Biosynthetic Processing of the Pro-α1(V)Pro-α2(V)Pro-α3(V)
Procollagen Heterotrimer*

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Type V collagen is a quantitatively minor fibrillar collagen comprised of different chain compositions in different tissues. The most widely distributed form, an α1(V)α2(V) heterotrimer, regulates the physical properties of type IV heterotypic collagen fibrils via partially processed NH2-terminal globular sequences. A less characterized α1(V)α2(V)α3(V) heterotrimer has a much more limited distribution of expression and unknown function(s). We characterized the biosynthetic processing of pro-α1(V)pro-α2(V) pro-α3(V) procollagen previously and showed it to differ in important ways from biosynthetic processing of the major fibrillar procollagens I–III. Here we have successfully produced recombinant pro-α1(V)pro-α2(V)pro-α3(V) heterotrimers. We use these, and mouse embryo fibroblasts doubly homozygous null for BMP-1, a gene that encodes the metalloproteinase bone morphogenetic protein-1 (BMP-1), and for a gene encoding the closely related metalloproteinase mammalian Tolloid-like 1, to characterize biosynthetic processing of pro-α1(V)pro-α2(V)pro-α3(V) heterotrimer, thus completing characterization of type V collagen biosynthetic processing. Whereas pro-α1(V) and pro-α2(V) processing in pro-α1(V)pro-α2(V)pro-α3(V) heterotrimers is similar to that which occurs in pro-α1(V)pro-α2(V) heterotrimers, the processing of pro-α3(V) by BMP-1 occurs at an unexpected site within NH2-terminal globular sequences. We also demonstrate that, despite similarities in NH2-terminal domain structures, pro-α3(V) NH2-terminal globular terminal sequences are not cleaved by ADAMTS-2, the metalloproteinase that cleaves the N-propeptide of the major fibrillar procollagen chains.

The major fibrillar collagen types I–III are synthesized as procollagens with N- and C-propeptides that are proteolytically removed to yield mature triple helical monomers capable of forming fibrils (1, 2). Impairment in the ability to cleave the N-propeptide of procollagen type I results in abnormal fibril morphology and forms of Ehlers-Danlos syndrome type VII (2), whereas failure to remove major fibrillar procollagen C-propeptides may be incompatible with fibrillogenesis (3). The N-propeptides of procollagens I–III are cleaved by the metalloproteinase ADAMTS-2 (4, 5) and perhaps by the closely related ADAMTS-3 and -14 as well (6, 7). The C-propeptides of procollagens I–III are cleaved by the metalloproteinase bone morphogenetic protein 1 (BMP-1) (8, 9) and by other members of a small family of metalloproteinases closely related to BMP-1 (10, 11). BMP-1 and related proteinases also process the prodomains of a number of other precursors to produce the mature functional forms of a variety of proteins involved in formation of the extracellular matrix, (12–16). BMP-1-like proteinases also process chordin (10, 11, 17), an extracellular antagonist of signaling by transforming growth factor-β-like Bmps, such as BMP-4 (18), and process within propeptide sequences to activate the transforming growth factor-β-like protein growth differentiation factor 8/myostatin (19). Thus, BMP-1-like proteinases may coordinate extracellular matrix formation with signaling by a subset of transforming growth factor-β-related proteins in morphogenesis and homeostasis.

Monomers of the minor, low abundance fibrillar collagen types V and XI are incorporated into growing type I and type II collagen fibrils, respectively, and play roles in regulating the shapes and diameters of the resultant heterotypic fibrils (20–25). Type V collagen is distributed broadly in type I collagen-containing tissues as a heterotrimer of the chain composition α1(V)α2(V) (26) and is found in a limited number of cell types and tissues as a rare α1(V)3 homotrimer (26–29). In addition, a relatively uncharacterized α1(V)α2(V)α3(V) heterotrimer has been isolated from human placenta (30–32) and has also been reported in uterus, skin, and synovial membranes (26, 33–35). Detection of α3(V) expression in nascent ligamentous attachments of developing joints, membranous linings of developing skeletal muscle, and in developing and regenerating peripheral nerves in mouse and rat (36, 37) suggests roles for the α1(V)α2(V)α3(V) heterotrimer in these tissues as well. Although type XI collagen was first described as an α1(XI)α2(XI)α3(XI) heterotrimer confined to cartilage (38), it is now apparent that the separately discovered collagen types V and XI may be viewed as constituting a single collagen type in which different combinations of chains associate in a tissue-specific manner. This conclusion is based on the finding of type XI chains in non-cartilaginous tissues (39), of type V chains in cartilage (40), and of cross-type heterotrimers composed of both type V and XI chains (41, 42) and on the extreme similarities in sequence and domain structure shown by certain of the type V and XI collagen chains. Unlike fibrillar collagens I–III, α1(V)α2(V) and α1(XI)α2(XI)α3(XI) heterotrimers retain partial NH2-terminal globular sequences (29, 28, 43–47). These protrude beyond the surface of heterotypic fibrils and may regulate fibrillogenesis by hin-

* This work was supported by National Institutes of Health Grants AR47746 and GM63471 (to D. S. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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† The abbreviations used are: N-propeptide, amino-terminal propeptide; ADAMTS, A Disintegrin And Metalloproteinase with Thrombospondin motifs; BMP, bone morphogenetic protein; C-propeptide, carboxy-terminal propeptide; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; pC, processing intermediate that retains the C-propeptide but from which the N-propeptide has been removed; pN, processing intermediate of procollagen chains that contains the N- but not the C-propeptide; PARP, proline/arginine-rich protein.

This paper is available online at http://www.jbc.org
adhering addition of collagen monomers to the fibril surface (45). Pro-\(\alpha2(V)\) closely resembles the major fibrillar procollagen chains in domain structure (50, 51), as does pro-\(\alpha3(XI)\), which is a modified product of the type II collagen pro-\(\alpha1(II)\) gene (40). In contrast, the pro-\(\alpha1(V)\), pro-\(\alpha1(XI)\), pro-\(\alpha2(XI)\), and pro-\(\alpha3(V)\) chains, which have triple helical and C-propeptides that resemble those of the other fibrillar procollagen chains, form a subgroup with a shared NH\(_2\)-terminal globular protein domain structure that differs in size and configuration from the NH\(_2\)-terminal globular sequences of the other chains (36). We reported previously the surprising findings that C-propeptides of pro-\(\alpha1(V)\) chains in either \(\alpha1(V)_1\) or \(\alpha1(V)_2\) trimers are cleaved by furin-like proprotein convertases, whereas BMP-1 cleaves at a specific site within pro-\(\alpha1(V)\) NH\(_2\)-terminal globular sequences (48, 49). Processing at similar sites and by similar proteinases appears to hold for pro-\(\alpha1(XI)\) as well (11, 52). Putative furin recognition sites in pro-\(\alpha2(XI)\) and pro-\(\alpha3(V)\) teleopeptides suggest that the C-propeptides of these chains may also be cleaved by furin-like proteinases, although the question has not been addressed experimentally. However, although residues flanking the BMP-1 cleavage site in pro-\(\alpha1(V)\) NH\(_2\)-terminal sequences are conserved in the NH\(_2\)-terminal sequences of pro-\(\alpha1(XI)\) and pro-\(\alpha2(XI)\), such conservation is lacking in pro-\(\alpha3(V)\) (49). Thus, it remains to be determined whether pro-\(\alpha3(V)\) NH\(_2\)-terminal globular sequences are processed and, if so, at which site(s), and by what type of proteinase(s).

Here we address experimentally in vitro and in vivo processing of the pro-\(\alpha3(V)\) chain and the question of whether the pro-\(\alpha2(V)\) N-propeptide is susceptible to cleavage by ADAMTS-2.

**EXPERIMENTAL PROCEDURES**

**Production of Recombinant Pro-\(\alpha1(V)\) Pro-\(\alpha2(V)\) Pro-\(\alpha3(V)\) Heterotrimers**—For production of recombinant pro-\(\alpha1(V)\), pro-\(\alpha2(V)\), and pro-\(\alpha3(V)\) heterotrimers, a previously described full-length human pro-\(\alpha1(V)\) cDNA (48) was excised from vector pBluescript II KS+ (Stratagene) with Sall and NotI and was inserted between the Xhol and NotI sites of the expression vector pc1-neo (Promega). A previously described full-length human pro-\(\alpha2(V)\) cDNA (49) was fitted with 5’-AflII and 3’-HaeIII ends and was inserted into the BglII and NotI sites of the expression vector pcDNA/TO/myc-His B (Invitrogen). The resulting vector thus expresses both pro-\(\alpha1(V)\) and pro-\(\alpha3(V)\) mRNAs, transcribed via two separate promotors. The pro-\(\alpha2(V)\) cDNA-containing pcDNA/TO/myc-His Zeo construct described above was cleaved with AgeI, then filled with dCTP using the Klenow fragment, and subsequently cut with AflII. The resulting full-length pro-\(\alpha2(V)\) cDNA was then inserted into a pcDNA5/T vector (Invitrogen) that had been cut with Bsp120I, filled with dGTP using the Klenow fragment, and subsequently cut with AflII. These vectors were cotransfected into T-REX-293 cells, as described below, and then selected for zeocin and hygromycin resistance. T-REX-293 cell cultures maintained in complete medium (Dulbecco’s modified Eagle’s modified Eagle medium containing 10% fetal bovine serum and 50 \(\mu\)g/ml ascorbate at 20 h before induction of collagen production. Cells were washed in phosphate-buffered saline (PBS), then incubated in serum-free Dulbecco’s modified Eagle’s medium containing 100 \(\mu\)g/ml soybean trypsin inhibitor, 50 \(\mu\)g/ml ascorbate, and 1 \(\mu\)M tetracycline (Sigma) in the absence or presence of 100 \(\mu\)M t-arginine or 20 \(\mu\)M high salt furin inhibitor decanoyl-RVKR-chloromethyl ketone (Bachem), as noted in the text. Conditioned media were recovered 24 h later and replaced with fresh media for a second harvest after another 24 h. Protease inhibitors were added to conditioned media to final concentrations of 0.2 \(\mu\)M aprotinin, 1 \(\mu\)M PMSF, and 1 mM p-aminobenzoic acid, 1 mM N-ethylmaleimide, and 1 mM p-aminobenzoic acid; media were centrifuged to remove debris and were then stored at -70 °C.

Stored samples were thawed and dialyzed against 50 mM Tris-HCl, pH 9.6, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 1 mM p-aminobenzoic acid at 4 °C, as described previously (48), for precipitation of type V procollagens. Precipitates were collected by centrifugation at 65,000 \(\times\) g for 30 min, and pellets were resuspended in buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). 

**Pepsinization**—Pro-\(\alpha1(V)\) pro-\(\alpha2(V)\) and pro-\(\alpha1(V)\) pro-\(\alpha2(V)\) heterotrimers were subjected to digestion with 100 \(\mu\)l pepsin (Sigma) in 0.5 M acetic acid, pH 2.0, for 6 h at 4 °C. The reaction was stopped by neutralizing with a final concentration of 0.5 M NaOH and by adding pepstatin to a final concentration of 30 \(\mu\)g/ml. Pepsin-resistant collagen chains were precipitated with ethanol and analyzed by SDS-PAGE.

**Immunoblots**—Samples were subjected to SDS-PAGE and subsequently electrophoresed to Immobilon-P membranes (Millipore) as described previously (54). Blots were blocked with 3% bovine serum albumin in T-PBS (PBS and 0.05% Tween) for 2 h and incubated with primary antibody at a 1:6,000 dilution for anti-\(\alpha1(V)\) antibodies, 1:5,000 for anti-\(\alpha2(V)\) antibodies, and 1:4,000 for anti-\(\alpha3(V)\) antibodies. Blots were then washed with T-PBS and blocked with 3% bovine serum albumin in T-PBS followed by incubation with secondary antibodies at 1:5,000 dilution. After eight washes with T-PBS, blots were incubated for 5 min in SuperSignal West Pico substrate (Pierce) and exposed to Scientific Imaging film (Kodak).

Affinity-purified polyclonal antibodies raised against the variable domain of pro-\(\alpha1(V)\) have been described previously (49), as has the anti-\(\alpha2(V)\) antibody raised against the rat \(\alpha2(V)\) homolog, P200 (53). The latter antibody was the generous gift of David J. Carey (Sigfried and Janet Weiss Center for Research).

**Immunoprecipitation**—Pro-\(\alpha1(V)\) pro-\(\alpha2(V)\) pro-\(\alpha3(V)\) heterotrimers were immunoprecipitated with anti-\(\alpha3(V)\) antibody using essentially the same methodology as described by Scott et al. (17). Briefly, 500 ng of each of the protein, precipitated under low salt conditions (see above), was incubated either with protein A-Sepharose beads (Amersham Biosciences) or with protein A-Sepharose beads pre-bound to antibody against the rat \(\alpha2(V)\) homolog, P200 (53). The latter antibody was the generous gift of David J. Carey (Sigfried and Janet Weiss Center for Research).

**Enzyme Cleavage Assays**—A human BMP-1 cDNA, which is full-length except that signal peptide sequences have been replaced by sequences encoding the BM40 signal peptide, for optimization of secretion (10) was inserted between the EcoRI and NotI sites of the pcDNA3-hyg expression vector. This construct was transfected into T-REX-283 cells, which were selected in Zeocin, and a clonal line expressing high levels of tetracycline-inducible BMP-1 was identified. Recombinant BMP-1 produced by this line was purified, as described previously (10), and 110 ng was incubated with 4 \(\mu\)g of pro-\(\alpha1(V)\) pro-\(\alpha2(V)\) pro-\(\alpha3(V)\) heterotrimer in total reaction volume.
Production of recombinant pro-α1(V)/pro-α2(V)/pro-α3(V) heterotrimers by a clonal line of transfected T-Rex-293 cells. Electrophoretic patterns are compared for proteins derived from the media of clonal lines of cells transfected with constructs for expression of pro-α1(V) and pro-α2(V) chains (lanes 1) or for expression of pro-α1(V), pro-α2(V), and pro-α3(V) chains (lanes 2). Shown are a Coomassie Blue-stained SDS-polyacrylamide gel (A), immunoblots probed with anti-pro-α1(V) (B) or anti-pro-α3(V) (C) antibodies, and D, a Coomassie Blue-stained gel of pepsin-treated material. All SDS-polyacrylamide gels comprised 3.5% acrylamide stacking and 5% acrylamide running gels.

Production of Stable Recombinant Pro-α1(V)/Pro-α2(V)/Pro-α3(V) Procollagen

RESULTS

Production of Stable Recombinant Pro-α1(V)/Pro-α2(V)/Pro-α3(V) Heterotrimers—Attempts to produce either recombinant pro-α3(V) homotrimers or heterotrimers composed solely of pro-α3(V) and pro-α1(V) chains failed to yield detectable pro-α3(V) chains (data not shown), suggesting that pro-α3(V) homotrimers and pro-α3(V)/pro-α1(V) heterotrimers are not formed, or are not stable. In contrast, cotransfection of cells with expression vectors for producing pro-α3(V), pro-α1(V), and pro-α2(V) chains was successful in yielding stable pro-α1(V)/pro-α2(V)/pro-α3(V) heterotrimers (Fig. 1). In addition to producing bands identical to those produced by cells transfected with expression constructs for pro-α1(V) and pro-α2(V) alone, clonal lines transfected with expression vectors for all three chains produced a novel ~190 kDa band as well (Fig. 1A). Because we have found previously that 293 cells transfected with pro-α1(V) eDNA vectors primarily produce the pN form of pro-α1(V)-derived sequences (Refs. 48 and 49 and Fig. 1A), the novel 190 kDa band in Fig. 1A was, by analogy, provisionally designated pNα3(V). Immunoblots using antibodies specific for pro-α1(V)-derived sequences (49) or antibodies specific for pro-α3(V)-derived sequences (33) confirmed that cells transfected either with expression constructs for pro-α1(V) and pro-α2(V) alone or with constructs for expression of all three type V chains had similar banding patterns for pro-α1(V)-derived proteins (Fig. 1B) but that only cells transfected with constructs for expression of all three type V chains expressed the 190-kDa species, which was pro-α3(V)-derived (Fig. 1C). Pepsin digestion of recombinant material from cells transfected either with constructs for pro-α1(V) and pro-α2(V) alone, or with constructs for expression of all three type V chains, demonstrated that the latter cells produced a novel pepsin-resistant chain and a pattern of three resistant chains identical with that of authentic α1(V)α2(V)α3(V) collagen heterotrimers isolated from tissues (Fig. 1D and Refs. 30 and 32). The ratio of pepsin-resistant α1(V), α2(V), and α3(V) chains in the sample suggests that the great majority of type V procollagen produced by cells transfected with constructs for expression of all three type V chains is in the form of stable pro-α1(V)/pro-α2(V)/pro-α3(V) heterotrimers, possibly admixed with lesser amounts of pro-α1(V)/pro-α2(V) heterotrimers and/or pro-α1(V) homotrimers.

To ascertain directly that heterotrimers with the composition pro-α1(V)/pro-α2(V)/pro-α3(V) heterotrimers are formed in the expression system, immunoprecipitations were performed with antibody specific for the pro-α3(V) chain, and it was determined whether or not pro-α1(V)- and pro-α2(V)-derived chains were communoprecipitated. As can be seen (Fig. 2), anti-pro-α3(V) antibody communoprecipitated both pro-α1(V) and pro-α2(V) chains from samples derived from cells transfected with expression vectors for all three chains. The specificity of the anti-pro-α3(V) antibody is demonstrated by the observation that neither pro-α1(V)- nor pro-α2(V)-derived bands were detectable upon immunoprecipitation using this antibody, and samples derived from cells transfected only with pro-α1(V) and pro-α2(V) expression vector. The most straightforward interpretation of the various data presented above is that heterotrimers with the composition pro-α1(V)/pro-
Pro-α3(V) are formed in the expression system described in this study.

Pro-α3(V) Chains Are Processed by Furin-like Proprotein Convertase Activity—Previously, we have demonstrated that the C-propeptides of pro-α1(V), but not pro-α2(V), chains are cleaved by furin-like proprotein convertase activity in cultures of transfected 293 cells (48, 49). To determine whether the pro-α3(V) C-propeptide is or is not cleaved by a similar activity, a clonal line of cells producing recombinant pro-α1(V) pro-α2(V) pro-α3(V) heterotrimers was incubated either in the presence of 100 mM arginine, which partially blocks such activity (48, 49), or in the presence of decanoyl-RVKR-chloromethyl ketone, a potent and highly specific furin inhibitor. As can be seen (Fig. 3), the shift of pNoα1(V) to full-length pro-α1(V) chains is evident in the presence of arginine or decanoyl-RVKR-chloromethyl ketone, whereas there is no shift in the mobility of pro-α2(V) chains, which have no furin recognition sites. In contrast, a slight but reproducible shift is detectable in the mobility of the novel 190 kDa band, consistent with its identity as the pNoα3(V) chain and consistent with the possibility that the pro-α3(V) C-propeptide is cleaved by furin-like proprotein convertase activity in cell culture.

Pro-α3(V) Sequences Are Cleaved by BMP-1 in Vitro—Previously, we have demonstrated that C-propeptides of pro-α2(V) chains and NH2-terminal globular sequences of pro-α1(V) chains are cleaved by BMP-1 in vitro (48, 49). To determine whether pro-α3(V) chains are processed by BMP-1, recombinant collagenous material from the media of a cell line expressing all three type V procollagen chains was incubated 20 h in the presence or absence of BMP-1. As can be seen (Fig. 4A), BMP-1 not only cleaves pNoα1(V) chains to produce mature α1(V) and cleaves pro-α2(V) chains to produce pNoα2(V), but also induces a mobility shift in pNoα3(V) chains to produce a faster mobility band. The most straightforward interpretation of the data is that the pNoα3(V) chain is cleaved within NH2-terminal globular sequences to produce what may correspond to the mature α3(V) chain. The band corresponding to the putative α3(V) chain was isolated and subjected to NH2-terminal sequencing via automated Edman degradation and was indeed found to have been cleaved within NH2-terminal globular sequences. The NH2-terminal sequence of AQAQAVLQQT identified the peptide bond between Gln-463 and Ala-464 of the human prepro-α3(V) amino acid sequence (Ref. 36; GenBank accession number AF177941) as the BMP-1 cleavage site. This site is within a short non-triple-helical region that lies between the major collagensous domain (COL1) and a small collagensous domain (COL2) just COOH-terminal of the variable subdomain of pro-α3(V) NH2-terminal sequences (Figs. 5 and 8).

To examine more closely the nature of processing of the pro-α3(V) chain, BMP-1-cleaved samples, similar to those of Fig. 4A, were electrophoresed on a 10% SDS-polyacrylamide gel for separation of the low molecular mass cleavage products (Fig. 4B) and subsequent determination of their NH2-terminal amino acid sequences by automated Edman degradation. As can be seen (Fig. 4B), four bands of approximate molecular masses of 52, 46, 41, and 38 kDa were visible by Coomassie Blue staining. It should be noted that molecular masses for cleaved pro-α3(V) N- and C-propeptides, pro-α1(V) N- and C-propeptides, and pro-α2(V) C-propeptides are 45,377, 27,301, 24,326, 28,166, and 27,198, respectively, based on amino acid sequences. However, each of these peptides is thought to be glycosylated, and in fact, we have previously estimated molec-
FIG. 4. Characterization of BMP-1 cleavage of pro-

α1(V) pro-α2(V) heterotrimers. Electrophoretic patterns are com-

pared for samples from T-REx-293 clones producing either recombinant pro-

α1(V) pro-α2(V) pro-α3(V) (lanes 1 and 2) or pro-α1(V) pro-α2(V) (lanes 4 and 5) heterotrimers. Samples were incubated in the absence

(−) or presence (+) of BMP-1 and were subjected to SDS-PAGE on a 3.5% acrylamide stacking and 10% acrylamide running gel (A), which

were stained with Coomassie Blue. Lane 3 in A contains size markers, whose approximate molecular masses are 250, 150, and 100 kDa. Bands in

B were shown by NH2-terminal sequencing to represent the cleaved pro-

α3(V) N-propeptide (N-α3(V)), pro-α3(V) C-propeptide (C-α3(V)), pro-

α2(V) C-propeptide (C-α2(V)), pro-α1(V) C-propeptide (C-α1(V)), or pro-

α1(V) N-propeptide (N-α1(V)).

FIG. 5. Alignment of pro-α3(V), pro-α1(XI), pro-α1(V), and pro-

α2(XI) sequences in the region corresponding to the human pro-

α3(V) BMP-1 cleavage site. Sequences for the human and mouse pro-

α3(V) (and rat pro-α3(V)), whose sequences are identical to mouse in this region, were aligned, using the Pileup program from Genetics Computer Group (56), as described previously (36). Dots represent gaps introduced by the program for optimal alignment of sequences. An arrow marks the site of BMP-1 cleavage between Gln-463 and Ala-464 of human pro-pre-α3(V) sequences, as determined in the present study. Possible COOH termini of the small collagenous domains (COL2) of the various chains and the beginnings of the major collagenous domains (COL1) are marked by brackets. Noncollagenous interruptions in COL2 domains are underlined, and an asterisk marks a conserved tyrosine, 24 residues NH2-terminal to COL1. Residues between COL2 and COL1 that are conserved in mouse and human pro-

α3(V), but not in pro-α1(XI) pro-α1(V) pro-α2(XI) chains, are in boldface type.

sample, gave a major NH2-terminal sequence of DPVDV-

KALG and a minor sequence of RFVPVPL. The major sequence represents the NH2 terminus of pro-α3(V), empirically showing that cleavage of the signal peptide occurs between residues Ala-29 and Asp-30 of the published human pre-pro-α3(V) se-

quence (36). The 52-kDa size of this N-propeptide and the absence of other bands found in BMP-1-cleaved pro-α1(V) pro-

α2(V) pro-α3(V)-, but not pro-α1(V) pro-α2(V)-derived material, both support the conclusion that BMP-1 cleaves pro-α3(V) NH2-terminal sequences solely at the peptide bond between Gln-463 and Ala-464, to produce the largest N-propeptide yet described for a fibrillar procollagen. The minor sequence derived from the 52 kDa band corresponds to the pro-α3(V) C-propeptide and demonstrates cleavage to have occurred between Arg-1501 and Arg-1502, immediately downstream of the furin recognition site RRRR, consistent with the probability that the pro-α3(V) C-propeptide is cleaved by a furin-like ac-

tivity. The larger than expected size of the pro-α3(V) C-propeptide may be related to the fact that the human pro-α3(V) C-propeptide contains three potential sites for Asn-linked gly-

cosylation, rather than the two found in other type V procollagen C-propeptides (36). Both the extra glycosylation site and apparent retarded mobility on SDS-PAGE are consistent with the possibility that the human pro-α3(V) C-propeptide has relatively high levels of glycosylation.

Cells Are Dependent on BMP-1- and Furin-like Proteinases for Cleaving Pro-α3(V) N- and C-propeptides, Respectively—

We next sought to determine directly the possible involve-

ment of endogenous BMP-1-like and furin-like proteinases in the processing of pro-α1(V) pro-α2(V) pro-α3(V) heterotrimers by cells. We have previously assayed for roles for BMP-1-related proteinases in the in vivo processing of various sub-

strates by comparing processing of those substrates in fibro-

blasts derived from wild type embryos to processing in fibroblasts derived from embryos doubly homozygous null for the Bmp1 gene, which encodes both BMP-1 and alternatively spliced mRNA for the related proteinase mammalian Tolloid, and for the Tll1 gene, which encodes the BMP-1-related proteinase mammalian Tolloid like-1 (mTLL-1) (11, 13, 14, 49). Use of the doubly null cells removes possible functional redundancy, as we have shown previously that products of the two genes overlap in their substrate specificities (10, 11, 13, 14). However, although we have demonstrated previously that such cells produce readily detectable levels of pro-α1(V) chains (49), presumably in the context of pro-α1(V) pro-α2(V)
heterotrimers, attempts in the present study to detect endogenous pro-α3(V)-derived chains in such cells by immunoblot were unsuccessful (data not shown). To overcome the inability to detect endogenous pro-α3(V)-derived chains in MEFs, a pro-α3(V) expression vector was electroporated into wild type MEFs and into MEFs derived from an embryo littermate doubly homozygous null for the Bmp1 and Tll1 genes, and mass cultures were selected that secreted stable recombinant pNα3(V) and mature α3(V) chains run on the same gel (not shown). When the wild type MEFs were cultured in the presence of the furin inhibitor decanoyl-RVKR-chloromethyl ketone, a new band, the size of intact pro-α3(V), was observed, concomitant with the disappearance of the putative mature α3(V) form. These results are consistent with a role for furin-like proprotein convertase activity in the processing of pro-α3(V) in MEFs. The fact that all traces of the putative α3(V) band disappear but that a strong band is still observable with about the same mobility of the pNα3(V) form is most consistent with the probability that the blockage of C-propeptide cleavage has resulted in replacement of α3(V) forms with pCoα3(V) forms that have a mobility similar to that of pNα3(V).

In contrast to wild type MEFs, only a single pro-α3(V)-derived chain is detectable in conditioned media of MEFs doubly null for the Bmp1 and Tll1 genes (Fig. 6A). This difference between wild type and Bmp1/Tll1-null MEFs indicates that endogenous BMP-1-like proteinases play a role in processing the pro-α3(V) chain in MEFs. Culturing the Bmp1/Tll1-null MEFs in the presence of the furin inhibitor decanoyl-RVKR-chloromethyl ketone results in disappearance of the single band and its replacement by intact pro-α3(V) chains. This latter result is consistent with the probability that the single chain in untreated Bmp1/Tll1-null MEF media is the pNα3(V) form and that blocking cleavage of the C-propeptide with furin inhibitor results in replacement of pNα3(V) chains with intact pro-α3(V) chains.

Immunoblots of the same wild type and Bmp1/Tll1-null MEF samples described above, using α1(V)-specific antibodies, gave results indistinguishable from those we have reported previously (49) (Fig. 6B). Thus, pro-α1(V) chains appear to be processed similarly by furin- and BMP-1-like proteinases whether or not the pro-α1(V) C-propeptide is cleaved by furin-like proprotein convertase activity (48, 49). Such processing results in replacement of α1(V)3 homotrimers (48) with pro-α1(V)pro-α2(V)pro-α3(V) heterotrimers. These results were radiolabeled, and autoradiography rather than staining was used for detection of bands to avoid possible confusion over the identity of possible pNα2(V) forms, which would be about the same mobility as ADAMTS-2. As can be seen (Fig. 7), neither pro-α2(V) nor either of the two other type V procollagen chains was detectably processed by ADAMTS-2.

**DISCUSSION**

We previously characterized biosynthetic processing of the pro-α1(V) chain in the context of pro-α1(V)3 homotrimers (48) and pro-α1(V)2 pro-α2(V) heterotrimers (49). In both contexts, pro-α1(V) NH2-terminal globular sequences are cleaved by BMP-1-like proteinases, resulting in removal of the PARP subdomain, whereas the pro-α1(V) C-propeptide is cleaved by furin-like proprotein convertase activity (48, 49). Such processing of the pro-α1(V) chain is clearly demonstrable in vitro and in cell cultures (48, 49). In contrast to pro-α1(V), but similar to the major procollagens I–III, it is the C-propeptide of pro-α2(V) that is cleaved by BMP-1-like proteinases (49). The latter result is not surprising because pro-α2(V) is closely related to the major fibrillar procollagen chains on the basis of sequence similarities, structures of cognate genes, and size and configuration of N-propeptides (50, 51).
pro-α2(XI), and pro-α3(V) chains, however, form a separate subgroup among fibrillar procollagen chains on the basis of the same criteria (57–63). Consistent with the relatedness of the pro-α1(V) and pro-α1(XI) chains, it has recently been demonstrated that pro-α1(XI) processing is similar to that of pro-α1(V), with processing of the pro-α1(XI) PARP domain by BMP-1-like proteinases (11, 52) and processing of the pro-α1(XI) C-propeptide by furin-like activity (11).

Resulting in part from low levels and limited distribution of expression, the recently cloned and sequenced pro-α3(V) chain (36) is the least characterized type V/XI chain, and α1(V)α2(V)α3(V) is the least characterized form of type V collagen. Studies have shown α1(V)α2(V) heterotrimeric chains to be incorporated into type I/V heterotrimers and to retain partial NH2-terminal globular sequences that regulate the shape and diameter of the resultant heterotypic fibrils (20, 21, 28, 45, 47), whereas α1(V) homotrimers may be localized to fibril surfaces and do not appear to regulate the geometries of heterotypic type I/V fibrils (64). In contrast, the nature of α1(V)α2(V)α3(V) macromolecular associations is relatively uncharacterized, and the nature of possible processing of a pro-α1(V)α2(V)α3(V) precursor is totally unknown. The somewhat challenging prospect of producing a heterotrimer composed of three different recombinant proteins has contributed to delay in elucidating the biology of the α1(V)α2(V)α3(V) heterotrimer.

Here we have successfully produced recombinant pro-α1(V)pro-α2(V)pro-α3(V) heterotrimer and have used biochemical, cell culture, and genetic means to characterize its proteolytic processing. Initial attempts at expressing the pro-α3(V) chain in T-REx 293 cells transfected only with a pro-α3(V) expression vector or with pro-α3(V) and pro-α1(V) expression vectors in the absence of a pro-α2(V) expression vector were unsuccessful. Thus, pro-α3(V) chains do not appear capable of forming stable homotrimers or heterotrimers composed solely of pro-α1(V) and pro-α3(V) chains.

We noted previously that the pro-α3(V) C-propeptide might be processed by a furin-like activity, based on the relatedness of pro-α3(V) and pro-α1(V) chains and on the conservation of a consensus site for cleavage by furin-like proprotein convertases in the pro-α3(V) telopeptide (36). Here, we provide evidence that pro-α3(V) C-propeptides are cleaved by furin-like activities in cultures of T-REx-293 cells and MEFs. These results and recent results showing cleavage of the pro-α1(XI) C-propeptide by a furin-like activity (11) support the conclusion that C-propeptides of the entire subfamily of pro-α1(V), pro-α1(XI), pro-α2(XI), and pro-α3(V) chains are likely to be cleaved by this class of proteinases. In contrast, residues flanking the BMP-1 cleavage site in pro-α1(V) NH2-terminal globular sequences, and conserved at the same positions in pro-α1(XI) and pro-α2(XI), are not conserved at similar positions in pro-α3(V) (36). This has previously raised the issue of whether pro-α3(V) NH2-terminal globular sequences are cleaved by BMP-1-like proteinases and, if so, at what site.

Here we demonstrate that BMP-1 cleavage releases a pro-α3(V) N-propeptide constituting essentially the entirety of NH2-terminal globular sequences, including PARP and variable subdomains, plus the small COL2 collagenous domain. The site of BMP-1 cleavage was surprising for several reasons. First, residues flanking the site show essentially no homology with the residues flanking any previously characterized site of cleavage by BMP-1-like proteinases (65). The pro-α3(V) BMP-1 cleavage site thus furthers the view that BMP-1-like proteinases, like other members of the astacin proteinase family (66), are not necessarily highly specific for residues immediately flanking the scissile bond and that other features must influence the recognition of such sites. A second reason for surprise regarding the pro-α3(V) BMP-1 cleavage site is that it is relatively far removed from the conserved position, between PARP and variable subdomains, at which pro-α1(V) and pro-α1(XI) chains are cleaved by BMP-1 (11, 48, 49, 52) despite similar NH2-terminal globular domain structures and sequences in these chains (Fig. 8). Despite a string of basic residues at the junction of the pro-α3(V) PARP and variable subdomains (36), there was no evidence for cleavage by a furin-like activity at this site.

We demonstrate here that α1(V) chains in α1(V)α2(V)α3(V) heterotrimers are resistant to cleavage by ADAMTS-2. An autoradiogram is shown of radiolabeled procollagens subjected or not subjected to incubation with ADAMTS-2. Shown is pro-α1(V)pro-α2(V)pro-α3(V) heterotrimer starting material (lanes 1), heterotrimer incubated without protease (lanes 2), incubation of 90 ng of ADAMTS-2/800 ng of heterotrimer (lanes 3), incubation of 180 ng of ADAMTS-2/2000 ng of heterotrimer (lanes 4), incubation of type II procollagen homotrimer without protease (lane 5), and incubation of 90 ng of ADAMTS-2/2210 ng of type II procollagen (lane 6). A longer (A) and shorter (B) exposure of the same gel is shown for lanes 1–4, so that all collagenous bands may be seen clearly.
sequences is a mechanism for tailoring the properties of both pro-collagenous sequences will be cleaved by cells that produce NH2-terminal to the pro-collagenous sequences, as reported by us are undoubtedly pro-

In tissues where essentially all pro-

The latter fibrils would thus differ from the former in having a lower density of pro- 

BMP-1 cleavage site. Thus, it is possible that, at least in some circumstances, cleaved pro-

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J. Biol. Chem. 2004, 279:30904-30912.
doi: 10.1074/jbc.M402252200 originally published online May 10, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402252200

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