Bone morphogenetic protein-1-like proteinases play key roles in formation of the extracellular matrix (ECM) in vertebrates via biosynthetic processing of precursors into mature functional proteins involved in ECM assembly. Such processing includes proteolytic activation of the zymogen for lysyl oxidase. Fibronectin (FN) is an abundant protein component of the ECM that is capable of regulating manifold cellular functions through its interactions with various ECM and cell surface proteins. It was previously shown that proteolytic activation of lysyl oxidase is much reduced in cultures of FN-null mouse embryo fibroblasts (MEFs). Here we demonstrate that cellular fibronectin, the form produced by fibroblasts and various other tissue cell types, and plasma fibronectin bind BMP1 with dissociation constants \( K_{\text{d}} \) of \( \sim 100 \) nm, consistent with a physiological role. Also consistent with such a role, cellular fibronectin FN is shown to positively regulate BMP1 processing activity against Chordin, probiglycan, and type I procollagen \textit{in vitro}. Endogenous FN and BMP1 are demonstrated to co-localize in cell layers and to form complexes in culture medium. In addition, processing of endogenous BMP1 substrates Chordin, probiglycan, and procollagen is demonstrated to be strikingly reduced in cultures of FN-/- MEFs compared with FN+/+ MEF cultures despite similar levels of endogenous BMP1. These data support the conclusion that FN binds BMP1-like proteinases \textit{in vivo} and that FN is an important determinant of the \textit{in vivo} activity levels of BMP1-like proteinases.

Fibronectin (FN) is a noncollagenous extracellular matrix (ECM) glycoprotein of relatively high abundance that regulates a wide variety of cellular functions, including adhesion, migration, proliferation, differentiation, and apoptosis \( (1–4) \). FN is secreted as a disulfide-bonded dimer, and each subunit comprises 12 type I, 2 type II, and 15–17 type III FN modules as well as a “variable” (V) region that lacks homology to other protein domains \( (3) \). FN is found as two different major forms, plasma fibronectin (pFN), a soluble form synthesized by hepatocytes, and cellular fibronectin (cFN), which is locally expressed by many other cell types in various tissues \( (5) \). Both forms can be assembled into a fibrillar ECM by cultured fibroblasts \( (6) \). Differences between cFN and pFN arise from alternative RNA splicing in three regions; two type III repeats (designated EDA and EDB) and the V region. EDA and EDB are present in cFN but absent from pFN, whereas although only one subunit of the pFN dimer contains the V region, almost all cFN subunits contain this region \( (7) \). These differences in domain structure contribute to distinct functions for pFN and cFN; cFN plays roles in the dynamic tissue modeling of early embryogenesis and wound healing \( (8) \), whereas pFN subserves roles in hemostasis and thrombosis and immune responses \( (3, 9–11) \) and provides a reservoir for deposition in tissue \( (12) \).

BMP1-like proteinases are evolutionary conserved extracellular metalloproteinases that play multiple roles in fostering ECM formation and activating TGFβ-like growth factors \( (13) \). These proteinases biosynthetically convert a variety of precursors into mature functional proteins with roles in ECM formation, including collagen types I-III, V, VII, and XI, laminin 332, and the small leucine-rich proteoglycans biglycan and osteoglycin. One important example is the zymogen for lysyl oxidase (LOX), an enzyme essential to formation of the covalent cross-links responsible for providing collagen and elastic fibers with much of their tensile strength \( (14) \). Recently, FN was reported to bind LOX \textit{in vitro} \( (15) \). It was also suggested to positively regulate the proteolytic activation of LOX, as dramatically decreased processing of the zymogen for LOX was observed in FN-/- mouse embryo fibroblast (MEF) cultures compared with FN+/+ MEF cultures even though equal amounts of BMP1 proteinase were produced by MEFs of the two different genotypes \( (15) \). These observations prompted the present study to determine whether FN might be involved in modulating the activities of BMP1-like proteinases. Herein, we provide evidence for direct interaction between FN and BMP1. BMP1 is shown to bind multiple FN sites via its non-protease domains, with affinities in the \( \sim 100 \) nm range for cFN and pFN. This is a range congruent with \( K_{\text{d}} \) values \( (30–800 \) nm) previously estimated for binding of FN to its integrin receptors \( (16, 17) \) and is,
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thus, consistent with the likelihood of in vivo FN-BMP1 interactions. Moreover, cFN is shown to positively regulate BMP1 processing activity against a number of substrates in vitro. Consistent with the in vitro evidence of FN-BMP1 interactions, we demonstrate FN-BMP1 co-localization and the existence of FN-BMP1 complexes in cell cultures. Also demonstrated is a striking decrease in the processing of various endogenous BMP1 substrates in cultures of FN−/− MEFs compared with FN+/− MEF cultures. Implications of the data, which support the conclusion that FN positively regulates BMP1 activities in vivo, are discussed.

EXPERIMENTAL PROCEDURES

In Vitro Cleavage Assays—For probiglycan and cleavage assays, the wells of 96-well microtiter plates were coated at 4 °C overnight with 4.5 μg/ml BSA as a control or with 4.5 μg/ml cFN (catalog no. F2518, Sigma-Aldrich) such that cFN in wells corresponded to 1× or 2× molar amounts of the 15 ng of BMP1 (9 nm) in a 20-μl reaction volume, prepared as previously described (18) in the used in the cleavage reactions. For procollagen cleavage assays, wells were coated with 6.0 μg/ml BSA or with 6 or 12 μg/ml cFN to correspond to 1× or 2× molar amounts of the 20 ng of BMP1 (12 nm) in a 20-μl reaction volume) used in the cleavage reactions. Wells were washed twice with PBS and then blocked with 1% BSA in PBS for 2 h at 37 °C. After washing with PBS, probiglycan, or procollagen I cleavage assays were carried out in the wells, as described below.

For probiglycan cleavage assays, 300 ng of recombinant probiglycan prepared as previously described (19) was incubated with 15 ng of BMP1 (9 nm, final concentration) in 20 μl of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM CaCl2 for 30 min at 37 °C. Subsequently, 4 μl of chondroitinase ABC (from a stock of 10 μl of 0.01 units/μl of protease-free chondroitinase ABC (Seikagaku Corp.). 40 μl of 6× chondroitinase buffer (100 mM Tris-HCl, pH 8.0, 240 mM NaOAc, 0.25 mM EDTA), and 10 μl 500 mM EDTA) was added for the dual purpose of quenching the cleavage reaction and removing glycosaminoglycan side chains for 14 h of additional incubation at 37 °C. Samples were then subjected to SDS-PAGE on a 10% gel, and Western blot analysis was performed using polyclonal anti-FLAG antibody anti-70K antibodies or monoclonal anti-FLAG antibody anti-70K antibodies (Sigma-Aldrich) diluted 1:1000 was used for Western blotting.

In Vitro FN Pulldown Assays—80 ng of FLAG-tagged BMP1 was preincubated overnight at 4 °C with/without 200 ng of pFN (catalog no. F2006, Sigma-Aldrich) or cFN in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ZnCl2, 1 mM CaCl2, 1 mM MgCl2, 0.1% Triton X-100, 0.1% CHAPS, 0.1% N-octylglucoside, 2% glycerol, and 1 mg/ml BSA (buffer A). Rabbit antibodies to the N-terminal 70-kDa catheptic fragment (70K) of FN (anti-70K) was then added, and the mix was allowed to rotate for 3 h at 4 °C followed by the addition of protein A-Sepharose beads (GE Healthcare) and then rotation for another 2 h. Beads were then washed for 15 min 3 times with PBS and boiled in 2× SDS loading buffer. Samples were subjected to SDS-PAGE on 7.5% gels, and Western blot analysis was performed using either monoclonal anti-FLAG antibodies or polyclonal anti-70K antiserum (21). Pulldown assays were similarly performed using either a previously described FLAG-tagged version of BMP1 comprising only pro- and protease domains (22) or a previously described FLAG-tagged version of BMP1 lacking only protease domain sequences (23).

Co-immunoprecipitation was performed with the following pieces of FN with BMP1; that is, the proteolytic N-terminal 70K fragment of human pFN (24), dimeric recombinant III1-C(EDA−)’ (encompassing FN repeat III1 to the C terminus and including EDA and variable region V89), III1-C(EDA−)’ and monomeric recombinant III1-14 (25). FLAG-tagged BMP1, 80 ng, was incubated with 70 ng of 70K or the molar equivalent of 11-C, FNIII1-C(EDA−)’, FNIII1-C(EDA−)’, or III1-14 overnight at 4 °C in buffer A. For immunoprecipitation of the 70K fragment, rabbit anti-70K was added, whereas for immunoprecipitating C-terminal FN fragments, previously described monoclonal anti-FN antibody LabMab (26) directed against an epitope in FN fragment III1-14 was added, and in both cases the mix was rotated 3 h at 4 °C. Protein A-Sepharose (GE Healthcare) or protein G-Sepharose (Sigma) was then added to the 70K or C-terminal mixes, respectively, and the mix was allowed to rotate for another 2 h at 4 °C. In each case Sepharose beads were then washed for 15 min 3 times with PBS, and 2× SDS loading buffer was then added. Samples were subjected to SDS-PAGE on a 7.5% gel, and Western blot analysis was performed using monoclonal anti-FLAG antibody anti-70K antibodies or rabbit anti-FN antibodies.

Immunostaining—AH1F neonatal foreskin fibroblasts (generously provided by Lynn Allen-Hoffmann, University of Wisconsin) and MG-63 human osteosarcoma cells (American Type Culture Collection, Manassas, VA) were grown on glass coverslips for 4–6 days at 37 °C in 24-well tissue culture plates in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum, and 2 mm L-glutamine with soybean trypsin inhibitor (100 μg/ml) included for non-permeabilized cells. After removal of media, cells were washed in PBS for 5 min and then fixed in 4% paraformaldehyde for 10 min. Cells to be per-
meabilized were then washed twice with PBS for 15 min and treated with 0.5% Triton X-100 for 5 min at room temperature followed by washing twice with PBS for 15 min. Coverslips were then blocked with 0.1% BSA-PBS for 30 min followed by the addition of goat polyclonal antibody against BMP1 (R&D Systems) in 1% BSA-PBS (1:50) and incubation for 1 h at room temperature. After rinsing cells twice with PBS for 5 min at room temperature, Alexa Fluor 594 donkey anti-goat antibody (Invitrogen) in 1% BSA-PBS (1:400) was applied to the coverslips for 40 min at room temperature in the dark. Subsequent steps were also performed in the dark. Coverslips were washed 3 times with PBS for 10 min and were then blocked again with 0.1% BSA-PBS for 30 min. Rabbit antibodies to full-length human pFN in 1% BSA-PBS (1:100) was then added followed by Alexa Fluor 488 donkey anti-rabbit secondary antibody (Invitrogen, 1:400), with incubation times and washing procedures the same as described above for BMP1 staining. Coverslips were then stained for 1 min with 4’,6-diamidino-2-phenylindole (Sigma, 2 μg/ml), washed 3 times with PBS for 10 min, and then mounted on glass slides using mounting medium (Immuno-mount, Thermo Shandon Inc.). Slides were examined with a Zeiss Axioshot 2 microscope. Pictures were captured and controlled in Axiovision AC. Exposure times were 10 and 80 ms for detection of FN and BMP1, respectively.

Co-immunoprecipitation of Endogenous Proteins from Cell Culture—MG-63 human osteosarcoma cells were purchased from the American Type Culture Collection and were maintained in DMEM, 10% fetal bovine serum, and 1% L-glutamine. Just-confluent MG-63 cells were rinsed twice with PBS and then rinsed once with serum-free media followed by the addition of serum-free DMEM containing vehicle (5 mM HCl) or 2 mg/ml TGFβ1 (R&D Systems) and 40 μg/ml soybean trypsin inhibitor (Sigma). Conditioned media were harvested after 24 h, protease inhibitors (2.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM p-aminobenzoic acid, 1 mM N-ethylmaleimide) were added, and harvested media were concentrated 40-fold in Amicon Centricron Plus-70 centrifugal filters (Millipore). To concentrated medium (1 ml) antibody directed against the C terminus of BMP1 (27) or a previously described antibody directed against the N terminus of Chordin (28) and against BMP1 (30) of 7D5 monoclonal anti-FN antibody was then added followed by incubation on a rotator for 2 h at 4 °C. Protein A-agarose beads (Roche Applied Science) for pull down of polyclonal antibodies or protein G-agarose for pull down of monoclonal antibodies were then added followed by incubation on a rotator overnight at 4 °C. Beads were then centrifuged and washed 3 times with buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 50 mM EDTA, and the protease inhibitors listed above. Proteins were then eluted from the beads by boiling for 5 min in SDS sample buffer, 1% β-mercaptoethanol. Boiled samples were subjected to SDS-PAGE and Western blotting using BMP1 C terminus antibody (27) or 4D1.7 monoclonal anti-FN antibody. The epitope of 4D1.7 has been localized to FN type 1 modules I₁₋₅ (25), and the epitope for 7D5 has been localized to FN modules I₁₋₅ (not shown).

BMP1-FN Binding ELISA—The binding target, cFN or pFN, was diluted to 5 μg/ml in TBS (10 mM Tris, 150 mM NaCl, pH 7.4) and used to coat 96-well microtiter plates (Costar 3590 high binding, Corning Inc., Corning, NY) with 50 μl per well for 16 h at 4 °C. Plates were washed once with TBS plus 0.05% Tween 20 (TBST) and blocked with 5% nonfat dry milk (NFDM) in TBST for 1 h. Control wells without antigen were also blocked. After washing the plates 3 times with TBST, BMP1 diluted to various concentrations in TBST plus 0.2% NFDM was added to wells coated with antigen as well as to a set of NFDM-blocked wells not coated with FN. The BMP1 dilutions were incubated in the wells for 2 h followed by washing of the plates 4 times with TBST. Anti-human BMP1/PCP antibody (R&D Systems, Minneapolis, MN) was diluted to 0.5 μg/ml in TBST plus 0.2% NFDM and incubated in the wells for 1 h. Plates were then washed four times with TBST. Horse-radish peroxidase-conjugated secondary antibody diluted 1/15,000 in TBST plus 0.2% NFDM was incubated in the wells for 1 h. The plates were then washed 5 times with TBST, and 50 μl per well of SureBlue TMB Microwell peroxidase substrate (KPL, Gaithersburg MD) was added and allowed to react. After 15 min, color development was stopped with 50 μl per well TMB stop solution. Absorbance was read at 450 nm.

Dissociation constants (KD) were determined from the plotted values of percent of maximum binding divided by the added BMP1 concentration (y axis) versus percent of maximum binding (x axis). Linear plots with R² > 0.97 that covered >90% dose-response range were obtained (data not shown). KD values were calculated from the equation KD = −1/slope. The KD values for BMP1 binding to cellular and plasma FNs are 110 ± 20 and 120 ± 30 nm, respectively (mean ± S.D. of three assays each).

Analyses of Processing of BMP1 Substrates in MEF Cultures—MEFs differentiated from FN-null (FN−/−) and heterozygous (FN+/−) stem cells (29) were cultured to confluence in DMEM, 10% FN-depleted fetal bovine serum. Cells were washed 3 times with PBS and incubated for 30 min in serum-free DMEM. For analysis of Chordin cleavage, cells were then washed with PBS again and switched to serum-free DMEM containing 40 μg/ml soybean trypsin inhibitor. Conditioned media were harvested after 24 h, and protease inhibitors were added to final concentrations of 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, 1 mM p-aminobenzoic acid, and 10 mM EDTA. Proteins from conditioned medium from MEFs of each genotype were either ethanol-precipitated or isolated using heparin-Sepharose (CL-6B; Amersham Biosciences). For the latter, 250 μl of 50% heparin-Sepharose slurry was added to 10 ml of conditioned medium, and the mix was allowed to rotate overnight at 4 °C. The medium was then centrifuged to remove the supernatant, and the Sepharose was washed 4 times with PBS. 2× SDS loading buffer was then added, and the samples were subjected to SDS-PAGE on 4–15% gradient gels. Western blotting was performed with previously described antibodies raised against the N terminus of Chordin (28) and against BMP1 (30) and with a commercial anti-sFRP2 antibody (R&D Systems). In addition, cell layers were scraped into hot SDS sample buffer, and Western blotting was performed with anti-α-tubulin antibody as previously described (31) to demonstrate similar numbers of cells in FN+/− and FN−/− MEF cultures.

To assay for the processing of probiglycan in MEF cultures, buffer exchange was performed on conditioned medium via
Centricon Plus-70 columns (Amicon), resulting in a final buffer composition of 1× chondroitinase ABC buffer (50 mM Tris-HCl, pH 8.0, 40 mM sodium acetate, 5 mM EDTA). Media were then concentrated to 100 µl followed by the addition of 10 µl of chondroitinase ABC and incubation overnight at 37 °C. 2× SDS loading buffer was then added to quench the reaction. Samples were subjected to SDS-PAGE on 10% gels, and Western blotting was performed with LF51, a previously described antibody raised against a peptide sequence (VPDLDVSPTF-SAMC) within the mature form of murine biglycan (20).

To compare procollagen processing and collagen deposition in cell-associated ECM, FN+/− and FN−/− MEFs were cultured in DMEM, 10% FN-depleted fetal bovine serum to 80% confluence and treated with 50 µg/ml ascorbate for 24 h. Cells were then washed twice with PBS and incubated for 15 min in serum-free DMEM at 37 °C. Cells were then washed once with PBS followed by the addition of serum-free DMEM containing 50 µg/ml ascorbate, 40 µg/ml soybean trypsin inhibitor. Conditioned media were harvested after 24 h, and cell layers were washed twice with ice-cold PBS and scraped into hot 2× SDS-sample buffer. Samples were run on 5% acrylamide SDS-PAGE gels, and Western blotting was performed with anti-α1(I) C-telopeptide polyclonal antibody LF67 (20), as described (28).

**RESULTS**

**cFN and pFN Bind BMP1 in Vitro**—To begin investigating the possibility of FN-BMP1 interactions, we first examined whether the two proteins can bind. Toward this end, a pull-down assay was conducted in which BMP1 was incubated with equimolar amounts of either pFN or cFN followed by immunoprecipitation with antisera raised against either solid-phase cFN and pFN. Both cFN and pFN were found to bind BMP1 in this non-quantitative assay (Fig. 1A). More BMP1 coprecipitated with cFN than with pFN despite equivalent input amounts of the two forms of FN, suggesting that cFN might bind BMP1 more readily than does pFN under the conditions of the pulldown assay. We next quantitatively examined the strength of interactions between BMP1 and cFN and between BMP1 and pFN by characterizing binding of BMP1 to solid-phase cFN and pFN via ELISA (Fig. 1B). BMP1 showed a sigmoidal dose-dependent binding to both cFN and pFN, with half-maximal binding at concentrations of 110 ± 20 and 120 ± 30 nM BMP1, respectively. BLAcore experiments (not shown) also indicate binding of fluid phase BMP1 to adsorbed cFN and pFN. It should be noted that the half-maximal binding concentration for pFN in the ELISA is markedly lower than the near µM concentration at which pFN is found in plasma, whereas calculated Kd values for both cFN and pFN are within the range of Kd values (30 to 800 nM) previously estimated for binding of FN to its integrin receptors (16, 17). Thus, the strength of FN-BMP1 interactions revealed by ELISA is consistent with the possibility that both forms of FN may bind BMP1 in vivo. The similar binding affinities of solid-phase cFN and pFN for BMP1 in the ELISA contrasts with the apparently greater binding of BMP1 to soluble cFN than to soluble pFN in the pulldown assay (Fig. 1A). Thus, it is possible that solid-phase pFN and/or cFN may bind BMP1 differently than does their soluble counterparts.

**BMP1 Binds FN via Its Non-protease Domains**—To gain further insights into the nature of BMP1-FN interactions, we next determined which portion of BMP1 is involved in binding FN. Toward this end, isolated BMP1 protease domain (BMP1-P) or a deleted version of BMP1 lacking the protease domain (BMP1-D) but containing all other domains (three complement-uegf-BMP1 domains interspersed with a single epidermal growth factor motif) were prepared (Fig. 2A) and then separately incubated with cFN followed by pulldown with anti-70K FN antibodies and blotting with anti-FLAG antibodies to detect BMP1-1 forms (Fig. 2B). As can be seen (Fig. 2B), cFN failed to bind BMP1-P, but bound BMP1-D at least as avidly as it did full-length BMP1. Thus, BMP1 binds cFN predominantly through its non-protease domains.

**BMP1 Has the Potential to Bind FN at Multiple Sites**—To begin locating the BMP1 binding site(s) on FN, full-length BMP1 was incubated with either the N-terminal 70K fragment of FN or the non-overlapping III1-C fragment, which extends from the first type III repeat to the C terminus (Fig. 3D), followed by immunoprecipitation with polyclonal anti-70K antibody. BMP1 binding of cFN and pFN in ELISA. BMP1 at 3.4, 11.4, 34.1, 113.6, 340.6, and 1136.4 nM was incubated with cFN or pFN adsorbed to microtiter wells, as described under “Experimental Procedures.” The calculated Kd values for BMP1 binding to cFN and pFN, as determined by Scatchard-type plots, were 110 ± 20 and 120 ± 30 nM, respectively. Values are expressed as the mean ± S.D. of three experiments.

![Figure 1](#) cFN and pFN bind BMP1. A, 80 ng of FLAG-tagged BMP1 was preincubated overnight with an equimolar amount of cFN or pFN. The complex was then immunoprecipitated (IP) with antisera raised against the N-terminal 70-kDa FN fragment (anti-70K), which recognizes both cFN and pFN. Immunoblotting (IB) was performed using either monoclonal anti-FLAG or polyclonal anti-70K antibody. B, BMP1 binding of cFN and pFN in ELISA. BMP1 at 3, 4, 11.4, 34.1, 113.6, 340.6, and 1136.4 nM was incubated with cFN or pFN adsorbed to microtiter wells, as described under “Experimental Procedures.” The calculated Kd values for BMP1 binding to cFN and pFN, as determined by Scatchard-type plots, were 110 ± 20 and 120 ± 30 nM, respectively. Values are expressed as the mean ± S.D. of three experiments.
pulldown assay (Fig. 3A), indicating the presence of at least two potential interaction sites.

pFN and cFN are alternatively spliced products of the same gene, with alternative splicing of three regions: the EDA, EDB, and V sequences (3). EDA is present in cFN but not in pFN. When C-terminal FN fragments III1-C with/without EDA (III1-C(EDA−) and III1-C(EDA+), respectively) were used for pulldown assays with BMP1, results showed similar amounts of BMP1 pulled down by III1-C(EDA−) and III1-C(EDA+) (Fig. 3B). Thus, alternative splicing of the EDA domain does not appear to affect FN-BMP1 binding, consistent with the similar binding constants of cFN and pFN for BMP1 (Fig. 1B).

To further localize BMP1 binding sites on the III1-C fragment, BMP1 was incubated with a fragment comprising the 1st to 14th FN type III modules and lacking the V region and all C-terminal type I repeats (fragment III1–14) followed by immunoprecipitation with anti-FN LabMab monoclonal antibody. As can be seen (Fig. 3C), immunoprecipitation of fragment III1–14 readily pulled down BMP1, consistent with the conclusion that FN region V and C-terminal type I repeats are not major determinants of binding between FN and BMP1 and indicating that binding occurs between BMP1 and the type III module sequences in the III1-C portion of FN.

**Cellular FN Can Increase BMP1 Activity in Vitro**—To determine how interaction with FN might affect BMP1 activity, in vitro cleavage assays for the processing of three substrates of BMP1-like proteinases (probiglycan, type I procollagen, and Chordin) were performed in the presence or absence of cFN. For probiglycan and type I procollagen, cFN was coated onto 96-well plates using BSA-coated wells as a control, and the wells were then used for in vitro BMP1 cleavage assays. For Chordin, in vitro cleavage assays were performed in the presence/absence of soluble fibronectin.
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cFN. As can been seen, processing of probiglycan (Fig. 4A) was markedly increased in cFN-coated wells in a dose-dependent manner. Similarly, procollagen I processing was increased in the presence of cFN to produce increased amounts of processing intermediates, and matureα1(I) chains were compared with a sample in which procollagen was incubated with BMP1 alone (Fig. 4B). This enhancement is shown to be dose-dependent in Fig. 4C. In the same figure (Fig. 4C) use of a different concentration polyacrylamide gel effectively separates pN- and pC-processing intermediates, thereby demonstrating that enhancement is specific to C-propeptide cleavage, which produces pN-α1(I) forms, as expected for specific cleavage by BMP1-like proteinases (13, 32). Finally, as in the probiglycan and procollagen I cleavage assays, in which exposure to solid-phase cFN coated on wells increased proteolytic processing (Figs. 4, A–C), exposure to soluble cFN was observed to increase in vitro processing of Chordin by BMP1 in a seemingly dose-dependent manner (Fig. 4D).

Co-localization of BMP1 and FN in the Extracellular Matrix—
To determine whether BMP1 and FN co-localize in vivo, immunofluorescent staining was performed on cultured human neonatal skin fibroblasts. As can be seen (Fig. 5), the signal for BMP1 co-localized in patches with fibrils of the FN ECM in non-permeabilized cultures. In permeabilized cultures, in addition to the patches of BMP1 signal that co-localize with fibrils of the FN ECM, additional punctate intracellular staining for BMP1 and a more diffuse intracellular staining for FN is seen. Interestingly, these intracellular signals do not co-localize and apparently represent BMP1 and FN in differently sized intracellular vesicles. Thus, there appears to be extracellular, but not intracellular, co-localization of BMP1 and FN in fibroblast cell cultures. Similar results were obtained upon immunofluorescent staining of another type of fibrogenic cell, the MG-63 osteosarcoma cell line (not shown).

Endogenous BMP1-FN Complexes Form in Cell Cultures—
The strength of BMP1-FN interactions, consistent with in vivo binding (Fig. 1), and immunofluorescent staining results showing co-localization of BMP1 and FN in cell cultures (Fig. 5), prompted an attempt to identify endogenous BMP1-FN complexes in cell cultures, as such complexes would provide additional evidence that these two proteins bind in vivo. Toward this end, immunoprecipitations from the conditioned media of MG-63 human osteosarcoma cells were performed with anti-BMP1 antibodies followed by immunoblotting to detect whether FN was co-precipitated. Immunoprecipitations were performed on cells pretreated with TGFβ1, as this growth factor has previously been shown to induce expression of both BMP1 (27) and FN (33). As can be seen (Fig. 6A), immunoprecipitation of BMP1 co-precipitated FN in a specific manner, consistent with the probability that these two proteins form complexes in vivo. Similarly, when the converse experiment was performed, immunoprecipitation of FN from cell culture media was found to specifically co-precipitate BMP1 as well (Fig. 6B), strengthening the likelihood that complexes of the two proteins exist in vivo.

Reduced Processing of BMP1 Substrates in FN-null MEFs—
To directly investigate the likelihood that FN modulates BMP1 activity in vivo, cultures of MEFs differentiated from FN-null (FN−/−) and FN-heterozygous (FN+/−) stem cells were examined for the processing of various endogenous substrates of BMP1. We have previously demonstrated that BMP1 and the closely related proteinase mammalian Tolloid-like 1 (mTLL1) are responsible for the in vivo processing of Chordin (chordi-
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Conditioned medium from cultures of MG-63 cells pretreated 24 h with TGFβ1 was subjected to immunoprecipitation (IP) with anti-BMP1 antibody (lane labeled IP α-BMP1 in panel A), anti-FN antibody (lane labeled IP α-FN in panel B), or IgG (IP IgG-labeled lanes in both panels A and B) followed by immunoblotting (IB) with anti-FN or anti-BMP1 antibody. Input lanes represent 30 μl conditioned media not subjected to immunoprecipitation.

FIGURE 6. Detection of endogenous BMP1-FN complexes in cell culture. Conditioned medium from cultures of MG-63 cells pretreated 24 h with TGFβ1 was subjected to immunoprecipitation (IP) with anti-BMP1 antibody (lane labeled IP α-BMP1 in panel A), anti-FN antibody (lane labeled IP α-FN in panel B), or IgG (IP IgG-labeled lanes in both panels A and B) followed by immunoblotting (IB) with anti-FN or anti-BMP1 antibody. Input lanes represent 30 μl conditioned media not subjected to immunoprecipitation.

FIGURE 5. Immunofluorescent co-localization of BMP1 and FN in fibroblast ECM. Paraformaldehyde-fixed cultures of human neonatal fibroblasts, either permeabilized or not permeabilized with Triton X-100, were immunostained with antibodies to FN (green) and BMP1 (red) and were stained with 4’,6-diamidino-2-phenylindole (DAPI, blue) for localization of nuclear DNA. The yellow signal indicates areas in which green and red signals overlap and, thus, areas of co-localization between FN and BMP1.

nase activity) in MEF cultures (18, 28). In our previous study (28), Chordin was difficult to detect above background in the conditioned media of wild type MEFs, and this was the case for FN+/− MEF cultures in the present study whether we attempted to concentrate Chordin with heparin-Sepharose or concentrate total media protein via ethanol precipitation (Fig. 7A). Thus, MEF FN+/− MEF cultures, like wild type MEF cultures, appear to have a relatively high level of chordinase activity. In contrast, full-length Chordin (∼100 kDa) was readily detected in the conditioned medium of FN−/− MEFs, similar to the previously reported easy detectability of full-length Chordin in the conditioned medium of MEFs doubly null for BMP1 and mTLL1 (28). Thus, FN is shown to be an important factor in determining levels of chordinase activity in MEF cultures.

We next assayed whether type I procollagen processing was affected in FN-null MEF cultures, as BMP1-like proteinases provide the procollagen C-proteinase (pCP) activity that cleaves C-propeptides from the procollagen precursors of the major fibrillar collagen types I-III in vivo (32), as previously demonstrated in MEF cultures (28). Toward this end, FN+/− and FN−/− MEF-conditioned media and cell layers were harvested and examined by immunoblot, employing antibody LF67 (20), which is directed against the C-telopeptide region of the collagen α1(I) chain and which, therefore, recognizes uncleaved procollagen precursor, processing intermediates, and fully processed mature collagen α1(I) chains. In FN+/− MEF cultures, type I collagen in any form was difficult to detect in medium, whereas type I collagen was readily apparent in FN−/− cell layers, predominantly in the form of fully processed, mature α1(I) chains in the cell-associated ECM (Fig. 7B). These results are consistent with a sequence of events in which efficient processing of procollagen to mature type I collagen monomers leads to efficient association of the latter into collagen fibrils and deposition of such fibrils into cell-associated ECM. In contrast to the apparent absence of forms of type I collagen in FN+/− media, type I collagen was readily detectable in FN−/− MEF media, primarily as processing intermediates (Fig. 7B). In FN−/− cell layer samples, in contrast to the large amounts of fully processed α1(I) chains found in FN+/− cell layers, predominant forms were unprocessed procollagen precursor and processing intermediates, with almost a complete absence of fully processed mature α1(I) chains. These results with FN−/− cultures are consistent with a sequence of events in which inefficient processing of procollagen to mature type I collagen monomers means that the latter are unavailable for fibril formation or deposition into ECM. Although processing intermediates pNα1(I) (which retain N-, but not C-propeptides) and pCa1(I) (which retain C-propeptides, but from which N-propeptides have been removed) were not well separated on our gels, the intermediate forms in both FN+/− and FN−/− cell layers are likely to be pNα1(I), as this form is much more efficiently incorporated into fibrils and cell-associated ECM than is pCa1(I) (27, 34). The processing intermediate in the FN−/− medium lane (Fig. 7B) may, thus, be pCa1(I), as it has a slightly
slower electrophoretic mobility than does the processing intermediate in the adjacent FN\(^{+/−}\) cell layer lane and as the pCa1(I) intermediate is somewhat larger than the pNa1(I) form. Persistence of pCa1(I) forms in FN\(^{+/−}\), but not FN\(^{+/−}\) medium would be consistent with the interpretation of a marked reduction in pCP activity in FN\(^{+/−}\) compared with FN\(^{+/−}\) MEF cultures, which would in turn be consistent with a role for FN in enhancing pCP activity in MEF cultures.

Finally, the processing of endogenous biglycan, another known substrate of BMP1-like proteinases (19), was assayed in FN\(^{+/−}\) and FN\(^{+/−}\) MEF cultures, with conditioned media samples first treated with chondroitinase ABC to remove chondroitin sulfate chains from biglycan, thus giving sharper bands on Western blots. As can be seen (Fig. 7C), although a \(\sim 48\)-kDa band of mature biglycan is clearly visible in the culture medium samples of both FN\(^{+/−}\) and FN\(^{+/−}\) MEF cultures, a \(\sim 50\)-kDa biglycan band was clearly detected in FN\(^{+/−}\), but not FN\(^{+/−}\) MEF media samples, thus clearly demonstrating reduced biglycan processing in FN\(^{+/−}\) MEF cultures.

As chordinase, pCP- and biglycan-cleaving activity have all previously been demonstrated to be provided by BMP1-like proteinases in MEF cultures (19, 28), and as the FN\(^{+/−}\) and FN\(^{+/−}\) MEFs examined here have been demonstrated to secrete similar levels of BMP1 (Fig. 7A, and Fogelgren et al. (15)), the data presented here show FN to have a marked enhancing activity on various activities of BMP1-like proteinases in vivo.

**FN Binds Chordin, Supporting a Scaffold Model for FN Enhancement of BMP1 Activity**—Although FN has previously been shown to bind BMP1 substrates biglycan (35) and the major fibrillar collagens (36, 37), it is unknown whether FN might bind Chordin. As FN binding to the latter substrate would support a model in which FN might act as an extracellular scaffold that enhances the activity of BMP1-like proteinases by facilitating their interaction with substrates (see “Discussion”), immunoprecipitations from the conditioned media of MG-63 cells were performed with anti-Chordin antibodies followed by immunoblotting to detect whether FN was co-precipitated. As can be seen (Fig. 8A), immunoprecipitation of Chordin co-precipitated FN in a specific manner, consistent with the probability that these two proteins form complexes in vivo. Similarly, when the converse experiment was performed, immunoprecipitation of FN from cell culture media specifically co-precipitated Chordin as well (Fig. 8B), strengthening the likelihood that complexes of the two proteins exist in vivo.

**DISCUSSION**

Previous observations (15) that proteolytic activation of LOXzymogen to active LOX is reduced in FN\(^{−/−}\) MEFs prompted the current study into possible FN-BMP1 interactions, as proteolytic activation of LOX has been demonstrated to be carried out by BMP1-like proteinases (38, 39). Here we demonstrate direct FN-BMP1 binding in vitro, with binding of BMP1 to multiple FN sites, as BMP1 is capable of binding both the FN N-terminal 70-kDa fragment and the non-overlapping C-terminal portion of FN, with binding to the latter via type III modules I-14. In contrast, FN EDA and V regions are shown to lack major determinants of BMP1 binding. FN is shown to bind
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BMP1 via the latter’s non-protease domains, with which BMP1-like proteinases are known to bind other proteins that modulate their activities (22, 23). This is also consistent with literature showing complement-uegf-BMP1 and epidermal growth factor motifs, which constitute the non-protease domains of BMP1-like proteinases (13), to be involved in protein-protein interaction in various proteins (40). However, additional studies will be needed to further pinpoint the locations of sites involved in interactions between FN- and BMP1-like proteinases.

BMP1 is bound half-maximally to cFN and pFN at concentrations of ~100 nM, within the range of $K_d$ values (30 to 800 nM) previously estimated for binding of FN to integrin receptors (16, 17) and consistent with the possibility of in vivo BMP1-FN interactions. In strong support of the latter possibility was the ready isolation of BMP1-FN complexes from conditioned media of cultured mammalian cells.

In addition to the binding studies described above, the current study presents in vitro data indicating that FN can directly affect the ability of BMP1 to process three of its known substrates, Chordin, biglycan, and procollagen. In strong support of the possibility that FN affects the activities of BMP1-like proteinases in vivo, proteolytic processing of the same three substrates of BMP1-like proteinases is shown to be dramatically decreased in FN−/− MEFs. Thus, FN appears able to directly affect BMP1 activity in vitro and is shown to be an important determinant of the in vivo activity levels of BMP1-like proteinases. It should be noted that the effect of FN on BMP1 activity was more striking in cell cultures than in the in vitro assays. A possible explanation for this observation is that the solid-phase FN bound to wells, and the soluble FN in the in vitro assays are unlikely to perfectly mimic all properties of an in vivo fibrillar FN matrix constructed by cells; one or more such properties lacking in the in vitro assays may be necessary for optimal enhancement of BMP1 activity by FN. Another possible explanation is that one or more additional proteins, available in cell cultures but lacking in the in vitro assays, are necessary for full enhancement of BMP1 activity by FN. Further studies may provide insights into the various components responsible for the striking effect of FN on BMP1 proteolytic activities in vivo.

In addition to the finding of co-immunoprecipitation of endogenous FN and BMP1 from cell cultures, as described above, the possibility that FN may affect BMP1 activity in vivo via direct interaction of the two proteins is also supported by immunofluorescence analysis in the current study, which demonstrates BMP1-FN co-localization in the ECM of cultured fibroblasts. Extracellular BMP1 was found to have a discontinuous, patchy distribution that co-localized with portions of the FN fibrillar matrix. Although not all matrix FN co-localized with BMP1, all BMP1 in the ECM co-localized with FN. Thus, FN binding may represent a major mechanism whereby BMP1-like proteinases are retained in ECM and presented to nascent ECM molecules. Interestingly, the patchy distribution of BMP1 that co-localized with FN is reminiscent of the “punctate” staining reported for LOX co-localized with FN (15). The discontinuous distribution of BMP1 and LOX in the FN matrix calls to mind reports that procollagen, a substrate of BMP1-like proteinases, can be deposited into ECM in a discontinuous manner via discrete specialized protrusions of the fibroblast plasma membrane, designated “fibripositors” (41, 42). BMP1 processing of procollagen and fibrillogenesis both occur in close proximity to fibripositors, co-incident with secretion (41, 42), suggesting the possibility that patchy deposits of BMP1 on the FN matrix may overlie fibripositors or similar specialized secretory structures and represent foci where procollagen, BMP1, LOX, and perhaps other co-secreted proteins interact in a facilitated fashion on the FN matrix. The FN matrix forms close to the cell surface (3, 4, 43), where BMP1-like proteinases are thought to operate (13, 44), and may help co-localize BMP1-like proteinases and their substrates near their points of secretion, where both proteinases and substrates are at relatively high concentrations, thus greatly enhancing the cognate proteolytic reactions. In fact, given the apparent ~100 nM dissociation constants reported here for BMP1-FN binding, high initial pericellular concentrations of BMP1 at points of secretion may help to drive such binding. It has previously been shown that deposition of fibrillar collagens into ECM can be dependent upon the presence of FN (45–48). Thus, enhancement of procollagen processing by BMP1-like proteinases, a rate-limiting step in fibrillogenesis, may be one mechanism by which FN influences collagen fibril formation in vivo.

It has previously been suggested that FN may enhance the proteolytic activation of LOX by acting as an extracellular scaffold that binds both substrate and proteinase, thereby facilitating their interaction (15). Data presented here suggest that FN may operate as an extracellular scaffold to enhance the activities of BMP1-like proteinases against additional substrates as well. In this regard it is relevant that FN has previously been shown to bind two of the substrates examined here, biglycan (35) and the major fibrillar collagens (36, 37). As it was relevant to the scaffold model described above, we ascertained whether FN also binds Chordin and demonstrate in Fig. 8 that endogenous FN and Chordin indeed bind each other in cell cultures.
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Thus, the FN matrix may act as an extracellular scaffold that binds and facilitates interactions of BMP1 with substrates that include LOX, procollagen, biglycan, and Chordin. In regard to this model, it is of interest that a precedent exists in that the protein ONT1 has recently been shown to act as an extracellular scaffold that binds both Chordin and BMP1-like proteinases in the Xenopus embryo, thereby facilitating their interaction (49). The finding in the current report that FN binds BMP1 and Chordin and facilitates their interaction, raises the issue of whether FN, like ONT1, may play some role in stabilizing BMP signaling gradients.

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