-protein containing culture medium of HEK-293T cells expressing the pNa1(V)-protein. Elution = eluate containing the 18G5 antibody, the pNa1(V)-protein and the putative interaction partner. *Protein band corresponding to the heavy chain of the 18G5 antibody; **protein band corresponding to the light chain of the 18G5 antibody.

Interestingly, although we used the monomeric α1(V)-N-propeptide in our initial Y2H screen, we show by our solid-phase binding assay that the heterotrimeric type V collagen interacts with type VI collagen, thereby supporting the interaction of type VI collagen with the physiological form of type V collagen. Previously, type VI collagen was shown to interact with various ECM components including types I, II, IV and XIV collagen, FN, fibromodulin, hyaluronic acid, perlecen, decorin, biglycan, tenasin-X and cell receptors such as the integrins and the NG2 transmembrane proteoglycan [33]. Type VI collagen is widely distributed in connective tissues and particularly abundant in the pericellular environment, where it functions to anchor the basement membrane to the underlying connective tissue [31]. Along this line, ultrastructural studies suggested that types V and VI collagen form intricate networks in the dermis that can be linked to collagen fibrils, elastic fibres and the basal lamina [34]. As such, the type V—type VI collagen protein complex may serve as a molecular bridge in the cell-matrix environment, and may be essential to maintain the architecture of the dermal matrix. Interestingly, UCMD (Ullrich congenital muscular dystrophy), which is caused by mutations in COL6A1, COL6A2 or COL6A3, shows clinical and ultrastructural characteristics overlapping with classic EDS, such as alterations of collagen fibril morphology (variation in size, loose packaging of fibrils and composite fibres) and increase in ground substance [35]. In view of the type V—type VI collagen interactions identified, cutaneous manifestations in UCMD may be mediated by disturbance of type V collagen function. In addition, as defects in type VI collagen are found only in approx. 40 % of the UCMD patients and since the involvement of type VI collagen has been excluded in a number of UCMD cases...
The function of this interaction, PCPE-1 enhancement of the N-propeptide cleavage by BMP-1 was assessed using an in vitro enzymatic assay. Recombinant \( \alpha 1(V) \)-N-propeptide was incubated with BMP-1 in the presence or absence of PCPE-1 (Figure 5A) and the reaction products were detected by immunoblotting. Densitometric measurements of the intensities of the bands corresponding to the TSPN-1 domain showed that the calculated ratio of the TSPN-1 band intensity in the presence of BMP-1 and PCPE-1 (Figure 5A, lane 4) over the TSPN-1 band intensity with BMP-1 alone (Figure 5A, lane 2) was 1.9. Subsequently, we performed a time course study using recombinant type V procollagen homotrimer as a substrate. The rates of release of the \( \alpha 1(V) \)-N-propeptide TSPN-1 fragment by BMP-1 in the presence or absence of PCPE-1 were compared after 0, 2, 4 and 6 h of incubation (Figure 5B). The intensity of the TSPN-1 band in the presence of PCPE-1 increased progressively with time (Figure 5B) and was higher than in its absence. Densitometric measurements for the BMP-1 digests indicated a 1.7-, 2.5- and 2.4-fold enhancement in the presence of PCPE-1 at 2, 4 and 6 h of incubation respectively. These results are consistent with the ratio obtained in Figure 5(A). The present study clearly indicates that PCPE-1 binding to the \( \alpha 1(V) \)-N-propeptide is required for the enhancement of BMP-1 activity, hereby providing a new biological function for PCPE-1. This finding was unexpected because, to date, PCPE-1-enhancing activity was thought to be restricted to the procollagen C-proteinase activity of BMP-1 [37]. Moreover, using a different approach, it has been reported previously that PCPE-1 has no effect on the in vitro BMP-1 processing of type V procollagen N-propeptide [37]. Consistent with our in vitro enzymatic activity, the binding sites identified are located within the PCPE CUB domains, also involved in the PCPE binding of fibrillar collagen C-propeptides [37]. As the type V collagen N-propeptide domain is of particular importance in regulating the collagen fibril diameter, we hypothesize that PCPE-1 can directly modulate collagen fibrillogenesis by regulating type V collagen N-propeptide processing.

Additional newly identified binding partners of the \( \alpha 1(V) \)-N-propeptide were TGF-\( \beta \)-1 and MMP-2, the most widespread matrix metalloproteinase, and its potent tissue inhibitor TIMP-1. TGF-\( \beta \)-1 is one of the most pleiotropic and multifunctional growth factors which up-regulates the expression of several ECM components (FN, biglycan, types I and V collagen, TNC) [38,39]. Additionally, it was shown that COL5A1 is a target of TGF-\( \beta \)-1 signalling with a potential role in osteogenesis [39]. Activation of the TGF-\( \beta \)-1 molecule results in the stimulation of many of its own activators, including furin [40], which is involved in the processing of the C-propeptide of the pro-\( \alpha 1(V) \)-collagen chain [4]. In the present study, we have shown that, besides these regulatory actions of TGF-\( \beta \)-1 on COL5A1, a physical interaction between the two proteins also exists. The downstream effects of this interaction so far remain elusive. However, it emphasizes further the complexity of the relation between TGF-\( \beta \)-1 signalling and the ECM. Moreover, it has been shown previously that TGF-\( \beta \)-1 binds to the CRR domain of the type II collagen N-propeptide [41]. Proteolytic removal of this CRR domain releases TGF-\( \beta \)-1 in cartilage and regulates chondrogenesis. Taken together, our findings suggest a novel function for the \( \alpha 1(V) \)-N-propeptide domain. As type V procollagen is synthesized, processed and deposited in the ECM, the \( \alpha 1(V) \)-N-propeptide could serve as a reservoir controlling the availability of TGF-\( \beta \)-1. The possibility that TGF-\( \beta \)-1 can bind to the \( \alpha 1(V) \)-TSPN-1 domain, whose release is mediated by BMP-1, suggests that this interaction may control ECM assembly and homoeostasis and needs further investigation. Remodelling of the ECM depends on

[35], type V collagen may be a candidate for unexplained cases of UCMD. Similarly, type VI collagen may be a good candidate gene for unresolved (classic) EDS patients.

Our findings also show that the \( \alpha 1(V) \)-N-propeptide interacts with PCPE-1, a potent protein enhancer of the C-terminal processing of type I, II and III procollagen by members of the procollagen C-proteinase family, notably BMP-1 [36]. However, BMP-1 cleaves the type V procollagen homotrimer at both ends, releasing the TSPN-1 domain of the N-propeptide very rapidly and the C-propeptide more slowly [25]. To examine
Figure 5 Effect of PCPE-1 on BMP-1 cleavage of recombinant pN\(\alpha(1)(V)\)-protein (A) and homotrimeric type V procollagen (B)

(A) Recombinant pN\(\alpha(1)(V)\)-protein was incubated with BMP-1 in the absence (lane 2) or presence (lane 4) of PCPE-1. The cleavage product of the pN\(\alpha(1)(V)\)-protein by BMP-1 that corresponds to the TSPN-1 N-terminal domain of the proc-\(\alpha(1)(V)\) chain is detected by Western blot analysis. Lanes 1 and 3 correspond to negative controls, incubations were performed without BMP-1 and PCPE-1 (lane 1) and without BMP-1 (lane 3) respectively. The upper unidentified band observed in all samples, whose migration, presence and intensity remains unchanged in all of the lanes, attests for the equal loading of proteins. (B) Time course of recombinant type V procollagen homotrimer processing by BMP-1. The rates of release of the TSPN-1 domain from the \(\alpha(1)(V)\)-N-propeptide by BMP-1 at the indicated time points (0, 2, 4 and 6 h), in the presence (+) or absence (−) of PCPE-1 is compared. Silver-stained SDS/PAGE analysis showed that the band corresponding to the TSPN-1 domain is more intense in the presence of PCPE-1 than in its absence. These results show that the activity of BMP-1 is enhanced by PCPE-1, regardless of the substrate used, either the \(\alpha(1)(V)\)-N-propeptide monomer (A) or the type V procollagen homotrimer (B).

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Identification of α1(V)-N-propeptide-interacting proteins


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SUPPLEMENTARY ONLINE DATA
Identification of binding partners interacting with the α1-N-propeptide of type V collagen

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SUPPLEMENTARY MATERIALS AND METHODS
Tissue extracts and recombinant proteins

To confirm the protein-protein interactions identified, several native and recombinant proteins were used. Type I collagen was extracted from embryonic bovine bones by pepsin digestion, and purified by repeated salt fractionation in acetic acid as described previously [1], freeze-dried, and a stock solution was prepared in 0.1 M acetic acid (2 mg/ml). Native human pepsinized type VI collagen was kindly provided by T. Sasaki (Martinsried, Germany), and acido-soluble type V collagen, retaining the α1(VI)-V AR domain, was extracted from fetal bovine cranial bones as described previously [2]. During purification of BMP-1 and PCPE-1 from the culture medium of 3T6 mouse fibroblasts, a fraction enriched with native mouse type I collagen C-propeptide was obtained as a by-product. Proteins remained soluble at 33 % saturation of ammonium sulfate [3], and were concentrated by ammonium sulfate precipitation (50 % saturation), followed by solubilization in and dialysis against 0.05 M Tris/HCl, 0.15 M NaCl and 0.005 M CaCl2 (pH 7.5). The concentration of the type I collagen C-propeptide in the resulting fraction was ~0.5 mg/ml; it also contained some residual PCPE-1 (~0.03 mg/ml). A small amount of C-propeptide was purified from this fraction by affinity chromatography on a column of Sepharose coupled to the CUB1CUB2 fragment of PCPE-1 (obtained from purified recombinant human PCPE-1 by trypsin treatment, followed by heparin-Sepharose chromatography to remove the NTR (netrin-like) domain [4]).

Recombinant α1(V)-N-propeptide protein was expressed in HEK-293-EBNA (Epstein-Barr nuclear antigen-expressing HEK-293) cells and purification of the recombinant protein was performed by two ion-exchange chromatography steps as described previously [5]. Protein purity was assessed by SDS/PAGE (Supplementary Figure S1A). The protein concentration was determined using a NanoDrop spectrophotometer (λ = 280 nm).

Unless specified otherwise, human recombinant PCPE-1 was expressed in HEK-293T cells and purified as described previously [6]. Another batch of human recombinant PCPE-1 was kindly provided by D. Hulmes (Lyon, France) and was used as a control for the obtained results. A third batch of PCPE-1 (His6-tagged; expressed in a baculovirus system and purified on a nickel column; gift from Dr P. Findell, Roche BioScience) was used in the enzymatic experiments to determine the rate of N-propeptide processing of the type V procollagen homotrimer. Recombinant human BMP-1 was expressed either in a baculovirus system [4,7] or in HEK-293-EBNA cells [6] and purified as described previously [6]. Recombinant human type V procollagen homotrimers were produced and purified as described previously [8]. Human type I collagen (C5483), human TGF-β1 (T7039) and murine laminin 1 (L2020) were purchased from...
S. Symoens and others

Figure S2  Co-immunoprecipitation assay: controls with untransfected HEK-293T cell lysates

The 18G5 antibody was bound on to Protein A Dynabeads®. Next, the pNα1(V)-protein was immobilized on Protein A Dynabeads®—18G5 antibody complex. The Protein A Dynabeads®—18G5—pNα1(V)-protein complex was incubated with the cell lysates of untransfected HEK-293T cells. The interaction complexes were eluted and analysed by SDS/PAGE and Western blotting. (A) The blot was probed with the mouse 18G5 antibody (directed against the pNα1(V)-protein) and the mouse anti-FLAG M2 antibody (directed against the FLAG tag epitope). Development of the blot was performed by using the secondary antibody ECLplex goat anti-mouse IgG-Cy5 (indodicarbocyanine). (B) The blot was probed with the mouse anti-TGFβ1 antibody. Development of the blot was performed by using the secondary antibody ECLplex goat anti-mouse IgG-Cy5. (C) The blot was probed with the polyclonal rabbit anti-(type V collagen) antibody and the rabbit anti-PCPE-1 antibody. Development of the blot was performed by using the Alexa Fluor® 488-conjugated goat anti-rabbit secondary antibody. (D) The blot was probed with guinea pig anti-(type I C-propeptide) antibody. Development of the blot was performed by using the Alexa Fluor® 594-conjugated goat anti-(guinea pig) secondary antibody. Fraction 1 = supernatant of the Protein A Dynabeads® after incubation with pNα1(V)-protein-containing culture medium of HEK-293T cells expressing the pNα1(V)-protein. Fraction 3 = supernatant of the Protein A Dynabeads®—18G5—pNα1(V)-protein complex after incubation with the cell lysate of untransfected HEK-293T cells. Fraction 4 = wash fraction of the Protein A Dynabeads®—18G5—pNα1(V)-protein complex. Elution = eluate containing the 18G5 antibody, the pNα1(V)-protein and the putative interaction partner. None of the interaction partners could be detected. This is expected as the cell lysate is isolated from untransfected HEK-293T cells. In (C), the band for PCPE-1 is visible, consistent with the observation [6] that HEK-293T cells express endogenous PCPE-1 (as also indicated in Figure 3B of the main text). *Protein band corresponding to the heavy chain of the 18G5 antibody; **protein band corresponding to the light chain of the 18G5 antibody.
TAP-binding protein (tapasin)
Proteasome (prosome, macropain) subunit
Clathrin
Perostin
Ribosomal protein L13a
Chromosome X clone
7S LR NA
Serine/threonine kinase Kp78
Splice variant arginine/leucine-rich peptide
Eph receptor B3
Ribosomal protein L15
N-myc downstream regulator gene 1
Tubulin
\(\beta\)
GEF (guanine-nucleotide-exchange factor) 1
Migration-inducing protein 6
\(\beta\)
Thymosin
CD248 antigen (endosialin)
Tubulin
\(\alpha\)
Sterol-regulatory-element-binding transcription factor 2
KIAA0892
Signal-induced proliferation associated gene 1
Mcl-1 ubiquitin ligase (MULE)
MAPK (mitogen-activated protein kinase) phosphatase like protein MK-STYX
Valosine-containing protein
Vascular endothelial growth factor and type I collagen-inducible protein (VCIP)
cAMP-responsive element-binding protein 3 like-1
NECAP endocytosis associated 2
Ferrochelatase (protoporphyria)
KIAA1002
NADH cytochrome b5 reductase
KIAA0323
C-terminal binding protein
\(\gamma\)
Actin
in the present study

Table S1  Primer sequences for plasmid generation

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<tr>
<td>COL5A1_Nreco_Sall_R</td>
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<td>5′-TAATTGTATGTCACACCAAGCCAAAAGAAGAAG-3′</td>
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Endonuclease restriction sites are shown in bold.

Table S2  Identified interaction partners that were not characterized further in the present study

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<td>cAMP-responsive element-binding protein 3 like-1</td>
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<td>Valosine-containing protein</td>
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<td>McII-1 ubiquitin ligase (MULE)</td>
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Sigma–Aldrich. Another batch of recombinant human TGF-\(\beta\)1 was used in the co-immunoprecipitation assay and was purchased from R&D Systems. The purity of the proteins used for binding assays was assessed by SDS/PAGE stained with Coomassie Blue (Supplementary Figure S1).

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