Bone Morphogenetic Protein-1 (BMP-1) Mediates C-terminal Processing of Procollagen V Homotrimer*

Efrat Kessler§, Agnès Fichard§, Hélène Chanut-Delalande§, Marina Brusel‡, and Florence Ruggiero§

From the §Tel-Aviv University Sackler Faculty of Medicine, Goldschleger Eye Research Institute, Sheba Medical Center, Tel-HaShomer 52621, Israel and ¶Institut de Biologie et Chimie des Protéines, Unité Mixte de Recherche CNRS 5086, 7 Passage du Vercors, 69367 Lyon Cedex 07, France

Received for publication, April 3, 2001, and in revised form, May 10, 2001 Published, JBC Papers in Press, May 17, 2001, DOI 10.1074/jbc.M102921200

The processing of the fibrillar procollagen precursors to mature collagens is an essential requirement for fibril formation. The enzymes involved in these events are known as the procollagen N and C proteinases. The latter, which cleaves the C-propeptides of the fibrillar procollagens I-III, is identical to the previously described bone morphogenetic protein-1 (BMP-1). Surprisingly, unlike the other fibrillar collagens, the processing of the C-propeptide domain of the procollagen V homotrimer was found to be mediated by furin rather than BMP-1. However, the presence of putative BMP-1 cleavage sites in the α1(V) C-propeptide sequence prompted us to reconsider the procollagen V C-propeptide cleavage by BMP-1. Using a recombinant system to produce substantial amounts of the proα1(V) homotrimer, we have previously shown that the C-propeptide is spontaneously released in the culture medium. The trimeric C-propeptide fragment, resulting from the furin cleavage, still encompassed the predicted BMP-1 cleavage sites. It was purified and tested as a substrate for BMP-1. In parallel, the release of the C-propeptide in the culture medium was inhibited by the addition of a specific furin inhibitor, allowing the re-examination of BMP-1 activity on the intact molecule. We showed that BMP-1 does cleave both substrates at one of the two predicted C-propeptidase cleavage sites. Our results favor a role for PCP/BMP-1 in a physiological C-terminal processing of procollagen V and imply a general mechanism for fibrillar collagen C-terminal processing.

The fibrillar collagens I, II, III, V, and XI are essential for the extracellular matrix scaffold integrity. They are synthesized as larger precursors, procollagens, in which the central uninterrupted triple-helical domain (COL1) is flanked by non-collagenous domains (NC), the N-propeptide and the C-propeptide (also referred to as the NC1 domain) (1). Both the C- and N-terminal extensions are processed extracellularly, an event that triggers the assembly of the mature protein into fibrils. Anomalies in processing of the propeptides can severely affect the aggregation of the molecules into fibrils leading to defective connective tissues (2).

Processing of procollagens I, II, and III results in an almost complete removal of both the N- and C-propeptides (3). The release of collagen V N-propeptide, however, appears to be partial, leaving a globular domain of functional importance. Indeed, collagens V and I co-aggregate to form heterotypic fibrils, and the N-terminal globule remaining on collagen V was suggested to serve as a sterical regulator of the heterotypic fibril accretion (4, 5). However, unlike collagen I, collagen V is rare in tissues, and this has greatly hampered its purification and characterization as an intact molecule, resulting in a controversial determination of the exact cleavage site of the N-propeptide (6–8). Concerning the collagen V C-propeptide, although little is known about its processing, its complete removal is generally agreed. Most of the studies on collagen V have dealt with the most abundant collagen V isoform, the heterotrimer [α1(V)2α2(V)] (9). Two other stoichiometries, α1(V)α2(V)α3(V) and [α1(V)]3, have also been described, but other chain associations might exist since a novel α4(V) chain has recently been described (10). One should therefore consider the possibility that the processing patterns of the various collagen V collagen molecules may differ.

Removal of the N- and C-propeptides from procollagens I, II, and III is catalyzed by specific Zn2+-dependent metalloendopeptidases, procollagen N- and C-proteinase, respectively (3). Procollagen C-proteinase, also known as bone morphogenetic protein-1 (BMP-1) (11–14), is a member of the astacin family of proteases (15, 16). In addition to procollagen processing, BMP-1 can cleave several other substrates (17–19) and, thus, play other roles central to extracellular matrix formation and early morphogenesis like embryo ventralization (20–22). Cleavages by BMP-1 of all the known substrates have been shown to occur at peptide bonds with Asp at the P1’ position, in particular Ala-Asp or Gly-Asp, sequences known as the consensus procollagen C-proteinase cleavage sites in procollagens I-III (3, 23). BMP-1 has also been reported to process the homotrimer of type V procollagen (24). However, the cleavage site, a Ser-Gln peptide bond, and its location within the N-propeptide domain, were both inconsistent with those predicted for C-proteinase cleavage.

Recombinant type V homotrimer was produced successfully in 293-EBNA cells; however, its C-propeptide domain was rapidly cleaved and accumulated in the medium (25). Subsequently, it has been reported that this cleavage was due to a furin-like activity (24). Considering the fact that two consensus C-proteinase cleavage sites do exist near the furin-like cleavage site (26, 27), the possibility that an enzyme other than BMP-1...
is responsible for physiological processing of the NC1 domain of procollagen V seemed puzzling. Moreover, the 293-EBNA cells are known to possess high furin-like activity. Thus, although processing of certain proteins in these cells appears to be physiological, as was shown for pro-fibrillin-1 (28), irrelevant cleavages have also been observed for collagen XVI (29).

To further investigate the role of BMP-1 in processing of the collagen V C-propeptide, in this study the trimeric NC1 fragment released by the furin-like activity (fur-Cpro) was isolated and tested as a substrate for BMP-1. Also, by adding a specific furin inhibitor, we were able to produce intact procollagen V homotrimer that served to re-examine BMP-1 action on the entire molecule. We found that BMP-1 can cleave one of the two predicted C-proteinase cleavage sites within both the isolated fur-Cpro fragment and the full-length procollagen V homotrimer. These results favor a role for procollagen C-proteinase/BMP-1 in physiological C-terminal processing of type V procollagen.

MATERIALS AND METHODS

Production of the Recombinant Procollagen V Homotrimer—Production of the recombinant procollagen V homotrimer in 293-EBNA cells followed the previously described procedure (25). Briefly, the full-length cDNA of the human proα1(V) chain was subcloned into the mammalian episomal expression vector pCEP-4 (InVitrogen), and the expression plasmid was transfected into human embryonic kidney 293-EBNA cells by electroporation. The transfected cells were selected by the addition of hygromycin (300 μg/ml) to the culture medium for 15 days. For protein production, hygromycin-resistant 293-EBNA cells were grown to confluence and then placed on serum-free medium containing sodium ascorbate (50 μM). The procollagen V were incubated for 19 h at 37 °C with 200 ng of BMP-1 in a total reaction volume of 80 μl. When indicated, the digests were performed in the presence of 1,10-phenanthroline (1.25 mM). The reactions were incubated at 20 °C until use.

Protein Purification—Large amounts of serum-free medium from transfected 293-EBNA cells were collected every 48 h for 2 weeks. To purify the procollagen V homotrimer, transfected cell media were dialyzed against 50 mM Tris/HCl, pH 8.6, and centrifuged, and the pellet containing the procollagen V homotrimer was resuspended in an appropriate volume of 50 mM Tris/HCl, pH 7.4, 500 mM NaCl. To recover the C-propeptide domain released from the procollagen V homotrimer by endogenous furin-like activity, the collected medium was dialyzed against 50 mM Tris/HCl, pH 7.4, 2 M urea and chromatographed on a DEAE-cellulose column (DE-52; Whatman) equilibrated with the same buffer. Adsorbed proteins were eluted with a linear 0–0.5 M NaCl gradient, and fractions were analyzed by SDS-PAGE (10% gels) in the presence or absence of a reducing agent. Fractions containing the C-propeptide fragment were subsequently applied to a reverse phase HPLC (C8 PR300) column and eluted with a gradient of acetonitrile (from 0 to 60%) in 0.1% trifluoroacetic acid. Fractions containing the purified C-propeptide were lyophilized and stored at −20 °C until use. The purified proteins were analyzed by SDS-PAGE (5 or 10% gels) and Coomassie Blue staining.

Enzyme Digests—Recombinant BMP-1 was produced in a baculovirus system and purified as previously described (13). The purified furin-C-propeptide (4 μg) was incubated for 8 h at 37 °C with 85 ng of BMP-1 in 40 μl of 50 mM Tris/HCl, 150 mM NaCl, 5 mM CaCl₂, 0.1% Brij-35, pH 7.5 (BMP-1 assay buffer). The reaction was stopped by heating (60 °C, 15 min) in Laemmli SDS sample buffer (30) containing 4% 2-mercaptoethanol. Procollagen V (2 or 3 μg of proα1(V) + pNα1(V) in a final volume of 32 or 48 μl of BMP-1 assay buffer, respectively) was incubated at 37 °C for 19 h with 60 or 100 ng of BMP-1, respectively. For N-terminal sequence analysis of cleavage products, −9 μg of procollagen V were incubated for 19 h at 37 °C with 200 ng of BMP-1 in a total reaction volume of 50 μl. When indicated, the digests were performed in the presence of 1,10-phenanthroline (1.25 mM). The reactions were terminated by heating at 100 °C for 1 min in Laemmli SDS sample buffer with or without reduction, as specified. Proteins were analyzed by SDS-PAGE on 10% gels and, unless otherwise stated, visualized by staining with Coomassie Blue.

Rotary Shadowing—For rotary shadowing, the recombinant procollagen V molecules and the NC1 fragments were diluted to 10 μg/ml with 0.1 M acetic acid and 0.2 M ammonium acetate, respectively. Samples in appropriate buffers were then mixed with glycerol (1:1), dropped onto freshly cleaved mica sheets and immediately placed on the holder of a MED 010 evaporator (Balzers). Rotary shadowing was carried out by evaporating platinum at an angle of 8°, followed by a 90° evaporation of carbon. Replicas were floated onto distilled water, picked up on copper grids and examined with a Philips CM120 microscope at the Center de Microscopie Electronique Appliquée à la Biologie et à la Géologie (Université Claude Bernard, Lyon I, France).

Protein Sequencing—Proteins were separated on 5 or 10% SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride membranes as previously described (25). Protein bands of interest were excised from the membrane, and N-terminal sequences were determined by automated Edman degradation on an Applied Biosystems Procise protein-sequencing system.

RESULTS

Purification of the C-propeptide Domain from Transfected Cell Medium—We have previously shown that expression of the proα1(V) homotrimer in 293-EBNA cells leads to the accumulation in the medium of a recombinant protein of about 250 kDa. This protein was shown to correspond to the pN form of the collagen V homotrimer, indicating that the NC1 domain was systematically and rapidly cleaved in the culture medium (25). The released fragment was recovered from the medium, and its purification was achieved by two steps of chromatography. Upon reduction of the disulfide bonds, electrophoresis of the purified NC1 fragment revealed a single ~40-kDa band that was not seen under nonreducing conditions. Without reduction, however, a higher molecular weight band of about 100 kDa was observed instead (Fig. 1A). This confirmed the presence of intersubunit disulfide bonds and the formation of trimers, as is the case for the C-propeptides of fibrillar collagens.

![Fig. 1. Purification and characterization of the C-propeptide domain released from the recombinant procollagen V homotrimer by the furin-like activity (A–C) and its cleavage by recombinant BMP-1 (D).](http://example.com/image)
Electron microscopy of rotary-shadowed purified NC1 fragments showed a homogenous population of small compact particles that ascertained the folding of this domain into a globular structure (Fig. 1B). N-terminal sequencing of the 40-kDa protein indicated that the release of the NC1 domain from the proα1(V) chain occurred by cleavage at a site immediately after an RTRR sequence corresponding to positions 1582–1585 of the proα1(V) chain, as was shown by others (24) (Fig. 1C). This cleavage, which involved the motif BXBB (B for basic residue), is typical for a furin-like enzyme; hence, the released C-terminal fragment was designated fur-Cpro. Interestingly, this latter processing occurred upstream of the two putative cleavage sites proposed for the C-proteinase (BMP-1) (26, 27). Thus, we assumed that the highly purified fur-Cpro fragment might be a good model substrate to test whether BMP-1 can process the NC1 domain of the procollagen V homotrimer.

**Cleavage of Purified fur-Cpro by BMP-1**—The fur-Cpro fragment was incubated with recombinant BMP-1, and the digestion products were analyzed by 10% SDS-PAGE followed by electrotransfer to polyvinylidene difluoride membrane and N-terminal amino acid sequencing. Incubation with BMP-1 resulted in the appearance of a band that showed a slight increase in mobility compared with the undigested protein (data not shown). N-terminal sequencing of this protein revealed the sequence DGNGENVY, which perfectly matches the sequence found for the corresponding band (XIDASQLLXD), X = an unidentified residue) started at Asn1586 indicating that no processing occurred in the absence of BMP-1. The BMP-1-cleaved C-propeptide fragment will be referred to hereafter as bmp-Cpro.

**Production of Intact Recombinant Procollagen V Homotrimer by Inhibition of the C-propeptide Cleavage in 293 Cell Medium**—To explore the ability of BMP-1 to cleave the C-propeptide from the intact molecule, we decided to produce and purify unprocessed recombinant procollagen V. With this aim, we cultured the transfected cells in the presence of decanoyl-Arg-Val-Lys-Arg-CH2Cl, a specific synthetic inhibitor of furin-like enzyme activity (31). In the absence of the inhibitor (control), a single polypeptide migrating at about 250 kDa accumulated in the medium that corresponded to the pN form of collagen V as previously described (25) (Fig. 2A). Electrophoretic analysis of culture media from cells incubated in the presence of the inhibitor showed an additional band migrating more slowly than the pN form. The difference in migration between the two bands was in good concordance with the size of the C-propeptide. Upon nonreducing conditions, the upper band migrated as a high molecular mass protein that was detected near the top of the gel, whereas the 250-kDa band migrated slightly faster (Fig. 2B). Such electrophoretic behavior is consistent with a successful inhibition of the C-propeptide cleavage. Indeed, the C-propeptide is the only domain that can form interchain disulfide bonds that generate the high molecular mass species.

**FIG. 2.** Inhibition of the spontaneous C-propeptide cleavage from the recombinant procollagen V in 293 cell medium. A, 6% SDS-PAGE electrophoretic patterns of the recombinant procollagen are compared when produced in the absence (lane 1) or presence (lanes 2 and 3) of 1 or 10 μM furin inhibitor (fur-Inh) decanoyl-Arg-Val-Lys-Arg-CH2Cl, respectively. Note the appearance of a band corresponding to the proα1(V) chain, denoted proα1(V), at the expense of the pN α1(V) band. B, 5% SDS-PAGE mobilities of the recombinant intact proα1(V) and the pN α1(V) bands are compared in the presence (lane 1) and absence (lane 2) of a reducing agent (dithiothreitol (DTT)). Note that only the proα1(V) chains migrate as disulfide-bonded trimers, attesting for the presence of the C-propeptide domain. A faint band corresponding to the C-propeptide generated by the furin-like enzyme in the 293-EBNA-transfected cell medium (reduced, fur-Cpro; unreduced, Cpro trimer) is also present in the preparation, further confirming that only a partial inhibition by the furin inhibitor was obtained. Running positions of protein standards are indicated in kDa on the left.

The undigested protein, incubated in buffer alone as a control, was also subjected to Edman degradation. The N-terminal sequence found for the corresponding band (XIDASQLLXD, X = an unidentified residue) started at Asn11586 indicating that no processing occurred in the absence of BMP-1. The BMP-1-cleaved C-propeptide fragment will be referred to hereafter as bmp-Cpro.
found upon nonreducing conditions. Likewise, the presence of intrachain disulfide bonds within the N-propeptide domain can explain the increased mobility of the 250-kDa band under nonreducing conditions. However, even in the presence of a 10-fold higher concentration of the inhibitor, complete inhibition was not achieved, and both the pN form and procollagen V homotrimer were always present in our preparation (Fig. 2A). The presence of the fur-Cpro band in the purified preparation, migrating at the bottom of the reduced gel and found as a trimer under nonreducing conditions, confirms that inhibition of the C-propeptide cleavage was incomplete (Fig. 2B). Rotary shadowing of the procollagen V homotrimers revealed 300-nm-long rod-like molecules flanked by two globular domains at their extremities. Based on their respective sizes, the large globular domain was assigned to the N-propeptide, and the smaller one, located at the opposite extremity, was assigned to the C-propeptide (Fig. 2C). The pN form presented the same structural characteristics but lacked the small globular domain (Fig. 2C, molecule on the right) as previously observed (25).

**Cleavage of the Procollagen V Homotrimer by BMP-1**—The preparation containing a mixture of the procollagen V homotrimer, the pN form, and the free 40-kDa C-propeptide fragment (fur-Cpro) was incubated with BMP-1, and the digestion products were analyzed by SDS-PAGE in the presence or absence of a reducing agent. Analysis under reducing conditions revealed that treatment with BMP-1 led to the appearance of two new bands migrating at ~38 and ~35 kDa (Fig. 3A). Edman degradation of the two additional bands was performed to identify the BMP-1 digestion products. The N-terminal sequence (not shown) of this band was identical to the sequence obtained for the band resulting from BMP-1 cleavage of the fur-Cpro fragment (see above, Fig. 3B). This cleavage is likely to derive from the procollagen V molecule since the corresponding band disappeared from the top of the gel and the intensity of the fur-Cpro band did not decrease much during incubation with BMP-1. This result suggests that the intact procollagen molecule is even a better substrate for BMP-1 than the fur-Cpro fragment. The 35-kDa band migrated faster under nonreducing conditions, which is consistent with the presence of intrachain disulfide bonds within the N-propeptide, whereas the doublet 40/38-kDa migrated together at about 110 kDa, as expected for the disulfide-bonded C-propeptide trimers.

As shown in Fig. 4, the formation of both the C- and N-propeptide fragments was inhibited by 1,10-phenanthroline, a metal chelator known to inhibit the activity of BMP-1 (13). This supported the involvement of BMP-1 in the processing of the N- as well as the C-propeptide domains of procollagen V. Intriguingly, whereas the release of the N- and C-terminal fragments was prevented in the presence of the inhibitor, the intensity of the fur-Cpro band was slightly increased under these conditions, perhaps reflecting the presence of some furin-like activity in the purified BMP-1 preparation. Consistent with this, the N-terminal sequence (not shown) of this band was identical to that of the authentic fur-Cpro fragment derived from the culture medium.

To gain more insight into the action of BMP-1 toward procollagen V, we performed a time course study in which the rates of release of the N- and C-propeptide fragments were compared by 10% SDS-PAGE analysis of samples removed at different time intervals during the incubation. The electrophoretic pattern showed a rapid appearance of the N-propeptide fragment that was almost complete within a 2-h digestion with BMP-1, whereas the intensity of the bmp-C-propeptide fragment started at Asp1509, and the sequence determined matched

---

**Fig. 3.** Cleavage by recombinant BMP-1 of the procollagen V homotrimer obtained in the presence of a furin inhibitor. A, 10% SDS-PAGE electrophoretic patterns of samples incubated with (lanes 2 and 4) or without (lanes 1 and 3) BMP-1. Samples were run either under nonreducing (nonred; lanes 1 and 2) or reducing (red; lanes 3 and 4) conditions (bme). Running positions of protein standards are indicated in kDa on the left. B and C, N-terminal sequences of the different cleavage products, fur-Cpro and bmp-Cpro (B) and N-propeptide (Npro) (C), as determined by Edman degradation. Human (hu) α1(V) sequences corresponding to positions 1573–1612 (B) and to positions 1–40 (C) deduced from the published cDNA (26, 27) are shown. The positions of furin (arrowhead) and BMP-1 (arrow) cleavages are indicated.
the absence (lane 3) or presence of 1.25 mM 1,10-phenanthroline (lane 3) and at the end (lanes 1 and 2) show as controls the electrophoretic patterns of the procollagen or without (lane 4) recombinant BMP-1. Lanes 3 and 4 correspond to the BMP-1 digestion products generated in lane 1 sample at the onset (lane 2) of the incubation. Lanes 1 and 2, 0 and 22 h time points.

DISCUSSION

Based on sequence similarities, structures of their cognate genes, and their low abundance, collagen V chains along with those of collagen type XI are classified as a subgroup of the fibrillar collagens known as the minor collagens. Within this group of collagens, the proα1(V), proα3(V), proα1(XI), and proα2(XI) chains are further distinguished by the characteristic structure of their N-propeptide domains, each containing a conserved N-terminal subdomain known as the PARP (proline/arginine-rich protein) domain, followed by a nonhomologous region referred to as the variable domain (26, 27, 32). There is evidence that the N-propeptides of the proα2(XI), proα1(V), and proα1(XI) chains are processed at a site just downstream of their PARP domain, and in some tissues, the released PARP domains may persist as intact molecules with potential functional importance (5, 33, 34). Another unique property of this subgroup of procollagen chains is that, with the exclusion of proα2(V), all contain a furin cleavage sequence (RXRR) in regions corresponding to the C-propeptide cleavage sites (Table I), which potentially can be processed by furin-like proprotein convertases (35). In comparison to the major fibril-forming collagen precursors, procollagens I-III, the mechanisms involved in processing of the minor procollagens are poorly understood.

The role of BMP-1, the enzyme that removes the C-propeptide from procollagens I, II, and III, in proteolytic processing of procollagen V was addressed previously (24). In contrast to type I-III procollagens, the C-propeptide of the proα1(V) homotrimer was not cleaved by BMP-1. Instead, BMP-1 released the N-terminal PARP domain, whereas the C-propeptide appeared to be cleaved by a furin-like proprotein convertase. Although the latter activity could be explained by the presence of the furin cleavage sequence in the vicinity of the predicted procollagen C-proteinase cleavage site (Table I), (i) the peptide bond cleaved within the N-propeptide domain (Ser-Gln) and the sequence surrounding this bond differed from the consensus cleavage site for procollagen C-proteinase processing site, the observations with BMP-1 were difficult to reconcile because (i) the peptide bond sequence surrounding this bond differed from the consensus sequence in the vicinity of the predicted procollagen C-proteinase cleavage site, and (ii) BMP-1 can process other extracellular matrix proteins, including prolyl oxidase (17), probiglycan (19), and the y2 chain of laminin-5 (18) and those cleaved in mouse chordin (20). B, hu, m, and r, human (26, 27, 32, 43), mouse (32), and rat (10) sequences, respectively; i and ii, the first and second potential BMP-1 cleavage sites in each, the proα1(V) and proα2(V) chains, respectively. Residues conserved in at least two documented BMP-1 substrates are shown in bold face.

A

| Procollagens | α1(V) | α2(V) | α1(I) | α1(II) | α1(III) |
|--------------|-------|-------|-------|--------|---------|
| α1(V)ho-i    | DAQQLD | DQEQNY |       |        |         |
| α1(V)hu-i    | ENYVDA | DQESIF |       |        |         |
| α2(V)ho-i    | HLTAALG | DINGSHY |       |        |         |
| α2(V)hu-i    | GDIYMHY | DRSNPD |       |        |         |
| α3(V)ho      | RRRSVTV | DTLEGGL |       |        |         |
| α3(V)hu      | RRRRFVF | . . . . | (none) |        |         |
| α4(V)2       | RRRSGLV | DTPESGL |       |        |         |

B

| Laminin 5 y2 chain | TGDCEYSG | DENFDEB |
|--------------------|----------|---------|

FIG. 4. Procollagen V homotrimer cleavage by BMP-1 is inhibited in the presence of 1,10-phenanthroline. 10% SDS-PAGE analysis stained with Coomassie blue of samples incubated for 19 h with (lanes 3 and 4) or without (lanes 1 and 2) recombinant BMP-1. Lanes 1 and 2 show as controls the electrophoretic patterns of the procollagen sample at the onset (lane 1) and at the end (lane 2) of the incubation. Lanes 3 and 4 correspond to the BMP-1 digestion products generated in the absence (lane 3) or presence of 1.25 mM 1,10-phenanthroline (OP; lane 4). Right, running positions of protein standards are indicated in kDa. Npro, N-propeptide.

FIG. 5. Time course of procollagen V homotrimer digestion by BMP-1. Lanes 2–6, electrophoretic patterns of the digestion products in the presence of BMP-1 (+) at the indicated time points. Lanes 1 and 7, controls without BMP-1 (−) are shown for 0 and 22 h time points. Samples were run on a 10% SDS-PAGE gel and silver-stained. The running positions of the different digestion products are indicated on the left. Npro, N-propeptide.
I). In particular, all BMP-1 cleavage sites except the one identified within the N-propeptide of the proc1(V) homotrimer were found to contain an aspartic acid residue at the P1′ position, a feature that appears to be indispensable for BMP-1 action. The surrounding sequences, including those residues that occupy the P1 position of the scissile bond, however, show a considerable degree of variability.

Similarly with probiglycan and chordin, the sequences of the two predicted BMP-1 cleavage sites in the proc1(V) chain (Table I) do not conform perfectly to the rules established for the corresponding sites in procollagenses I-III. However, both contain at least one aspartic acid residue, and one of them contains an A-D bond, a typical BMP-1 cleavage site. In view of these similarities and the relative sequence variability demonstrated for other BMP-1 cleavage sites, we were intrigued by the reported (24) resistance of the type V procollagen NC1 domain to BMP-1, which led us to re-examine the procollagen V homotrimer as a substrate for BMP-1.

In addressing processing of the NC1 domain, we took advantage of the availability of the purified fur-Cpro fragment that was released spontaneously in the culture medium as a result of the furin-like activity and contained the two potential BMP-1 cleavage sites. The results with this fragment clearly indicated that BMP-1 cleaved the D-D peptide bond within the first putative BMP-1 cleavage site (Table I). Although this suggested that BMP-1 may be involved in physiological processing of procollagen V, additional experiments were required to demonstrate that such cleavage can also occur in the intact procollagen molecule. To produce the intact procollagen V homotrimer in cultured 293 cells, it was necessary to inhibit the endogenous furin-like activity. Toward this, we chose to add a specific irreversible furin inhibitor instead of arginine as was used by others (24), because this compound is far more potent as a furin inhibitor, and thus, effective inhibition of the enzyme can be achieved at concentrations that are not harmful to the cells. This approach has been used previously to block furin-dependent processing of pro-MMP-2 in cultured human fibrosarcoma cells (36), in which system an almost complete inhibition of processing was achieved at an inhibitor concentration of 100 μM. Because of the large scale of our experiments, the cells were incubated in the presence of lower amounts of this costly inhibitor, sufficient to slow down the spontaneous processing of the C-propeptide and allowing partial accumulation of intact procollagen to be tested as a substrate for BMP-1. With this substrate preparation, which contained a mixture of pro- and pNa1 chains as well as some free fur-Cpro, we found that in addition to the previously reported processing of the N-propeptide domain, BMP-1 also released the C-terminal propeptide domain, and this resulted from cleavage of the same Asp-Asp peptide bond as observed with the free fur-Cpro fragment. Since the N-terminal sequence of the free N-propeptide fragment generated in our hands as well as its size and electrophoretic properties were the same as those reported previously (24), we presume that this fragment resulted from the same cleavage. The demonstration that the release of the N-terminal fragment and that of the C-terminal propeptide were both inhibited by the zinc chelator, 1,10-phenanthroline, provided supportive evidence that both reactions resulted from the action of BMP-1 itself. A time course study showed that cleavage of the N-propeptide occurred more rapidly than that of the C-propeptide. This, together with the small difference in migration of the fur-Cpro and bmp-Cpro bands, can explain why cleavage of the C-propeptide by BMP-1 was not observed previously (24). Given the unusual location in the procollagen molecule and nature of the specific processing site within the N-propeptide domain, the rapid release of the N-propeptide fragment was surprising. Perhaps this region within the procollagen V molecule is fully exposed and, thus, more sensitive to proteolysis. The marked difference in the progress of the two reactions, however, may also reflect the importance of secondary interactions of the enzyme with its substrate.

In addition to the immediate interactions of the enzyme active site with residues surrounding the cleavage site, the noncatalytic domains of BMP-1 may contribute to substrate recognition, thereby affecting the efficiency and specificity of cleavage. Indeed, in common with other family members, BMP-1 consists of a proteolytic (astacin-like) domain followed by a number of CUB (37) and epidermal growth factor-like domains. Although detailed kinetic studies on BMP-1 processing of the various substrates are lacking, there is evidence that the noncatalytic domains of BMP-1 and its alternatively spliced variant, mTld, are important in this regard. Indeed a number of BMP-1 variants have been identified that are either products of alternative splicing from the Bmp1 gene (mTLD, BMP-1/His) (38) or products of different genes (mTll1, mTll2) (20, 39). When BMP-1 and mTld activities are compared, mTld appears to cleave probiglycan less efficiently than BMP-1 (19) and appears inactive on chordin (20). In addition, early determinations of the kinetic constants for processing of type I procollagen by BMP-1 and mTld (40, 41) showed that the K_m value for processing by BMP-1 is about 20-fold less than that for mTld. Thus, the presence of two additional CUB domains in the latter procollagen C-proteinase variant has marked effects on substrate specificity. Consistently, these additional CUB domains do not bind to procollagen type I (42), suggesting that they may interfere with enzyme-substrate recognition.

Together, our observations and those made by Imamura et al. (24) suggest that BMP-1 may play a dual role in proteolytic maturation of procollagen V, namely, removal of the C-propeptide domain as well as cleavage of the N-terminal PARP domain of the proc1(V) chain. Clearly, detailed quantitative studies with BMP-1 as well as mTld and procollagen V molecules of other chain stoichiometries are required for better understanding of the role(s) of BMP-1/mTld in proteolytic maturation of collagen V. The demonstration that BMP-1 can cleave the C-propeptide of the type V homotrimer does not exclude a role for furin-like proprotein convertases in this process. Alignment of the C-propeptide sequence of the known procollagen V chains indicates that most of these chains contain candidate BMP-1 cleavage sites as well as a conserved furin cleavage sequence at the end of their triple helical domain (Table I). However, two outstanding exceptions exist; one exception is that the human a3(V) chain does not present an obvious BMP-1 cleavage site, and second and more striking, the o2(V) chain does not contain the conserved furin cleavage sequence (Table I). It is thus conceivable that C-terminal processing of procollagen V chains may depend on both types of processing enzymes, zymogen- and furin-like proteases. This possibility offers an explanation for the complete C-terminal processing of the a1(V)a2(V) heterotrimeric molecule, the most abundant molecular form of type V collagen found in tissues. The distribution of the different molecular forms of procollagen V among tissues is not uniform. Although the distribution of the a1(V)a2(V) heterotrimer is relatively broad, the a1(V)v2(V)v3(V) form is found mainly in placenta, the a1(V)v2a4(V) variant is essentially produced by Schwann cells, and the a1(V)a3 was described in some embryonic tissues. Consistent with this diversity, the results favor the view that processing of the various type V procollagen chains may vary depending on the cell type and tissue.

Acknowledgments—We thank M. Courteau for amino acid analysis and A. Bosch for expert art work.
C-terminal Procollagen V Processing by BMP-1

REFERENCES

1. Keilty, K., Hopkinson, I., and Grant, M. E. (1993) in Connective Tissue and Its Heritable Disorders (Royce, P. M., and Steinman, B., eds) pp. 103–147, Wiley-Liss, Inc., New York.
2. Steinman, B., Royce, P. M., and Superti-Furga, A. (1993) in Connective Tissue and Its Heritable Disorders (Royce, P. M., and Steinman, B., eds) pp. 351–407, Wiley-Liss, Inc., New York.
3. Prockop, D. J., Sieron, A. L., and Li, S. W. (1998) Matrix Biol. 16, 399–408.
4. Birg, D. E., Fitch, J. M., Barbia, J. P., Deane, K. J., and Lissemayer, T. F. (1990) J. Cell Sci. 95, 649–657.
5. Lissemayer, T. F., Gilkey, E., Ilges, F., Gordon, M. K., Fitch, J. M., Fessler, L. I., and Birg, D. E. (1993) J. Cell Biol. 121, 1181–1189.
6. Broek, D. L., Madri, J., Eikenberry, E. F., and Brodsky, B. (1985) J. Biol. Chem. 260, 555–562.
7. Niyibizi, C., and Eyre, D. R. (1993) Biochim. Biophys. Acta 1203, 304–309.
8. Moradi-Ame, M., Rousseau, J. C., Klem, J. P., Champliaud, M. F., Boutillon, M. M., Bernillon, J., Wallach, J., and van der Rest, M. (1994) Eur. J. Biochem. 211, 967–975.
9. Fichard, A., Klem, J. P., and Ruggiero, F. (1994) Matrix Biol. 14, 515–531.
10. Chernousov, M. A., Rothblum, K., Tyler, W. A., Stahl, R. C., and Carey, D. J. (2000) J. Biol. Chem. 275, 28208–28215.
11. Kessler, E. (1998) in Handbook of Proteolytic Enzymes (Barrett, A. J., Rawlings, N. D., and J. F. Woessner, ed) pp. 1236–1240, Academic Press, Inc., London.
12. Wenzey, J. M., Rosen, V., Celeste, A. J., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988) Science 242, 1528–1534.
13. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) Science 271, 360–362.
14. Li, S. W., Sieron, A. L., Pertula, A., Hajima, Y., Arnold, W. V., and Prockop, D. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5127–5130.
15. Bond, J. S., and Beynon, R. J. (1995) Protein Sci. 4, 1247–1261.
16. Stoeck, W., Grans, F., Baumann, U., Reinemer, P., Genrom-Roth, F. X., McKay, D. B., and Bode, W. (1995) Protein Sci. 4, 823–840.
17. Panchenko, M. V., Stoker-Stevenson, W. G., Trupetskoy, O. V., Gachere, S. N., and Kagan, H. M. (1996) J. Cell Biol. 275, 7113–7119.
18. Amano, S., Scott, I. C., Takahara, K., Koch, M., Champliaud, M. F., Gerecke, D. R., Keene, D. R., Hudson, D. L., Nishijama, T., Lee, S., Greenspan, D. S., and Burgeson, R. E. (2000) J. Biol. Chem. 275, 22728–22735.
19. Scott, I. C., Imamura, Y., Pappano, W. N., Troedel, J. M., Recklies, A. D., Roughley, P. J., and Greenspan, D. S. (2000) J. Biol. Chem. 275, 36504–36511.
20. Scott, I. C., Blitz, I. L., Pappano, W. N., Imamura, Y., Clark, T. G., Steiglitz, B. M., Tomas, C. L., Maas, S. A., Takahara, K., Cho, K. W. Y., and Greenspan, D. S. (1999) Dev. Biol. 213, 283–300.
21. Piccolo, S., Agius, E., Lu, B., Goodman, S., Dale, L., and De Robertis, E. M. (1997) Cell 91, 407–416.
22. Blader, P., Restegar, S., Fischer, N., and Strahl, U. (1997) Science 278, 1937–1940.
23. Oren, G., Joule, J. A., and Kadler, K. E. (2000) J. Peptide Sci. 6, 489–495.
24. Imamura, Y., Steiglitz, B. M., and Greenspan, D. S. (1998) J. Biol. Chem. 273, 27511–27517.
25. Fichard, A., Tillet, E., Delacoux, F., Garrone, R., and Ruggiero, F. (1997) J. Biol. Chem. 272, 30083–30087.
26. Takahara, K., Sato, Y., Okazawa, K., Okamoto, N., Noda, A., Yaoi, Y., and Kata, I. (1991) J. Biol. Chem. 266, 13124–13129.
27. Greenspan, D. S., Cheng, W., and Hoffman, G. G. (1991) J. Biol. Chem. 266, 24727–24733.
28. Lonnqvist, L., Reinhardt, D., Sakai, L., and Peltonen, L. (1998) Hum. Mol. Genet. 7, 2039–2044.
29. Grassel, S., Timpl, R., Tan, E. M. L., and Chu, M. L. (1996) Eur. J. Biochem. 242, 578–584.
30. Laemmli, U. K. (1970) Nature 227, 680–685.
31. Cui, Y., Jean, F., Thomas, G., and Christian, J. L. (1998) EMBO J. 17, 4735–4743.
32. Imamura, Y., Scott, I. C., and Greenspan, D. S. (2000) J. Biol. Chem. 275, 8749–8759.
33. Neame, P. J., Young, C. N., and Treep, J. T. (1990) J. Biol. Chem. 265, 20401–20408.
34. Rousseau, J. C., Farnajel, J., Boulleau, M. M., Harnabart, D. J., van der Rest, M., and Moradi-Ameli, M. (1996) J. Biol. Chem. 271, 27433–27435.
35. Nakayama, K. (1997) Biochem. J. 327, 625–635.
36. Marqui, X., Noel, A., Frankenne, F., Angliker, H., Murphy, G., and Foidart, J.-M. (1998) FEBS Lett. 424, 262–266.
37. Bork, P., and Beckmann, G. (1993) J. Mol. Biol. 231, 539–545.
38. Takahara, K., Lyons, G. E., and Greenspan, D. S. (1994) J. Biol. Chem. 269, 32572–32578.
39. Takahara, K., Brevard, R., Hoffman, G. G., Suzuki, N., and Greenspan, D. S. (1996) Genomics 34, 157–165.
40. Hugina, Y., van der Rest, M., and Prockop, D. J. (1985) J. Biol. Chem. 260, 15966–16003.
41. Adar, R., Kessler, E., and Goldberg, B. (1986) Collagen Relat. Res. 6, 267–277.
42. Sieron, A. L., Tretiakova, A., Jameson, B. A., Segall, M. L., Lund-Katz, S., Khan, M. T., Li, S. W., and Stoeck, W. (2000) Biochemistry 39, 3231–3239.
43. Weil, D., Bernard, M., Gargano, S., and Ramirez, F. (1987) Nucleic Acids Res. 15, 181–198.
Bone Morphogenetic Protein-1 (BMP-1) Mediates C-terminal Processing of Procollagen V Homotrimer

Efrat Kessler, Agnès Fichard, Hélène Chanut-Delalande, Marina Brusel and Florence Ruggiero

J. Biol. Chem. 2001, 276:27051-27057.
doi: 10.1074/jbc.M102921200 originally published online May 17, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M102921200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 22 of which can be accessed free at http://www.jbc.org/content/276/29/27051.full.html#ref-list-1