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**ENZYMATIC CLEAVAGE SPECIFICITY OF THE Pro α 1(V) CHAIN
PROCESSING ANALYZED BY SITE-DIRECTED MUTAGENESIS**

**Christelle Bonod-Bidaud, Mickaël Beraud, Elisabeth Vaganay, Frédéric Delacoux*,
Bernard Font, David J.S. Hulmes and Florence Ruggiero**

**Université de Lyon; Université Lyon 1, France; CNRS, Institut de Biologie et Chimie des
Protéines, UMR 5086; IFR128 BioSciences Lyon-Gerland, 7 Passage du Vercors, 69367
Lyon cedex 07, France.**

Short title: Processing of Pro α 1(V) chain and derived mutants.

Address correspondence to: Dr. Florence RUGGIERO, Institut de Biologie et Chimie des
Protéines, UMR 5086 CNRS - Université Lyon1, 7, passage du Vercors, 69367 Lyon Cedex
07 – France, e-mail: f.ruggiero@ibcp.fr, Phone: 33 (0)4 72 72 26 67 Fax: 33 (0)4 72 72 26 02

*Present address: Université de Reims Champagne-Ardenne; UFR de Sciences Exactes et
Naturelles; CNRS UMR 6198; Laboratoire de Biochimie Moulin de la Housse BP1039 F-
51687 Reims Cedex 2.

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transient cell transfection, site-directed mutagenesis.

Abstract

The proteolytic processing of procollagen V is complex and depends on the activity of several enzymes among which the BMP-1/tolloid metalloproteinase and the furin-like proprotein convertases. Few of these processing interactions could have been predicted by analyzing the presence of conserved consensus sequences in the pro α 1(V) chain. In this study we opted for a cell approach that allows a straightforward identification of processing interactions. A construct encompassing the complete N-terminal end of the pro α 1(V) chain, referred to as N α 1, was recombinantly expressed to be used for enzymatic assays and for antibody production. Structural analysis showed that N α 1 is a monomer composed of a compact globule and an extended tail which correspond respectively to the non-collagenous N α 1 subdomains, TSPN and the variable region. N α 1 was efficiently cleaved by BMP-1 indicating that the triple-helix is not required for enzyme activity. By mutating residues flanking the cleavage site, we showed that the aspartate residue at position P'2 is essential for BMP-1 activity. BMP-1 activity at the C-terminal end of the procollagen V was assessed by generating a furin double mutant (Arg1584/1585Ala). We showed that, in absence of furin activity, BMP-1 is capable of processing the C-propeptide even though less efficiently than furin. Together, our results provide new relevant informations on this complex and poorly understood mechanism of enzymatic processing in procollagen V function.

INTRODUCTION

Most extracellular matrix proteins are synthesized by cells as precursors that undergo subsequent proteolytic processing into mature and functional molecules. Among these proteins are members of the collagen superfamily. Collagens undergo a great variety of proteolytic modifications involved in biosynthesis, fibrillogenesis, functional activation, shedding of transmembrane collagens and production of functional collagen-derived fragments also called matricryptins. The fate and functions of the released fragments derived from collagens, i.e. propeptides, collagen ectodomains and other cryptic non collagenous domains, are still under intensive investigation [1]. A large repertoire of proteinases is responsible for these processing interactions. Included among such enzymes are the ADAMTS and the BMP-1/tolloid families of metalloproteinases, and more recently the furin-like proprotein convertases [2]. Tolloid metalloproteinases play key roles in the control and orchestration of extracellular matrix deposition, cohesion, mineralization and cross-linking through proteolytic processing of the various extracellular matrix proteins. Their enzymatic activities include processing of fibrillar procollagens [3], laminin 5 and collagen VII [4, 5], maturation of small leucine-rich proteoglycans [6, 7], proteolytic activation of lysyl oxidase [8] and the release of bioactive domains from SIBLING proteins (dentin sialophosphoprotein and dentin matrix protein-1) [9] and perlecan [10].

The proteolytic processing of procollagen V is complex and depends on the activity of several enzymes. Collagen V is a minor fibrillar collagen with a broad distribution in tissues such as dermis, tendons, bones, blood vessels and cornea in which it can assemble in three different known stoichiometries. The heterotrimer $\alpha 1(V)_2\alpha 2(V)$ is the most abundant and is widely distributed, whereas the heterotrimer $\alpha 1(V)\alpha 2(V)\alpha 3(V)$ and the homotrimer $\alpha 1(V)_3$ show more restricted distributions [11, 12]. The physiological relevance of these three molecular forms is unclear. However, a wealth of evidence favors the role of the heterotrimer $\alpha 1(V)_2\alpha 2(V)$ in the control of fibril assembly [13], a function that is not shared with by the homotrimer $\alpha 1(V)_3$ [14]. A key step in collagen fibril formation is the proteolytic removal of the non collagenous N- and C-propeptides. BMP-1 cleaves $\text{pro}\alpha 1(V)$ at both N- and C-termini. These processing cleavages release a part of the N-propeptide at the N-terminus, the TSPN-1 domain, and the C-propeptide at the C-terminus [15, 16]. ADAMTS-2 was recently shown to cleave the heterotrimer $\text{pro}\alpha 1(V)_2\alpha 2(V)$ extracted from skin and the recombinant homotrimer $\text{pro}\alpha 1(V)_3$ [17]. From the determination of the cleavage site, it was deduced that the released fragment includes the TSPN-1 domain and the variable region [17]. The C-

propeptide of the recombinant homotrimer pro α 1(V)₃ was shown to be cleaved rapidly in the conditioned medium of transfected HEK-293 cells [12]. It was further shown that an endogenous furin-like proprotein convertase was responsible for this cleavage [15]. Interestingly, not all of these processing interactions could have been predicted by analyzing the presence of conserved consensus sequences in the N- and C- terminal regions of the pro α 1(V) chain or based on our knowledge about the processing of the fibrillar procollagens. Here, the proteolytic processing specificity of the pro α 1(V) chain by BMP-1 and by furin-like proprotein convertases was analysed in details by site directed mutagenesis. *In vitro* digestion assays are commonly used to investigate matrix protein processing and give, in many cases, satisfactory results. However, fastidious extraction and purification steps are often necessary to obtain limited amounts of unprocessed proteins and active enzymes since most of them are present in trace amounts in tissues. These problems have been partly solved by expressing recombinant matrix proteins and enzymes, though this alternative method does not avoid purification procedures. Therefore, in the present study, the proteolytic processing of procollagen V was approached by using transient cell transfection for allowing rapid and straightforward analysis of processing interactions. Using this method, we provide new and reliable informations on the enzymatic cleavage specificity of procollagen V.

MATERIALS AND METHODS

Plasmids

Plasmids pCEP4-pro α 1V containing the full-length cDNA of the human pro α 1(V) chain, and pCEP4-BMP-1 were already described [12, 18]. pCEP4-N α 1(V) construct was obtained from pCEP4-pro α 1V, after a KpnI/XhoI digestion (from nt 1 to nt 1783) and a subcloning in pCEP4 previously digested with the same enzymes. N α 1(V) is a small domain of 64 kDa containing the NC3, COL2 and NC2 domains and eleven triplets from COL1 (Fig. 1A).

Mutagenesis

For mutagenesis, N α 1(V) and pro α 1(V) had to be subcloned into the pcDNA3 plasmid (Invitrogen). After KpnI/XhoI digestion of N α 1(V) from PCEP4-N α 1(V), and KpnI/KpnI digestion of pro α 1(V) from pCEP4-pro α 1V, fragments were introduced into pcDNA3 linearized with KpnI/XhoI or KpnI/KpnI digestions, respectively. Mutation of the putative cleavage site of the α 1(V) C-propeptide by furin (Fig. 1B) was done on pcDNA3-pro α 1(V), and different simple, double or triple mutations of the BMP-1 cleavage site of N α 1(V) (Fig.

1B) were done on pcDNA3-N α 1(V). All mutations were done using the Quick-change II XL site-directed mutagenesis kit (Stratagen) according to the manufacturers' instructions, using the following oligonucleotides:

5'GCATCCAGGACGGCGGCGAACATCGACGCC3'	R1584/1585A	forward
5'GGCGTCGATGTTTCGCCGCCGTCCTGGATGC3',	and	reverse
5'CCTGACACCCACAGGCGCAGGACCCCAATCC3'	S254A	forward
5'GGATTGGGGTCCTGCGCCTGTGGGGTGTGTCAGG3',	and	reverse
5'GACACCCACAGTCGGCGGACCCCAATCC3'	Q255A	forward
5'GGATTGGGGTCCGCCGACTGTGGGGTGTGTC3',	and	reverse
5'CCACAGTCGCAGGCCCCCAATCCAGATG3'	D256A	forward
5'CATCTGGATTGGGGGCCTGCGACTGTGG3',	and	reverse
5'GAATATTACACGGAAGGAGCCGGCGAGGGTGAG3'	D267A	forward
5'CTCACCCCTCGCCGGCTCCTTCCGTGTAATATTC3',	and	reverse
5'GACACCCACAGGCGAACGACCCCAATCC3'	S254A-Q255N	forward
5'GGATTGGGGTTCGTCCTGTGGGGTGTGTC3',	and	reverse
5'GACACCCACAGTCGGCGGCCCCCAATCC3'	Q255A-D256A	forward
5'GGATTGGGGGCGCCGCGACTGTGGGGTGTGTC3',	and	reverse
5'GACACCCACAGGCGGCGGCCCCCAATCC3'	S254A-Q255A-D256A	forward
5'GGATTGGGGGCGCCGCCGCTGTGGGGTGTGTC3'.	and	reverse

The cDNA sequences of the mutants were thoroughly checked.

Cell culture

Human embryonic kidney (HEK) 293 EBNA cells were grown in DMEM medium supplemented with 10% fetal calf serum and penicillin-streptomycin cocktail (all from Sigma Aldrich, USA) at 37°C in a 5% CO₂ incubator. Stable cell lines were selected and grown in the same medium supplemented with 200 μ g/ml hygromycin B (Calbiochem, USA).

Production and purification of recombinant proteins

pCEP4-BMP-1 and pCEP4-N α 1(V) were transfected in HEK 293-EBNA cells by electroporation and the transfected cells were selected by adding hygromycin B (200 μ g/ml) during 7-10 days to DMEM culture medium supplemented with 10% fetal calf serum [12]. Resistant 293-EBNA cell media were tested for expression of the recombinant proteins by 6% SDS-PAGE followed by Coomassie blue staining. Large amounts of serum-free medium from

transfected HEK-293 EBNA cells were collected every 48 h and stored at -20°C until used. The purified BMP-1 was obtained as previously described [18]. The $\text{N}\alpha 1(\text{V})$ fragment was purified from conditioned media by 2 steps of ion exchange chromatography. Conditioned medium (360 ml) was dialyzed against 50 mM Tris/HCl pH 7.6, 100 mM NaCl, 2 M Urea. After centrifugation, the supernatant was passed over a DEAE column (DE 52, Whatman) and subsequently eluted with a linear 0-0.6 M NaCl gradient. Pools containing purified recombinant $\text{N}\alpha 1(\text{V})$ fragment were recovered and dialyzed against 50 mM Tris/HCl pH 7.6, 100 mM NaCl. Samples were then subjected to a HitrapQ column (Pharmacia, Biotech) and eluted by a NaCl gradient. Recombinant protein-containing fractions were analyzed by SDS-PAGE on a 6% gel and dialyzed against a 50 mM Tris/HCl pH 7.6, 100 mM NaCl. Purified $\text{N}\alpha 1(\text{V})$ fragment was stored at -20°C until used.

Polyclonal and monoclonal antibodies

Polyclonal antibodies to BMP-1 were kindly provided by Dr. Efrat Kessler [19]. For production of monoclonal antibody against the N-propeptide of the $\text{pro}\alpha 1(\text{V})$, mice were immunized with the purified recombinant $\text{N}\alpha 1(\text{V})$ fragment. Among the tested hybridomas, three clones produced an antibody able to recognize $\text{N}\alpha 1(\text{V})$ both by ELISA and Western blotting. Using the various recombinant N-propeptide domains, it was determined that the clones named 6A7 and 18G5 were specific for the variable region and to for the NC2 domain, respectively. Neither of them recognized the TSPN-1 domain (Fig. 2C).

To obtain antibodies able to recognize specifically the TSPN-1 region, rabbits were immunized with the recombinant TSPN-1 domain produced in *E. Coli*. The TSPN-1 cDNA (nt 258-723) was generated by polymerase chain reaction (PCR) using the $\text{N}\alpha 1$ clone as template. Two oligonucleotides flanking the desired sequence were designed, forward: 5'-TATGAATTCCCACCAAGCAGCTGTACCCTG-3' and reverse: 5'-TATCTGCAGGCTGTAGTGCTCACAGTAATC-3', the underlined sequences introducing a EcoRI site at the 5' end and a PstI site at the 3' end. The resulting PCR product was subcloned in the EcoRI and PstI sites of a pT7/7-6His expression vector [20]. The resulting plasmid, named pTSPN1-6His, encoded the TSPN-1 domain of the human $\text{pro}\alpha 1(\text{V})$ chain, under the control of the *E. coli* phage T7 promoter. *E. coli* host strain BL21 (DE3) was transformed with pTSPN1-6His and cells were harvested after 2 hours of IPTG induction as previously described [21]. Purified TSPN-1 domain was obtained by subjecting cell lysates to a Ni^{2+} -NTA agarose column (Qiagen). The recombinant protein was analyzed by 15% SDS-

PAGE followed by Coomassie blue staining. Polyclonal antibodies obtained after immunization with the recombinant protein were purified on a CNBr-activated Sepharose 4B column coupled with the purified recombinant N α 1(V) fragment as indicated by the manufacturer (Pharmacia Biotech). The purified antibodies were able to recognize N α 1(V) (Fig. 2C lane 1) and the recombinant TSPN-1 domain (data not shown) both by ELISA and Western blotting. Rabbit polyclonal antibodies to human pepsinized collagen V were obtained from Novotec (Lyon, France).

Transient cell transfection

For transient transfection experiments, wild-type HEK-293 EBNA cells or HEK-293 EBNA cells stably transfected with pCEP4-BMP-1 were plated in 100 mm Petri dishes with 1.5×10^6 cells and transfected the following day with the different plasmids using the calcium phosphate transfection kit from Invitrogen. 24 hours after transfection, cells were washed three times with PBS and placed in serum-free medium supplemented with 50 μ g/ml sodium ascorbate. Media were collected 48 hours after transfection for protein analysis. Efficiency of transfection was analyzed by transfecting the cells with a plasmid that expresses green fluorescent protein (GFP). The percentage of transfection was calculated by counting green cells 48 hours after transfection with an inverted microscope equipped with fluorescence (Nikon). The values varied from 50% to 70%.

Protein analysis

Proteins were recovered from transfected cell media by addition of 1% Triton X-100 followed by precipitation with 10% trichloroacetic acid (TCA). After centrifugation, pellets were washed twice with ethanol and acetone, resuspended in Laemmli buffer and analyzed by 12%, 10% or 6% SDS-PAGE as indicated, under reducing conditions. Proteins were electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, France) overnight in 10 mM CAPS pH 11, 5% methanol. Then, membranes were saturated for 2 hours in PBS supplemented with 10% non-fat milk. For N α 1 digestion by BMP-1, double immunolabelling using two different colorimetric detection kits was performed to discriminate the BMP-1 band from the N α 1 bands. Membranes were first probed with polyclonal primary antibodies against the TSPN-1 domain followed by incubation with alkaline phosphatase coupled goat anti-rabbit secondary antibodies (BioRad) and detection using an alkaline phosphatase colorimetric kit (AP Color kit, BioRad). Then membranes were

probed with polyclonal primary antibodies to BMP-1 followed by incubation with peroxidase coupled goat anti-rabbit secondary antibodies (DAKO A/S, Denmark) and the signal was detected using a peroxidase colorimetric kit (Sigma Aldrich). For the furin site mutants, membranes were probed with the monoclonal 6A7 antibody followed by alkaline phosphatase coupled goat anti-mouse secondary antibodies incubation (BioRad) and detected using an alkaline phosphatase colorimetric kit. For the BMP-1 mutants, N α 1 and TSPN fragments were detected with polyclonal primary antibodies against the TSPN-1 domain, followed by incubation with horseradish peroxidase coupled goat anti-rabbit secondary antibodies (DAKO A/S, Denmark) and signals were detected using an ECL reagent (Amersham Biosciences) and X-ray film exposure. When indicated, 18G5 antibody was used to detect the N α 1 fragment after BMP-1 cleavage. Membranes were then de-hybridized according to the manufacturer's instructions and probed with antibodies to BMP-1 and treated as described above.

Analytical and electron microscopy methods

Amino acid sequence analysis was performed by automated Edman degradation using an Applied Biosystems 473A protein sequencer. For N-terminal sequencing of the TSPN-1 released fragment, the membrane, after immunotransfer, was treated with pyroglutamate aminopeptidase (Boehringer-Mannheim) to remove the pyroglutamic acid blocking groups before performing automated Edman degradation.

For rotary shadowing, purified N α 1 fragment was diluted to 5-10 μ g/ml with 0.1 M ammonium acetate. Samples were mixed with glycerol (1:1), sprayed onto freshly cleaved mica sheets and immediately placed on the holder of a MED 010 evaporator (Balzers). Rotary shadowing was carried out as previously described [22]. Observations of replicas were performed with a Philips CM120 microscope at the "Centre Technique des Microstructures" (Université Lyon 1, Villeurbanne, France).

The possible triple helix folding of N α 1 was ascertained by pepsin digestion experiments and circular dichroism. Pepsin digestion experiments were performed on the N α 1 fragment and the recombinant homotrimeric α 1(V) as positive control [12]. Samples were treated with pepsin for 2 hrs at room temperature at an enzyme/substrate ratio of 1:10 in acetic acid 0.5 M. Digestion products were analyzed by 5-20% gradient (for N α 1) or 6 % (for the procollagen V homotrimer) SDS-PAGE, under reducing conditions, followed by Coomassie blue staining. The N α 1 fragment was analysed by circular dichroism before and after pepsin treatment. The pepsinized recombinant homotrimer collagen V, referred to as α 1TH [12], was analyzed as

control. Spectra were recorded at 20°C in 50 mM acetic acid (for $\alpha 1\text{TH}$) or 20 mM sodium phosphate pH 7.0 (for $\text{N}\alpha 1$) on a Chirascan apparatus (Applied photophysics). Measurements were performed with either 0.5 mm or 0.2 mm path length cells. Spectra were collected at 0.5 nm intervals over the wavelength range from 260 to 180 nm.

RESULTS

Expression and characterization of recombinant $\text{N}\alpha 1(\text{V})$ fragment

The $\text{Npro}\alpha 1(\text{V})$ fragment, referred to as $\text{N}\alpha 1$, was designed to encompass the complete N-propeptide of the human $\alpha 1(\text{V})$ chain, including the NC3, COL2 and NC2 domains plus eleven triplets of COL1 (Fig. 1A). The fragment contains the BMP-1 cleavage site, which is located between the TSPN-1 domain and the variable region (Fig. 1A). The theoretical molecular weight of the corresponding fragment is 64 kDa but electrophoretic analysis of serum-free medium from $\text{Npro}\alpha 1(\text{V})$ transfected 293-EBNA cells demonstrated a protein band at 116 kDa (Fig. 2A, lane 1), which was absent in non-transfected cell medium (data not shown). The identity of the recombinant protein was confirmed by N-terminal sequencing after electrotransfer. The determined sequence, Ala-Gln-Pro-Ala, starts with the first amino-acid of the $\text{pro}\alpha 1(\text{V})$ chain after the peptide signal cleavage site [12]. This indicates that the recombinant protein, even though it migrated much slower than expected, corresponds to the $\text{Npro}\alpha 1(\text{V})$ fragment. This is in agreement with previous results on the recombinant production of the entire $\text{pro}\alpha 1(\text{V})$ chain. Collagenase digestion of the recombinant $\text{pro}\alpha 1(\text{V})$ chains revealed a 86 kDa fragment corresponding to the entire N-propeptide [12]. The difference in migration (116 kDa vs 86 kDa) matches correlates with the presence in the construct of an additional sequence encoding the complete COL2 and NC2 domains and the 11 triplets of the COL1 domain (Fig. 1A). Two steps of anion exchange chromatography were performed to purify the recombinant fragment (Fig. 2A). Rotary-shadowing of the purified $\text{N}\alpha 1$ fragment revealed an homogenous preparation of 50 nm long molecules with a globular domain at one extremity, the TSPN-1 domain, and an extended tail corresponding to the variable region (Fig. 2B). This observation attests for the proper folding of the NC3 domain. The COL2 and COL1 domains were not visible in our preparations indicating that these domains do not fold into stable triple helices. Moreover, formation of a trimer should be visualized as a fish-spear shape with 3 TSPN globular domains at one end of the molecule. We definitively excluded the possibility that $\text{N}\alpha 1$ folds into a triple helix from pepsin digestion experiments and circular dichroism analysis. As shown in Figure 2C, $\text{N}\alpha 1$ was

completely digested by pepsin (Fig. 2C, lane 2) whereas pepsin digestion of the $\alpha 1(V)$ homotrimer in the same conditions showed a pepsin-resistant band (Fig. 2C, lane 4) corresponding to the triple helix domain referred to as $\alpha 1TH$ [12]. CD spectra were monitored for $N\alpha 1$ before and after pepsin digestion and compared to the spectrum of $\alpha 1TH$ which was typical of a triple helix conformation (a negative minimum peak at 197 nm and a positive peak around 222 nm) (Fig. 2D). The spectrum obtained for $N\alpha(1)$ before pepsinization corresponded to signals from the different sub-domains of the fragment, particularly the non collagenous domains, TSPN and the variable region (Fig. 2D). After pepsin digestion, no particular signal was monitored for $N\alpha 1$ (Fig. 2D) corroborating the results obtained with SDS-PAGE analysis (Fig. 2C).

The purified fragment $N\alpha(1)$ was used to obtain monoclonal antibodies to different regions of the N-propeptide. None of the hybridomas obtained recognized the TSPN-1 domain. The monoclonal antibody to the N-propeptide, called 6A7, was selected for this study, whose epitope was located in the variable region. It was shown to recognize the complete pro $\alpha 1(V)$ chain and the $N\alpha 1$ fragment (Fig. 3, lanes 4 and 5) but not the released BMP-1 fragment, the TSPN-1 domain (data not shown). Polyclonal antibodies to the TSPN-1 domain were obtained by immunizing rabbits with the purified recombinant domain produced in bacteria. These antibodies, pAbTSPN, recognized the complete pro $\alpha 1(V)$ chain, the $N\alpha 1$ fragment (Fig. 3, lanes 1 and 2) and the recombinant TSPN-1 domain (data not shown).

$N\alpha 1$ is processed by BMP-1

HEK 293 cells were transiently transfected with both $N\alpha 1$ and BMP-1 constructs, in order to visualize digestion products immediately after SDS-PAGE analysis or immunoblotting. We chose the HEK 293 cell line for several reasons. These cells have been successfully used in the past for production of both matrix proteins and enzymes because correct protein folding and high expression level are achieved. In addition, they express endogenous furin [23], a proprotein convertase enzyme involved in functional processing of metalloproteinases, notably BMP-1 and ADAMTS. BMP-1 processing of the C-propeptide of fibrillar collagens is known to be independent of the triple helical conformation [16]. To test whether the N-propeptide cleavage by BMP-1 is also conformation independent, we chose to perform experiments using the monomeric N-propeptide recombinant fragment as substrate. Processing experiments using cell factory showed that a released 34 kDa fragment is detected

in conditioned medium from cells co-transfected with $N\alpha 1$ and BMP-1 constructs using pAbTSPN (Fig. 4A, lane 2). This band was not detected when cells were transfected with $N\alpha 1$ construct alone (Fig. 4A, lane 1). The production of active BMP-1 by cells after co-transfection was assessed by probing the same membrane with BMP-1 antibodies as described in Materials and Methods (Fig. 4A lane 2). In contrast to the control (Fig. 4A lane 1), a clear band migrating at the expected position was detected in cells co-transfected with both constructs (Fig. 4A lane 2). N-terminal sequence of the released fragment confirmed that it corresponds to the N-terminal portion of the $N\alpha 1$ fragment. The processed $N\alpha 1$ fragment was identified by probing the membrane with the 18G5 antibody (not shown) and the N-terminal sequence, X-Asp-Pro-Asn-Pro, was determined after deblocking with pyroglutamate endopeptidase. It corresponds to the sequence previously described by others [15]. *In vitro* experiments were carried out in parallel with the purified recombinant fragment and enzyme. Western blot analysis of samples after overnight incubation at 37°C of the purified $N\alpha 1$ and BMP-1 also showed the appearance of a 34 kDa fragment recognized by pAbTSPN. The same blot was probed with BMP-1 antibodies to reveal the presence of the enzyme band in the assay contrary to the control in which BMP-1 was omitted (Fig. 4A, lanes 3 and 4). However, contrary to the processing assays in cells, considerable protein breakdown was observed after overnight incubation. A similar experiment was performed using cells stably transfected with the BMP-1 construct (Fig. 4B, lane 1). Analysis of conditioned media after single transfection with the $N\alpha 1$ construct revealed the presence of a dense band corresponding to the released TSPN-1 domain (Fig. 4B, lane 2). Our results indicate that, as for C-propeptide processing, triple helical conformation is not necessary for BMP-1 cleavage of collagen V N-propeptide.

BMP-1 cleavage specificity of $N\alpha 1$ processing

With the exception of procollagens V and XI N-terminal processing, all the previously identified BMP-1 cleavage sites contain an aspartic acid residue at the P1' position that appears to be indispensable for BMP-1 activity [16]. The BMP-1 cleavage site in the N-propeptide of the pro $\alpha 1(V)$ chain is different from the consensus site described for other matrix proteins [24] and corresponds to the Ser-254/Gln-255 peptide bond between the TSPN-1 domain and the variable region (Fig. 5A). The P1' residue indispensable for the cleavage is thus not an aspartic acid residue but a glutamine. To investigate which residue is essential for BMP-1 activity, six different mutants were generated from the $N\alpha 1$ wild type

construct (Fig. 1B): three single mutants S254A, Q255A and D256A, two double mutants S254A/Q255N and Q255A/D256A and the triple mutant S254A/Q255A/D256A. Mutation to Ala of Gln-255 at the P1' position of the cleavage site might introduce a known cleavage site for BMP-1, Ala-Asp [16], with regard to the presence of an Asp residue at the P2' position (Fig. 1B). To address this possibility, Gln-255 was mutated to Asn in the double mutant S254A/Q255N. The D267A mutant located downstream to the cleavage site was also generated as a control (Fig. 1B). A screening for BMP-1 cleavage efficiency was assayed by transfection of mutant and wild type constructs into BMP1-293 EBNA cells (Fig. 5C) for which BMP-1 expression level was checked using antibodies against BMP-1 (Fig. 5B). Alternatively, co-transfection of both $\text{N}\alpha 1$ and BMP-1 constructs in HEK-293 EBNA cells was performed to confirm the results (not shown). Conditioned media were analyzed by Western blotting using anti-TSPN-1 antibodies. The results showed that N-propeptide cleavage was either unaffected or only slightly affected in cells expressing the single mutants S254A and Q255A and the double mutant S254A/Q255N (Fig. 5C lanes 3, 4 and 6) as well as in D267A control (not shown). In striking contrast, the data showed that the P2' residue Asp is crucial for BMP-1 activity (Fig. 5C, lane 5). In agreement with this result, BMP-1 cleavage of the double Q255A-D256A mutant was greatly affected (Fig. 5C, lane 7). Transfection with the triple mutant construct completely abolished the N-propeptide cleavage by BMP-1 (Fig. 5C, lane 8). Our results highlight the importance of the Asp residue at the P2' position in the $\alpha 1(\text{V})$ N-propeptide cleavage by BMP-1.

Processing of the procollagen V C-propeptide by BMP-1

BMP-1 and furin are both capable of *in vitro* processing of the pro $\alpha 1(\text{V})$ C- propeptide chain [16]. Proteolytic C-propeptide removal by furin occurred between Arg-1585 and Asn-1586 (Fig. 1B), a cleavage site located upstream to the previously identified cleavage site of BMP-1 (Asp-1594/Asp-1595) [15, 16]. *In vitro*, processing of the pro $\alpha 1(\text{V})$ C-propeptide by furin is more efficient than processing by BMP-1. We thus took advantage of the "in cellulo" method to study $\alpha 1(\text{V})$ C-propeptide processing by BMP-1 in the absence of furin cleavage. The use of decanoyl-RVKR-chloromethyl ketone to protect from furin activity in HEK-293 cells only inhibited furin cleavage by about 50-70% [16]. We thus sought to abolish the furin cleavage site of the pro $\alpha 1(\text{V})$ chain by mutating to alanines the two arginines, Arg-1584 and Arg-1585, located at positions P2 and P1 of the cleavage site (Fig. 1B). The furin mutant and wild type constructs were transiently transfected into HEK-293 cells and conditioned media were

analyzed by SDS-PAGE. As can be seen (Fig. 6A), the R1584/1585A mutations efficiently protected the C-propeptide cleavage from furin activity (Fig. 4A, lane 2). Only a very faint band migrating at the pN α 1(V) position was observed in conditioned medium of mutant cells by Western blotting with the 6A7 antibody (Fig. 6B, lane 3). The pro α 1R1584/1585A construct was then transfected into BMP-1 293 cells to analyze C-propeptide cleavage efficiency compared to the wild type (Fig. 6C and D). BMP-1 processing of pro α 1R1584/1585A homotrimers yielded two bands recognized by the 6A7 antibody (Fig. 6C, lane 2). The largest corresponds to the pC α 1(V) form that migrated with a mobility faster than that of pN α 1(V) (Fig. 6B, lane 3). In the same experimental conditions, processing of the wild type homotrimer by BMP-1 gave a unique band corresponding to the mature α 1(V) chain (Fig. 6C, lane 1). In agreement with these results, 12% SDS-PAGE gel analysis (Fig. 6D) revealed the absence of the band corresponding to the furin cleavage in BMP-1 293 cells transiently transfected with pro α 1R1584/1585A construct (Fig. 6D, lane 3 compared to lanes 1 and 2). The BMP-1 activity on the N-propeptide in the cell factory assay was attested by the presence of a faster band of same intensity (Fig. 6D, lanes 2 and 3), which corresponds to the N-terminal portion of the pro α 1(V) homotrimer released by BMP-1. As can be seen (Fig. 6D), the C-propeptide cleavage by BMP-1 was not enhanced in the absence of furin cleavage. The corresponding band migrating at about 38 kDa was of similar intensity in wild type and mutant samples (Fig. 6D, lanes 2 and 3). These data confirm *in vitro* experiments [16] and indicate that BMP-1 is capable of processing the C-propeptide even though less efficiently than furin.

DISCUSSION

Removal of the N- and C-propeptides of fibrillar collagens is accomplished by procollagen proteinases of the ADAMTS and BMP-1/TLD families respectively. This post-translational event plays a crucial role in fibril assembly [25] and proteolytic products of extracellular matrix proteins may have important biological functions [2]. Collagen V shows a partial retention of N-propeptide that inhibits lateral fibril growth [26]. The excision of the N-propeptide can occur at different sites in a tissue specific manner and be ensured by BMP-1 and/or ADAMTS-2 [15, 17]. Another feature of the minor fibrillar collagen V is that enzyme activities responsible for C-propeptide cleavage (BMP-1 and/or furin) are chain-dependant [16, 27].

In order to analyze processing of the procollagen V at the molecular level, we took the advantages of a cell approach based on the transient transfection of cells with plasmids that each encode one of the partners (enzyme and substrate) followed by direct analysis of digestion products. The major advantages of this procedure are the rapid screening of processing interactions, the lack of protein breakdown during purification and enzyme digestion steps and the analysis of enzyme activity in a cellular environment.

Procollagen V can be processed at the C-terminal end by two enzymes, furin and BMP-1. The consensus recognition sequence for furin cleavage is Arg-Xaa-Arg/Lys-Arg (Xaa for any other residue). Excision of the C-propeptide from the pro α 1(V) chain by BMP-1 activity occurs at a site (Asp-1594/1595) next to the furin cleavage site (Arg-1585/Asn-1586) [15]. Kessler et al. [16] showed that the C-propeptide processing by BMP-1 was much less efficient than furin cleavage and 4-fold less efficient than the cleavage of the N-propeptide by BMP-1. Partial inhibition of furin activity, using enzyme inhibitors, showed that BMP-1 is capable of cleaving the C-propeptide from the intact molecule [16]. To accurately analyze the physiological relevance of BMP-1 cleavage, it was important to carry out experiments in a cellular environment and in the complete absence of furin activity. The double mutation (Arg1584/1585Ala) completely abolished furin activity and showed that the C-propeptide fragment released by BMP-1 activity did not result exclusively from further cleavage of the furin-derived fragment. However, a consistent finding over the course of several experiments was the lower efficiency of the C-propeptide cleavage by BMP-1 compared to furin and no enhancement of BMP-1 activity was obtained after mutation of the furin cleavage site. Given the strong conservation of this domain in all the fibrillar procollagens [28], a common processing mechanism was expected. However, this does not turn out right. It has been shown that the C-propeptides of the α 1 and α 3 chains of collagen V, a member of the fibrillar collagen family, are efficiently cleaved by furin activity [15, 27]. The procollagen C-proteinase enhancer (PCPE) is a potent stimulator of the BMP-1 processing of the major fibrillar collagens [24]. But the possible role of PCPE as an enhancer of BMP-1 cleavage of the fibrillar collagen V C-propeptides remains an open question. Low amounts of PCPE were detected in the 293 HEK cell media [18]. The data presented here indicate either that PCPE enhancement of BMP-1 processing is not targeted to all members of the collagen fibrillar family or that the cells produce insufficient amount of PCPE to efficiently increase BMP-1 activity. The later hypothesis is likely since maximum enhancement activity appears to occur at a PCPE:procollagen molar ratio of 1:1 [29].

The pro α 1(V) N-propeptide BMP-1 cleavage site, which occurs at the peptide bond Ser-254/Gln-255, differs from those previously described [16], most remarkably in the absence of an invariant aspartate at the P1' position. Proteolytic processing of pro α 1(XI) by BMP-1 occurs at an analogous cleavage site (Fig. 7) [30]. The pro α 3(V) chain was shown to be cleaved at a previously unreported site [31]. Though the primary sequence of the pro α 3(V) chain is highly homologous to pro α 1(V), BMP-1 cleavage occurs at a different peptide bond located between Gln-463 and Ala-464 (Fig. 7). In that case, the complete N-propeptide is released. Again, the cleavage sequence lacks an aspartate at the P1' position and residues adjacent to the site showed no homology with the residues flanking any previously characterized cleavage sites of BMP-1 (Fig. 7). Along this line, Colige and collaborators [17] showed that ADAMTS-2 processes the aminopropeptide of pro- α 1(V) homotrimer at the end of the variable domain at a cleavage site (Pro-Ala) different from the previously described sites (Pro/Ala-Glu) for ADAMTS-2. In generating mutants to the previously identified cleavage site, we demonstrate that the residue crucial for BMP-1 cleavage is the Asp at the P2' position of the cleavage site whereas the P1' Gln does not influence BMP-1 activity. This result was unexpected. First, the P1' Gln is conserved in the pro α 1(V) and pro α 1(XI) chains of various species [15] but the mutation Q255A and the double mutation S254A/Q255N BMP-1 only slightly affect the activity of BMP-1. Second, the presence of a conserved Asp residue is known to be important for BMP-1 cleavage but at the P1' position. The presence of a conserved P3' Pro adjacent to the Asp residue can play an important role in BMP-1 activity although prolines are not found at similar positions in all previously identified BMP-1 cleavage sites. The fact that mutation of the P1 Ser has no effect on BMP-1 activity is less surprising since this residue is not conserved in the pro α 1(XI) chains [15].

Fibrillar collagens are the most abundant structural proteins in the extracellular matrix. They undergo N- and C- propeptide processing that implicates specific proteinases. Collagen V is a minor fibrillar collagen that can be distinguished from the others by its capacity to control fibrillogenesis. In addition, this molecule is submitted to a particular processing and is involved in fundamental processes such as development and human connective tissues disorders. Here, in exploring the N- and C- terminal proteolytic processing of pro α 1(V) chain using several mutants, our results provide new relevant informations on this complex and poorly understood mechanism essential to the function of the extracellular matrix proteins.

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The abbreviations used are: BMP-1, bone morphogenetic protein- 1; ADAMTS, a disintegrin and metalloproteinase domain with thrombospondin type 1 repeats; N-pro, N-propeptide; C-pro, C-propeptide.

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FIGURE LEGENDS

Figure 1: Constructs and mutants of the human pro α 1(V) chain. *A*, schematic representation of the N-propeptide construct referred to as N α 1. It consists of NC3, COL2, NC2 and eleven triplets from COL1. *B*, Sequences of the C-propeptide encompassing the furin cleavage site and of the R1584/1585A mutant. The two arginines at positions 1584 and 1585 were mutated to alanine. *C*, Sequences of the N-propeptide encompassing the BMP-1 cleavage site and of the BMP-1 cleavage site mutants. COL, collagenous domain; NC, non-collagenous domain. Mutated residues are underlined. Arrows indicate cleavage sites.

Figure 2: Production and characterization of N α 1(V) fragment and antibodies. *A*, 6% SDS-PAGE analysis of recombinant N α 1(V) fragment produced in 293-cells. Electrophoretic patterns of serum-free medium from transfected cells (lane 1) and of the purified N α 1(V) fragment (lane 2). *B*, Rotary-shadowing images of recombinant purified N α 1(V) fragment revealing the presence of a compact globular domain (TSPN-1 domain) and an extended rod-like domain (variable region). *C*, 5%-20% gradient SDS-PAGE analysis of N α 1(V) (lane 1) and N α 1(V) digested with pepsin (lane 2) and 6% SDS-PAGE analysis of α 1(V) homotrimeric molecule treated without (lane 3) and with pepsin (lane 4). *D*, Circular dichroism spectra of α 1(V)TH, N α 1 and N α 1 after pepsin digestion. Buffer baselines were systematically subtracted.

Figure 3: Western-blot characterization of the polyclonal antibodies to the TSPN-1 domain (pAbTSPN) and the monoclonal antibody (mAb6A7) directed against the variable region of the N α 1(V) fragment. pAbTSPN (lanes 1 and 2) and the monoclonal antibody 6A7 (lanes 4 and 5) recognized the complete pro α 1(V) chain (lanes 2 and 5) and the N α 1(V) fragment (lanes 1 and 4). Lane 3 shows pro α 1(V) chain recognition by polyclonal antibodies directed against the collagen V triple helix (from Novotec). Epitopes of the different antibodies are indicated on the schematic representation of the N α 1(V) fragment presented below. Left, running positions of protein standards are indicated in kDa.

Figure 4: BMP-1 cleavage of the N-propeptide domain of the pro α 1(V) chain. *A*, Western blot analysis of BMP-1 processing of the N-propeptide of the pro α 1(V) chain in 293-EHK cells (lanes 1 and 2) compared to *in vitro* experiments with purified proteins (lanes 3 and 4). Electrophoretic patterns of 293-HEK cell media transfected with N α 1 construct alone (lane 1)

and co-transfected with BMP-1 and N α 1 constructs (lane 2). Electrophoretic patterns of purified recombinant N α 1(V) fragment incubated without (lane 3) or with (lane 4) recombinant BMP-1. *B*, Western blot analysis of BMP-1 processing of the N-propeptide of the pro α 1(V) chain in HEK-293 cells expressing BMP-1. Electrophoretic patterns of HEK-293 BMP-1 cell medium (lane 1) and of medium from HEK-293 BMP-1 cells transfected with N α 1 construct (lane 2). Membranes were probed with pAbTSPN and re-probed with anti-BMP-1 polyclonal antibodies. Left, running positions of protein standards are indicated in kDa.

Figure 5: BMP-1 cleavage specificity of N α 1 processing analysed by site-directed mutagenesis. *A*, Sequence for the N-propeptide encompassing the BMP-1 cleavage site. The mutated residues S254, Q255, D256 and D267 are in *boldface*, the BMP-1 cleavage site is underlined. *B*, Western blot analysis showing the level of BMP-1 expression in HEK-293 transfected with BMP-1. Electrophoretic patterns of cell media transfected with a mock plasmid as negative control (lane 1), and with BMP-1 construct (lanes 2 and 3). A low level of proBMP-1 is observed in wild type HEK-293 cells transfected with a control plasmid, while the high expression level of the mature form of BMP-1 is reproducibly observed in transfected HEK-293 cell media. *C*, Electrophoretic patterns of cleavage products obtained by processing wild type N α 1(V) and mutant constructs using cell factory assays. Western blots were performed to monitor efficiency of BMP-1 cleavage. Membranes were probed with pAbTSPN. Cells expressing BMP-1 (*B*, lane 2) were transfected with a mock plasmid as negative control (lane 1), wild-type N α 1 construct (lane 2) or with the mutant constructs (lane 3) S254A; (lane 4) Q255A, (lane 5) D256A, (lane 6) S254A-Q255N, (lane 7) Q255A-D256A and (lane 8) S254A-Q255A-D256A. Mutants D256A, S254A-Q255A-D256A and Q255A-D256A showed no or weak BMP-1 activity. Right, running positions of the different processed and unprocessed forms.

Figure 6: Effect of furin site mutagenesis on the α 1(V) C-propeptide cleavage by BMP-1.

A, 6% SDS-PAGE analysis of transfected HEK-293 cell medium with the human pro α 1(V) construct (lane 1) and with the mutant construct pro α 1R1584/1585A (lane 2). *B*, Western blot analysis of (lane 1) purified pN α 1(V) homotrimer as running position standard, transfected HEK-293 cell medium with the human pro α 1(V) construct (lane 2) and with the mutant

construct pro α 1R1584/1585A (lane 3). Membranes were probed with mAb 6A7. Western blot shows that the C-propeptide cleavage is completely abolished in HEK-293 cells transfected with the furin mutant. *C*, Western blot analysis (mAb 6A7) of the cleavage products in 293-HEK cells expressing BMP-1, transfected with the pro α 1(V) construct (lane 1) and with the mutant construct pro α 1R1584/1585A (lane 2). Right panel, schematic representation of the different pro α 1(V) processed forms and cleavage sites. N, N-propeptide; C, C-propeptide; TH, triple helix domain. *D*, Electrophoretic patterns of cleavage products obtained by processing pro α 1(V) (lanes 1 and 2) and the furin mutant pro α 1R1584/1585A (lane 3) in 293-HEK cells expressing recombinant BMP-1 and endogenous furin (lanes 2 and 3) and in wild type HEK-293 cells (lane 1). Samples were run on a 12% SDS-PAGE and stained with Coomassie blue. Right, running positions of the different processed forms.

Figure 7: Alignment of sequences corresponding to the BMP-1 cleavage site of the N-propeptide of the human pro α 1(V) (A) and pro α 3(V) (B) chains with human pro α 1(XI). COL, collagenous domain, NC, non collagenous domain. Conserved residues are *boldface*, arrows indicate known BMP-1 cleavage sites in (A) human pro α 1(V) and pro α 1(XI) and (B) pro α 3(V) (accession numbers are NM000093, NM001854 and NM015719 respectively).

Figure 1

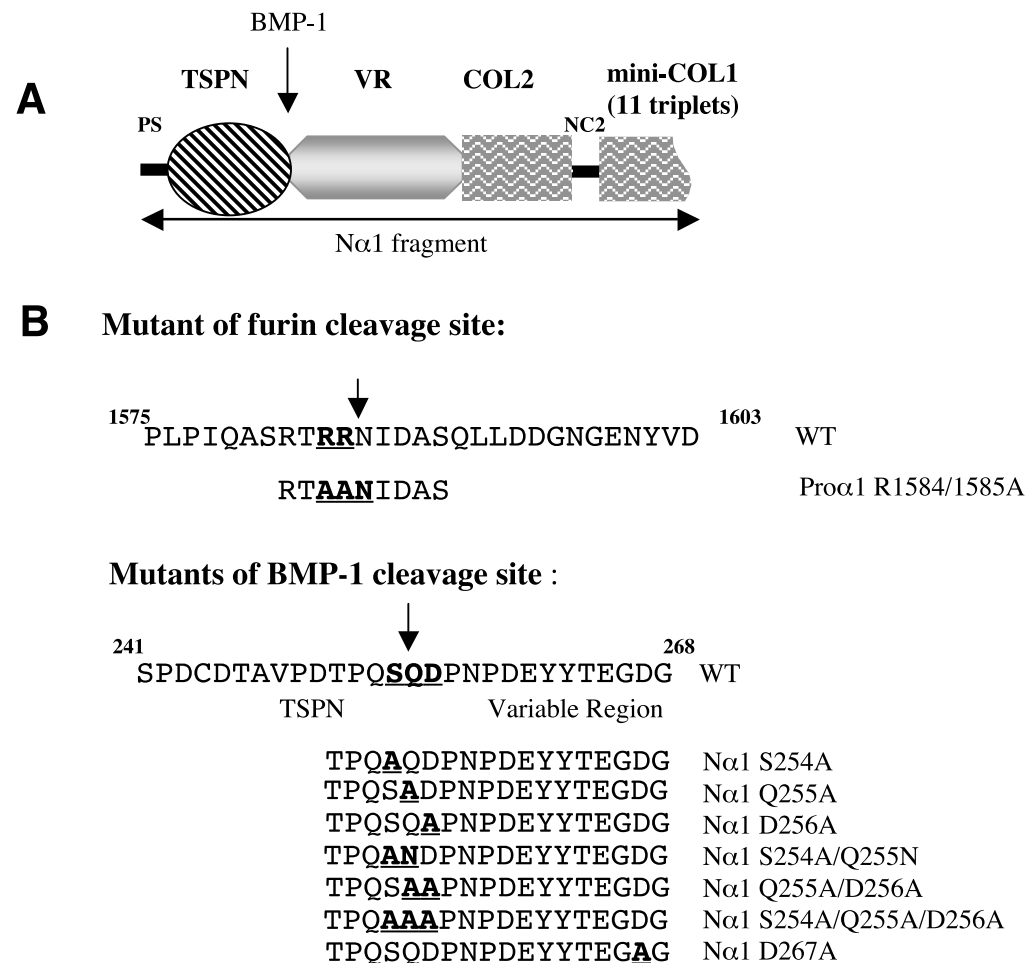


Figure 2

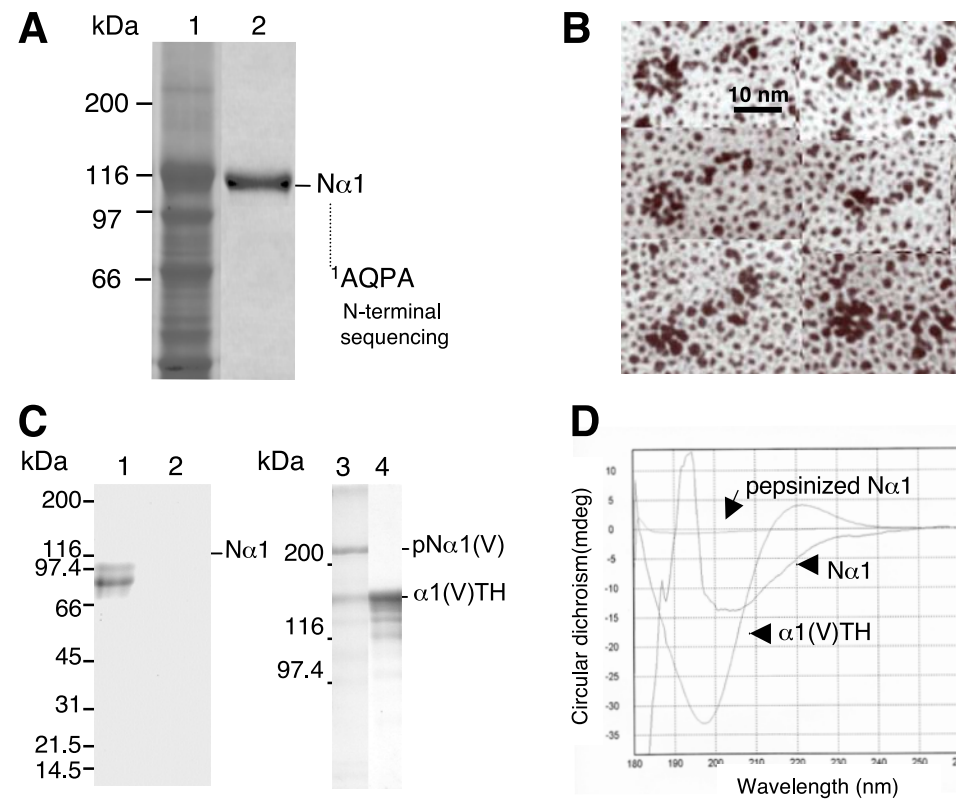


Figure 3

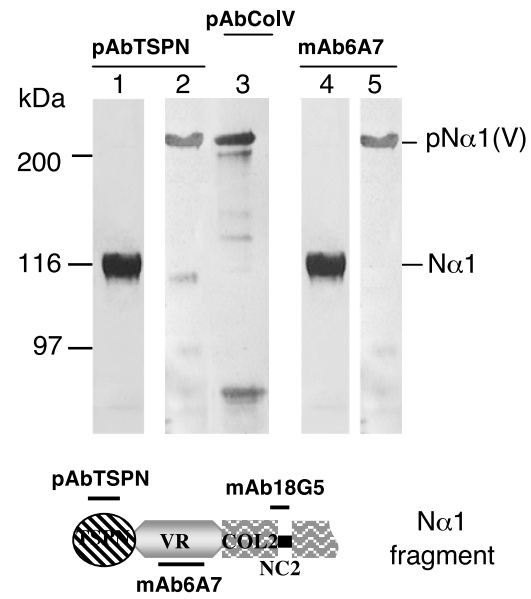


Figure 4

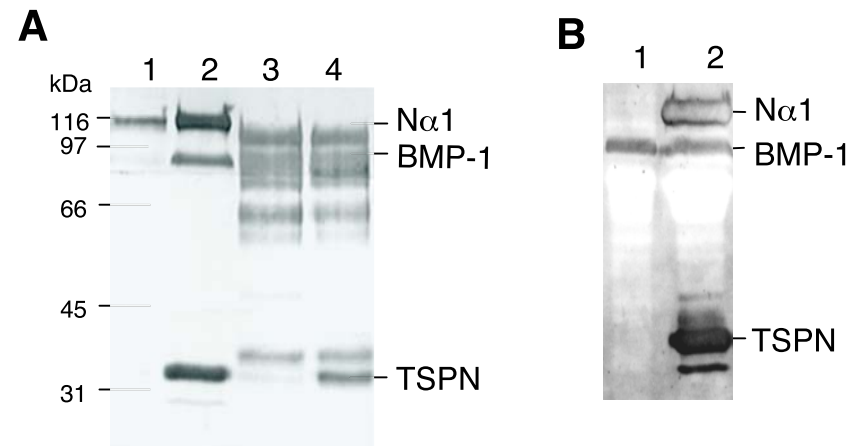


Figure 5

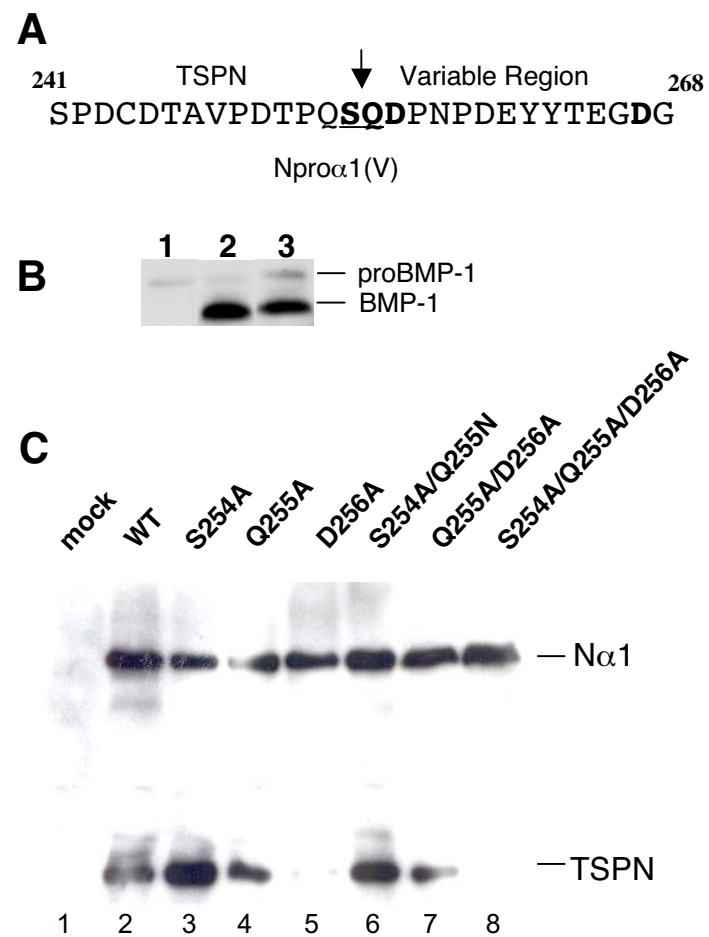


Figure 6

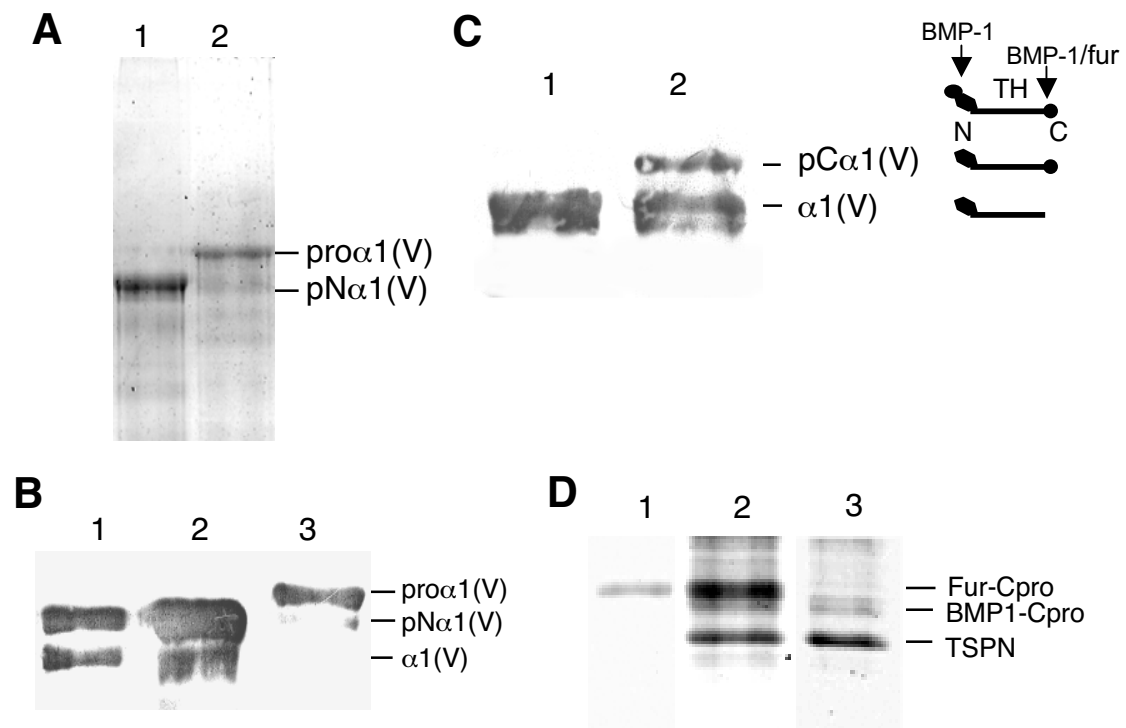


Figure 7

