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18. Affinity-purified Rad53p antibody was prepared from glutathione-S-transferase–Rad53 as described (*19*). For immunoblot analysis, 150 μg of cell extracts was run on 8% SDS–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) (Dupont) membrane as described (*19*), and visualized by enhanced chemiluminescence (Amersham). For immunoprecipitations, 1 mg of protein extract was incubated with Rad53p affinity-purified antibody in 25 mM Hepes (pH 7.6), 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 200 μg/ml of bovine serum albumin, 0.1% Tween-20, 0.1 mM benzamidine, 1 μM aprotinin, 0.1 μg/ml each of leupeptin, soybean trypsin inhibitor, pepstatin, and antipain, 0.1 mM Na_3VO_4, and 30 mM NaF for 1 hour at 4°C. Protein-antibody complexes were precipitated with protein Sepharose A and washed with 25 mM Hepes (pH 7.6), 1% (w/v) Triton X-100, 10% (v/v) glycerol, 1 μM aprotinin, 0.5 M NaCl, 0.1 mM Na_3VO_4, and 30 mM NaF.
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Bone Morphogenetic Protein–1: The Type I Procollagen C–Proteinase

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Bone morphogenetic proteins (BMPs) are bone-derived factors capable of inducing ectopic bone formation. Unlike other BMPs, BMP-1 is not like transforming growth factor–β (TGF-β), but it is the prototype of a family of putative proteases implicated in pattern formation during development in diverse organisms. Although some members of this group, such as *Drosophila* tolloid (TLD), are postulated to activate TGF-β–like proteins, actual substrates are unknown. Procollagen C–proteinase (PCP) cleaves the COOH-propeptides of procollagens I, II, and III to yield the major fibrous components of vertebrate extracellular matrix. Here it is shown that BMP-1 and PCP are identical. This demonstration of enzymatic activity for a BMP-1/TLD-like protein links an enzyme involved in matrix deposition to genes involved in pattern formation.

After the isolation of BMP-1 peptides from osteogenetic fractions of bone (*1*), proteins involved in morphogenetic patterning, such as *Drosophila* TLD and TLR-1 (*2–4*) and sea urchin BP10 and SpAN (*5*), were isolated and found to be structural homologs of BMP-1. Each contains an NH_2-terminal astacin-like metalloprotease domain (*6*) followed by varying numbers of epidermal growth factor (EGF) motifs and CUB protein-protein interaction domains (*7*). Substrates have not been demonstrated for any member of this family of putative proteases. Although évidence suggests they may function in morphogenesis by activating TGF-β–like molecules (*1*, *8*), it has also been suggested that BMP-1/TLD-like proteases may modify matrix components, thus influencing cell fate decisions by altering cell-matrix interactions (*3*).

Collagen types I, II, and III are the major fibrous components of vertebrate matrix, and their orderly deposition is critical for normal morphogenesis. They are synthesized as procollagens, precursors with NH_2- and COOH-terminal propeptides that must be cleaved to yield mature monomers capable of forming fibrils (*9*). PCP, the physiological activity that cleaves procollagen I, II, and III COOH-propeptides, shares a number of features with BMP-1. It is a secreted N-glycosylated metalloprotease that requires calcium for optimal activity (*10–12*), and it is crucial for cartilage and bone formation, because collagen types II and I, respectively, are the major protein constituents of these tissues. Similarly, BMP-1 is implicated in de novo endochondral bone formation (*1*) and is also a metalloprotease with multiple sites for potential N-linked glycosylation and EGF-like sequences that may confer calcium dependence (*13*) on binding activities of adjacent CUB domains. The molecular mass of mammalian PCP (*11*) is close to that expected for mature BMP-1 (*1*). PCP activity is stimulated by the procollagen C–proteinase enhancer (PCPE), a glycoprotein that binds the type I procollagen COOH-propeptide by means of CUB domains (*11*, *14*). Because PCP also binds the COOH-propeptide (*11*, *12*) and PCPE itself (*15*), we noted that PCP may contain CUB domains and, thus, be BMP-1–like (*14*).

To investigate possible correlations between BMP-1 and PCP, we compared the properties of secreted recombinant BMP-1 (rBMP-1) produced by a baculovirus system (*16*) with those of purified mouse PCP (*17*). We first examined whether rBMP-1 has PCP-like activity (*17*). Type I procollagen was incubated with conditioned media from cells infected with baculovirus containing a BMP-1 complementary DNA (cDNA) insert. The type I procollagen was processed to yield the disulfide-linked COOH-propeptide and pNα1 and pNα2 chains as the major products (Fig. 1A) (*18*). No nonspecific cleavages were evident. The SDS–polyacrylamide gel electrophoresis (SDS-PAGE) mobilities of rBMP-1–generated products were identical to those of products generated by mouse PCP. Cleavages did not occur when procollagen was incubated with conditioned media from cells infected with wild-type virus (Fig. 1, A and B) or from uninfected cells (*19*). Electrophoretic mobilities of reduced propeptide subunits C1 and C2 from human (Fig. 1B) or chick (Fig. 1C) type I procollagen were also indistinguishable when released by rBMP-1 or PCP.

These results were consistent with the

Table 1. Inhibition profiles of rBMP-1 and procollagen C–proteinase (PCP). DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor. ND, not done.

Inhibitor	Concentration (mM)	Inhibition (%) with	
		rBMP-1*	PCP†
EDTA	10	100	100
EGTA	10	100	100
1,10-phenanthroline	1	100	98
1,7-phenanthroline	1	0	ND
1,4-phenanthroline	1	89	ND
L-Lysine	10	89	80
L-Arginine	10	92	86
ε-Amino caproic acid	10	81	80
Dithiothreitol	1	100	100
DFP	1	10	11‡
PMSF	0.4	0	13
SBTI	10§	0	10
Leupeptin	10§	0	0

*rBMP-1 activity was determined with the assay for PCP activity described in Kessler *et al.* (*12*). †PCP data are from Kessler *et al.* (*12*). ‡Value for inhibition of PCP by DFP is from Hojima *et al.* (*10*). §10 μg/ml.

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cleavage of procollagen by rBMP-1 and PCP at identical sites. NH_2-terminal amino acid sequencing (*20*) of the propeptide subunits released by rBMP-1 from human procollagen I (Fig. 1D) indicated that cleavages had occurred at the physiological PCP sites (*10, 12*). In addition, the human procollagen substrate contained some type III procollagen (Fig. 1B), and the C1(III) sequence also demonstrated cleavage at the physiological site (Fig. 1D).

Processing activity of rBMP-1, like that of PCP, was stimulated by the PCPE (Fig. 1 A, B, and C). A quantitative activity assay (Fig. 1E) showed that PCPE enhanced rBMP-1 processing activity sevenfold, a level comparable to the amount of enhancement obtained for mouse PCP (*11*). The same activity assay was also used to study inhibition properties of rBMP-1, which were found to be the same as those displayed by PCP (Table 1) (*10, 12*).

PCP specifically binds the type I procollagen COOH-propeptide (*11, 12*), a property utilized for purification of mouse PCP with COOH-propeptide-Sepharose chromatography (*11, 12*). Purification of PCP is improved by chromatography on lysyl-Sepharose before binding to COOH-propeptide-Sepharose (*11*). When conditioned media from insect cells expressing rBMP-1 were subjected to sequential chromatography on lysyl- (Fig. 2A) and COOH-propeptide-Sepharose (Fig. 2B), rBMP-1 bound and eluted from these resins with a profile similar to that observed for PCP (*21*). Moreover, the chromatography yielded an rBMP-1 preparation with only two protein bands detected by SDS-PAGE and

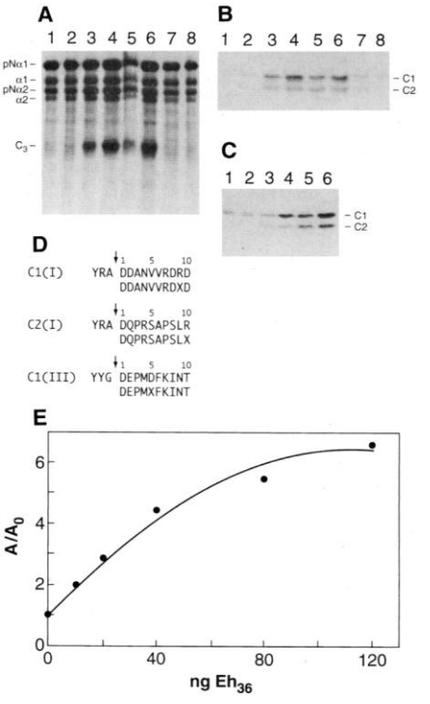

Fig. 1. rBMP-1 has PCP activity. (**A**, **B**, and **C**) rBMP-1 cleaves procollagen COOH-propeptides. Human type I procollagen labeled with [^{3}H]tryptophan and [^{14}C]proline and [^{14}C]glycine (A and B) and [^{3}H]tryptophan-labeled chick procollagen type I (C) were incubated with rBMP-1 and samples analyzed by SDS-PAGE without (A) and with (B and C) reduction. Reactions were performed in the presence (even lanes) or absence (odd lanes) of PCPE. Lanes 1 and 2, procollagen substrate; 3 and 4, substrate plus medium from insect cells infected with recombinant virus; 5 and 6, substrate plus mouse PCP; 7 and 8, substrate plus medium from insect cells infected with wild-type virus. pNα1 and pNα2, collagen processing intermediates containing NH_2-, but not COOH-propeptides; α1 and α2, mature collagen chains; C_3, disulfide-linked COOH-propeptide; C1 and C2, COOH-propeptide subunits of proα1 and proα2 chains, respectively. In (B) the faint band in front of C1 is C1(III). (**D**) NH_2-terminal sequences of COOH-propeptides cleaved from human procollagens I and III by rBMP-1. Shown are the C1(I), C2(I), and C1(III) propeptide subunits of the proα1(I), proα2(I), and proα1(III) chains, respectively. Sequences obtained (bottom lines) are compared to those obtained by cleavage with mouse PCP (*12*) (top lines). Arrow, peptide bond cleaved by PCP and rBMP-1. X, unresolved residue. (**E**) rBMP-1 processing activity is enhanced by PCPE. Procollagen processing activity of rBMP-1 was measured in the absence and presence of increasing amounts of PCPE and the relative increase of enzyme activity (A/Ao) determined. Eh36, 36-kD form of PCPE (*11*); A and Ao, enzyme activity with and without PCPE, respectively.

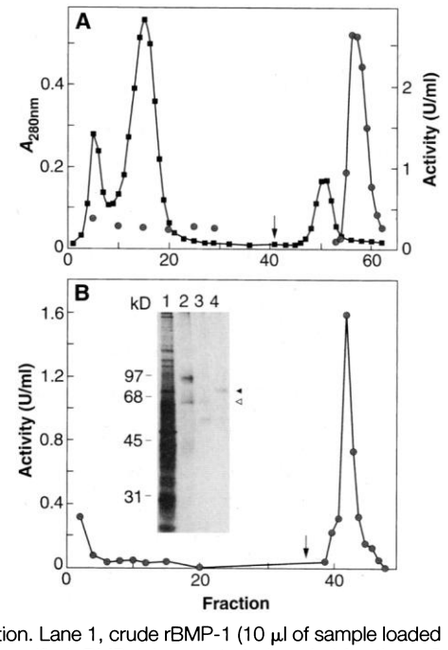

Fig. 2. rBMP-1 binds lysyl- and C-propeptide-Sepharose. (**A**) Lysyl-Sepharose chromatography of rBMP-1. Concentrated conditioned media from insect cells expressing rBMP-1 (~17 units/6 ml; equivalent to 32 ml of media) was chromatographed on lysyl-Sepharose as described for mouse PCP (*11*). Arrow, initiation of elution with 0.1 M ε-aminocaproic acid in buffer B (*21*). Fractions 5 to 30 were directly assayed for rBMP-1 activity, whereas fractions 53 to 62 were dialyzed against buffer A before assaying activity. (■), absorbance at 280 nm; (●), rBMP-1 activity. (**B**) COOH-propeptide-Sepharose chromatography of rBMP-1. Fractions 55 to 62 from the lysyl-Sepharose column were pooled, concentrated, and dialyzed against buffer B. 1.1 ml (~13 units) was chromatographed on COOH-propeptide-Sepharose as described (*11*). Arrow, initiation of elution with buffer B made 1 M in guanidine hydrochloride and NaCl. Fractions 2 to 20 were directly assayed for rBMP-1 activity, whereas fractions 39 to 48 were dialyzed against buffer A before assay. Absorbance at 280 nm was not measured because of the very low protein concentrations in column fractions. Active fractions 40 to 45 were pooled, concentrated to 1 ml, dialyzed against buffer A, and stored at −80°C. (Inset) Electrophorogram showing progress of rBMP-1 purification. Lane 1, crude rBMP-1 (10 μl of sample loaded on the lysyl-Sepharose column); 2, lysyl-Sepharose purified rBMP-1 (concentrated pooled fraction, 15 μl); 3, COOH-propeptide-Sepharose purified rBMP-1 (concentrated pooled fraction, 25 μl); 4, purified mouse PCP (~20 ng). Electrophoresis in a 10% polyacrylamide gel was without reduction, and protein bands were visualized by silver staining (*29*). Left, migration positions of (reduced) standard proteins with indicated molecular masses. ◀ and ◁, migration positions of PCP and rBMP-1, respectively.

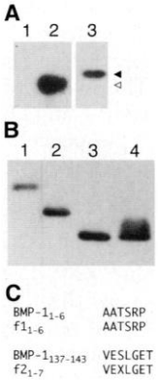

Fig. 3. (**A**) When immunoblotted, rBMP-1 and PCP cross-react immunologically with antibodies to a BMP-1 fusion protein. Lane 1, conditioned media from insect cells infected with wild-type virus (25 μl; concentrated 50-fold); 2, conditioned media from insect cells infected with recombinant virus (25 μl; concentrated 50-fold; ~0.6 units); 3, purified PCP (~0.25 units). All samples were reduced with 2-mercaptoethanol. ◀ and ◁, PCP and rBMP-1, respectively. (**B**) Migration of deglycosylated rBMP-1 and PCP is identical on SDS-PAGE. rBMP-1 and PCP (~250 ng; purified as in Fig. 2) were treated with PNGase F (*22*) and analyzed by immunoblotting with antibody to BMP-1 fusion protein. Lane 1, PCP (25 ng); 2, rBMP-1 (250 ng); 3 and 4, deglycosylated PCP and rBMP, respectively. Samples were unreduced. SDS-PAGE (A and B) was on 10% polyacrylamide gels. (**C**) Comparison of peptide sequences of mouse PCP (bottom lines) with the corresponding amino acid sequences of BMP-1 (top lines) (*1, 25, 26*). f1 and f2, fragments derived from mouse PCP by V8 protease treatment; X, unresolved residue. Subscript numbers, positions of residues within respective sequence. Analyses were performed on 6 and 2 pmol of f1 and f2, respectively.

silver staining (Fig. 2B, inset). The band corresponding to rBMP-1 migrated somewhat ahead of mouse PCP (open and closed arrowheads, respectively) and was identified by immunoblotting with BMP-1–specific antibodies. SDS-PAGE mobility of this protein was identical to that of the only positive band in immunoblots (*22*) of the crude rBMP-1 preparation, whereas the same band was absent from control media of insect cells infected with wild-type virus (Fig. 3A). Immunoblotting also showed that mouse PCP is recognized by BMP-1–specific antibodies (Fig. 3A), demonstrating, in spite of the mobility difference, the relatedness of the two proteins. N-glycosylated mammalian proteins expressed in baculovirus systems are generally smaller than native counterparts as a result of differences in N-glycosylation by insect and mammalian cells (*23*). Removal of N-linked saccharides of mouse PCP and rBMP-1 with peptide-N-glycosidase F (PNGase F) (*22*) caused a pronounced increase in the mobility of both, such that the two proteins migrated identically (Fig. 3B).

To further establish the relatedness of PCP and BMP-1, we performed NH_2-terminal amino acid sequencing on two fragments, f1 and f2, generated from mouse PCP by V8 protease treatment (*24*). The sequences obtained (Fig. 3C) both lie within the published amino acid sequences of human and mouse BMP-1 (*1*, *25*, *26*) and correspond to residues 1 to 6 (f1) and 137 to 143 (f2) of the metalloprotease domain. Consistent with V8 protease specificity of cleavage, position 136 in BMP-1 is a Glu. These results provide strong evidence that BMP-1 and PCP are the same protein. The f1 sequence corresponds precisely to the beginning of the metalloprotease domain, immediately downstream of the dibasic processing motif RSRR (*1*, *2*, *25–27*). It, thus, likely represents the NH_2-terminus of mature BMP-1, providing experimental evidence that the peptide bond following the RSRR sequence is the site for cleavage of the proregion.

Previous biochemical (*1*) and genetic (*8*) evidence suggested that BMP-1/TLD–like proteases may affect cell fate decisions through activation of TGF-β–like proteins. The identification of BMP-1 as PCP does not preclude the possibility that BMP-1/PCP is a multifunctional enzyme that also activates TGF-β–like proteins. Alternatively, because the BMP1 gene produces alternatively spliced transcripts for BMP-1 and for a longer protein, mammalian tolloid (mTld) (*25*), with a domain structure identical to that of *Drosophila* TLD, these two proteins may serve separate functions. Because BMP-1 peptides that copurify with TGF-β–like BMPs (*1*) are common to both BMP-1 and mTld, it remains to be determined whether BMP-1, mTld, or both actually interact with TGF-β–like molecules. Some TGF-β–like molecules can stimulate matrix production (*28*). Thus, a product or products of the BMP1 gene may activate TGF-β–like molecules and then process induced procollagens into insoluble matrix, the latter further influencing cell fate decisions by altering cell-matrix interactions. The parsimonious use of the BMP1 gene for these dual roles would make it a key player in morphogenesis and wound repair and in pathological conditions involving fibrosis and scarring.

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16. To produce rBMP-1, we isolated a 2199-bp Sph I–Bgl I cDNA fragment containing the full-length human BMP-1 coding sequence from clone KT11 (*25*), ligated it into vector pBacPAK9 (Clontech), and recombined it with Bsu36 I–digested BacPAK6 (Clontech) viral DNA by co-transfection into Sf21 insect cells. After 60 to 72 hours, resultant virus was plaque-purified and amplified 4 days on Sf21 monolayers in 10% fetal calf serum and Grace's medium (Sigma). Virus stocks were then reamplified in Sf21 suspension cultures in serum-free Sf-900 media (Gibco-BRL). After 6 days, fresh cells were infected with reamplified virus and grown in fresh Sf-900 media, which was harvested for recombinant protein 4 days after infection. Media was concentrated eightfold with Aquacide (Calbiochem), dialyzed against buffer A [0.05 M tris-HCl, 0.15 M NaCl, and 5 mM $CaCl_2$ (pH 7.5)], and stored at −20°C.
17. Procollagens, PCP, and PCPE were prepared as described (*11*, *12*) [E. Kessler and B. Goldberg, *Anal. Biochem.* **86**, 463 (1978)]. Enzyme digests and SDS-PAGE analyses (in 6 and 12% gels for unreduced and reduced samples, respectively) were as described [S.-T. Lee, E. Kessler, D. S. Greenspan, *J. Biol. Chem.* **265**, 21992 (1990)].
18. Undigested procollagen and pCα chains (processing intermediates that contain COOH- but not NH_2-propeptides) migrated at the top of the gel (Fig. 1A, not shown). The slight increase in α chain levels reflects cleavages by rBMP-1 of COOH-propeptides from pCα chains already present in the procollagen substrate.
19. E. Kessler, K. Takahara, L. Biniaminov, M. Brusel, D. S. Greenspan, unpublished data.
20. To prepare COOH-propeptides for NH_2-terminal sequencing, human procollagen type I (*12*) [500 μg in 900 μl of buffer A containing 25 μg of PCPE, 1 mM DFP, and SBTI (10 μg/ml) and leupeptin] was incubated with 1 unit of affinity-purified rBMP-1 (Fig. 2) for 17 hours at 37°C. Proteins were then precipitated with trichloroacetic acid (TCA) (*12*), isolated by SDS-PAGE [M. W. Hunkapiller, E. Lujan, F. Ostrander, L. E. Hood, *Methods Enzymol.* **91**, 227 (1983)], and electrotransferred to polyvinylidene difluoride (PVDF) [P. J. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)]. NH_2-terminal amino acid sequencing was performed with the ABI 475A liquid phase protein sequenator.
21. Lysyl- and propeptide-Sepharose chromatography was as described (*11*). Propeptide-Sepharose was equilibrated with buffer B [0.05 M Hepes, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.1% Brij 35, (pH 7.5)], whereas lysyl-Sepharose was equilibrated with buffer B with 1 M NaCl. rBMP-1 activity was determined as described (*11*). One unit of activity is the amount of enzyme that cleaves 1 μg of procollagen in 1 hour at 37°C.
22. For Fig. 3A, proteins in the media of insect cells were concentrated by TCA precipitation (*12*). For Fig. 3B, affinity-purified rBMP-1 and PCP were incubated (3 hours; 37°C) with PNGase F (New England Biolabs; 140 ng) in buffer A containing SBTI (100 μg/ml), benzamidine (10 mM), leupeptin (10 μg/ml), SDS (0.1%), and NP-40 (1%). Immunoblots were developed with guinea pig antiserum to BMP-1 fusion protein (1:1000), with the ECL system (Amersham). To prepare BMP-1 fusion protein, we subcloned a 1040-bp Apa I–Hinc II cDNA fragment into expression vector pRSET B (Invitrogen) and expressed it in BL21 (DE3) *Escherichia coli*. The fusion protein, which includes human BMP-1 residues 197 to 543 (*1*) fused to a polyhistidine domain, was purified on nickel-Sepharose (Invitrogen) followed by SDS-PAGE (*11*). Gel suspensions in phosphate-buffered saline (*11*) containing 15 (first immunization), 7.5 (second and third boosts), or 20 μg of fusion protein (fourth boost) were injected subcutaneously into a guinea pig at 2-week intervals. Sera were collected 10 days after the third and fourth immunizations.
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24. To generate mouse PCP peptides, enzyme (~8 μg) from 40 liters of 3T6 fibroblast conditioned media was purified as described (*11*), concentrated by TCA precipitation (*12*), heat denatured in SDS-sample buffer [U. K. Laemmli, *Nature* **227**, 680 (1970)], and electrophoresed in a 10% polyacrylamide gel. The gel region containing the enzyme band (~4 μg) was applied to a 12% polyacrylamide gel for fragmentation with V8 protease (Sigma, 7.5 ng) and electrophoretic separation of resulting peptides [D. W. Cleveland, S. G. Fisher, M. W. Kirschner, U. K. Laemmli, *J. Biol. Chem.* **252**, 1102 (1977)] and electroblotting to PVDF [P. J. Matsudaira, *J. Biol. Chem.* **262**, 10035 (1987)]. Two major fragments with molecular masses of ~20 kD (f1) and ~40 kD (f2) were subjected to NH_2-terminal sequencing as in Fig. 1D.
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