The Prodomain of BMP-7 Targets the BMP-7 Complex to the Extracellular Matrix*

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Biochemical and biophysical methods are used to show that BMP-7 is secreted as a stable complex consisting of the processed growth factor dimer noncovalently associated with its two prodomain propeptide chains and that the BMP-7 complex is structurally similar to the small transforming growth factor β (TGFβ) complex. Because the prodomain of TGFβ interacts with latent TGFβ-binding proteins, a family of molecules homologous to the fibrillins, the prodomain of BMP-7 was tested for binding to fibrillin-1 or to LTBP-1. The BMP-7 prodomain and BMP-7 complex, but not the separated growth factor dimer, interact with N-terminal regions of fibrillin-1. This interaction may target the BMP-7 complex to fibrillin microfibrils in the extracellular matrix. Immunolocalization of BMP-7 in tissues like the kidney capsule and skin reveals co-localization with fibrillin. However, BMP-7 immunolocalization in other tissues known to be active sites for BMP-7 signaling is not apparent, suggesting that immunolocalization of BMP-7 in certain tissues represents specific extracellular storage sites. These studies suggest that the prodomains of TGFβ-like growth factors are important for positioning and concentrating growth factors in the extracellular matrix. In addition, they raise the possibility that prodomains of other TGFβ-like growth factors interact with fibrillins and/or LTBP-1 and are also targeted to the extracellular matrix.

BMP-7 (bone morphogenetic protein-7), also known as OP-1 (osteogenic protein-1), is a member of the transforming growth factor β (TGFβ)1 superfamily. Ectopic bone formation can be induced by either purified preparations of authentic BMP-7 from bovine bone (1) or by recombinantly expressed BMP-7 (2). However, the role of BMP-7 in developing tissues is not restricted to bone formation. BMP-7 is widely expressed during development, performing functions in skeletal muscle, nerves, blood vessels, cartilage, and perichondrium (3). Gene targeting experiments in mice have demonstrated that BMP-7 is crucial for normal development of the kidney, eye, and autopod (4, 5).

Members of the TGFβ superfamily of growth factors share amino acid sequence similarities in the C-terminal active growth factor domains of these proteins. Crystal structures of these domains from TGFβ (6) and BMP-7 (7) demonstrated the structural similarities between these growth factors and predicted structural similarities between other superfamily members. However, less is known about the structure and function of the prodomains of TGFβ superfamily members.

The prototype member of the superfamily, TGFβ, is synthesized as a molecule containing a large N-terminal prodomain and a C-terminal growth factor domain. During the secretory process, two growth factor domains fold into a disulfide-linked dimer. Subsequently, proteolytic processing occurs at a furin-type consensus sequence. However, the cleaved propeptides remain noncovalently associated with the growth factor dimer and confer latency to the growth factor (8–10). The secreted complex containing the active growth factor dimer noncovalently associated with its cleaved propeptides is called the small latent complex. Significant structural rearrangements have been shown to occur when the prodomain (called β1-latency associated peptide or β1-LAP) complexes with TGFβ (11, 12). Therefore, latency may result either from LAP blocking the interaction of TGFβ with its receptors or from LAP inducing a conformational change in TGFβ such that it no longer interacts with its receptors (12).

In addition, β1-LAP is disulfide-linked to a much larger binding protein, which does not itself confer latency to TGFβ (13). This large protein, called LTBP-1 (latent TGFβ-binding protein-1), facilitates the secretion of the small latent complex (14), and it targets the small latent complex to the extracellular matrix (15). A specific modular domain with eight cysteine residues (8-Cys domain) in LTBP-1 becomes disulfide-bonded to β1-LAP during the secretory process (16–18). This complex of LTBP-1:β1-LAP:TGFβ is called the large latent complex.

In contrast to the homologies identified between the active growth factor domains, the N-terminal prodomains of TGFβ superfamily members appear to be dissimilar. Some of these regions, like the prodomain of TGFβ, contain cysteine residues, whereas others, like the BMP-7 prodomain, are devoid of cysteines. It is not known whether the BMP-7 prodomain functions like TGFβ-LAP. In the studies reported here, we provide extensive biochemical characterization of the BMP-7 complex, comparing it with TGFβ. Furthermore, we investigate whether the prodomain of BMP-7 interacts with fibrillin-1, a protein highly homologous to LTBP-1, and whether the prodomain targets the BMP-7 complex to extracellular fibrillin microfibrils.

**EXPERIMENTAL PROCEDURES**

Production of Recombinant BMP-7—cDNA encoding human BMP-7 (GenBank™ accession NM_001719) was amplified by reverse tran-
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Expression of Human BMP-7—BMP-7 (375 amino acid residues) was recombinantly expressed in human 293 cells. The expression construct consisted of the BM-40 signal peptide, the growth factor dimer, and the prodomain-growth factor complex, which were separated by endogenous signal peptide cleavage sites.

Recombinant BMP-7-pcDNA3.1(+) vector was transfected into human embryonic kidney 293 cells using calcium phosphate precipitation. After 2 days, the cells were selected by addition of the antibiotic G418 (500 μg/ml) to the culture medium, DMEM, with 10% fetal calf serum. Resistant clones were picked and analyzed for protein expression. The proteins present in 1 ml of serum-free medium were precipitated by addition of 10% trichloroacetic acid, 0.1% Triton X-100 and analyzed using 12.5% SDS-PAGE. Western blotting using monoclonal antibodies specific for the His tag and for the BMP-7 growth factor domain (both from R & D Systems, Minneapolis, MN) was used to identify recombinant BMP-7. Clones expressing substantial amounts of BMP-7 were identified using Coomassie Blue-stained gels. These highly expressing cell lines were then expanded to triple-layer flasks for serum-free medium collection.

Purification—N-terminal histidine-tagged BMP-7 was purified from serum-free medium by chelating chromatography followed by molecular sieve chromatography. Two liters of serum-free media were treated (Centriplus-10, Amicon, Billerica, MA) and applied to a Superose 12 gel filtration column. The intensity of scattered light was measured (Beckman) as recombinant BMP-7 was chromatographed using a Superose 12 gel filtration column. The instrument.

Protein concentrations were determined by amino acid analysis. Samples were gas-phase-hydrolyzed using 300 μl of 6 N HCl, 2% phenol at 110 °C for 22 h, and the hydrolysates were analyzed on a Beckman 6300 amino acid analyzer using sodium citrate buffers. In some cases, concentrations were estimated using the micro BCA assay (Pierce) with bovine serum albumin as a standard.

Determination of Molecular Mass by Laser Light Scattering—To determine precisely the molecular weight of the BMP-7 complex, quasielastic laser light scattering was measured (Dawn Eos, Wyatt Technology Corp.) as recombinant BMP-7 was chromatographed using a Superose 12 gel filtration column. The intensity of scattered light was simultaneously detected at 15 different angles. The molecular weight of the sample was then calculated using the Astra software provided with the instrument.

False-Color Analysis of BMP-7—1 × 10^2 293 cells, stably transfected with BMP-7, were seeded in 100-mm plates and grown for 2 days to reach confluency. After washing with phosphate-buffered saline, 6 ml of 0.2% trimethoxybenzol, 0.1% met cell labeling mixture (ICN Biomedicals, Irvine, CA) was added to each plate. Following 1 h of labeling, the medium was removed (time point = 0); the cell layer was washed with phosphate-buffered saline, and non-radioactive DMEM was added. The medium was harvested at 30 min and 1, 2, 4, 8, and 24 h after labeling, and 1 mm phenylmethylsulfonyl fluoride was added. One ml of medium was trichloroacetic acid precipitated and analyzed by 12.5% SDS-polyacrylamide gels. Gels were fixed, treated with Amplify (Amersham Biosciences) for 30 min, dried, and exposed to autoriadiograph film at ~80 °C.

Rotary Shadowing and Electron Microscopy—Purified BMP-7 complex (100 μg/ml) was dialyzed against 0.2 M NH4HCO3, or 0.1 M acetic acid. The isolated growth factor and propeptide domains (~100 μg/ml each) were dialyzed against 0.2 M NH4HCO3. Each sample was diluted to 70% glycerol and spotted onto freshly cleaved mica, and rotary-shadowed with Pt-C using a Balzers BAE 250 vacuum evaporator. Photomicrographs were taken using a Philips EM410 transmission electron microscope operated at 80 kV. The circumference of the molecules was measured from photomicrographs, enlarged 150,000 times, using a digitizing tablet and quantitated using Bioquant imaging software.

Circular Dichroism Analyses—Purified samples of the BMP-7 prodomain, the growth factor dimer, and the prodomain-growth factor complex (0.5–1.0 μg/ml) were dialyzed extensively against 5 mM HClO4. The concentration of each sample was determined in triplicate by amino acid analysis. Each sample was analyzed in the far-UV range (260–180 nm) at 25°C by a Jasco J-500 spectropolarimeter couple to a computer, using a quartz cell with a path length of 100 μm. Spectra were smoothed, and buffer base lines were subtracted. The secondary structure was estimated using the variable selection method with an initial set of 33 basis spectra. To estimate the secondary structure of the prodomain when it is associated with the growth factor domain, the difference between the normalized spectra of the prodomain/growth factor complex and the growth factor (GF) dimer was obtained, using a calculation that accounted for the different number of amino acids (aa): prodomain = (complex × 412 aa − GF × 139 aa)/273 aa. This difference spectrum of the prodomain in the complex was then solved and compared with the spectrum of the separated propeptide.

Production and Characterization of Monoclonal Antibodies to BMP-7—BMP-7–BMP-7(BALB/cJ mice were immunized with purified BMP-7 complex, and monoclonal antibodies were generated as we described previously (20). Medium from hybridomas was screened by ELISA, using either the separated prodomain or growth factor domain of BMP-7 as the coated substrate (5 μg/ml). Selected hybridomas were cloned by limiting dilution and then expanded and grown in DMEM containing IgG-free fetal calf serum. Antibodies were purified and concentrated by protein-G affinity chromatography. Two monoclonal antibodies, mAb 2 and mAb 6, were generated that were specific for the prodomain, and one, mAb 6, was specific for the growth factor domain.

To test whether mAb 6 cross-reacted with other BMPs, 1 μg/ml recombinant BMP-2, -4, -5, -6, and -7 (R & D Systems) was coated onto ELISA plates, and titered concentrations of mAb 6 were incubated for 2 h at room temperature. Bound antibody was detected using a horse-radish peroxidase-conjugated goat anti-mouse secondary antibody, visualized after addition of substrate (100 μl of 3,3′,5′-tetramethylbenzidine (Sigma) to each well. Absorbance was measured at 450 nm on an Emax microplate reader (Molecular Devices, Sunnyvale, CA).

ELISA Binding Assays—Recombinant fibrillin-1 polypeptides rF11, rF6, rF23, rF31, rF18 and rF45 and recombinant LTBP-1 polypeptides rL1-N, rL1-M, and rL1-C were used as soluble ligands. These polypeptides were chosen because the recombinant BMP-7 complex, separated propeptide chains, or separated growth factor dimers were used to coat ELISA wells. Protein concentrations were determined by amino acid analysis. ELISA wells were coated with BMP-7 peptides or bovine serum albumin at a concentration of 0.1 or 0.2 μg/ml and incubated overnight at 4 °C. The wells were then washed (three times for 5 min) with TBS/Tween 20 (0.25%) and blocked with 5% nonfat dry milk in TBS for at least 2 h. After washing, the soluble ligands of fibrillin-1 or LTBP-1 peptides (titrated in a buffer of 2% milk in TBS) were added at the indicated concentrations and incubated for 3 h at room temperature. Wells were then washed again. Primary antibody was added and incubated for 2 h at room temperature. Mouse monoclonal antibodies (mAb 15, 26, and 201; anti-His (R & D Systems)) were diluted to 5 or 10 μg/ml, and rabbit polyclonal sera (pAb 9543, pAb 39, and pAb 6164) were diluted at a ratio of 1:1000 in buffer. BMP-7 complex, monoclonal antibody (Sigma) were added at a dilution of 1:1000 and incubated for 1 h at room temperature. After final washes, the peroxidase substrate, tetramethylbenzidine, was added, and absorbance was read at 450 nm on an Emmax microplate reader.

Immunofluorescence Microscopy—Immunofluorescence microscopy was performed as described (23). For double labeling experiments, mAb 6 or mAb 2 (1:100 dilutions of a 1 mg/ml solution) was incubated first on sections for 3 h at room temperature, followed by a 2-h incubation of pAb 9543 or pAb 8162. Secondary antibodies (Alexa488 goat anti-mouse and Alexa546 goat anti-rabbit, Molecular Probes, Eugene, OR) were diluted 1:250 and applied together for 1 h at room temperature. Sections were coverslipped with ProLong Gold anti-fade mounting medium (Alexa) and viewed using a Zeiss Axiostar 200M microscope with Axiovision software.

RESULTS

Recombinant Expression of Human BMP-7—Full-length human BMP-7 was recombinantly expressed in human 293 cells. The expression construct consisted of the BM-40 signal peptide, a 6-histidine tag, and the coding sequence of BMP-7 3′ to the predicted signal cleavage site (Fig. 1A). Recombinant BMP-7 was secreted into the medium, and high expression of the recombinant protein was demonstrated by SDS-PAGE and Coomassie Blue staining of serum-free medium proteins (Fig. 27971
BMP-7 propeptide (dithiothreitol (DTT)) construct is given reducing agent (20 mM dithiothreitol). Positions of molecular weight markers are shown. Following transfection, major bands were found that were consistent with the calculated sizes of the processed propeptide (15,681 Da). The presence (+) or absence (−) of dithiothreitol (DTT) is indicated. Contaminants of the bound and eluted fractions were then removed by Superose 12 gel filtration chromatography. Concentrated serum-free medium from BMP-7 transfected 293 cells was applied to a cobalt chelating column, and bound proteins were eluted. SDS-PAGE (12.5% acrylamide), followed by Coomassie Brilliant Blue staining, showed proteins present in the starting material (lanes 1 and 4), proteins in the fraction that flowed through the column (lanes 2 and 5), and bound proteins (lanes 3 and 6). The presence (+) or absence (−) of dithiothreitol (DTT) is indicated. Contaminants of the bound and eluted fractions were then removed by Superose 12 gel filtration chromatography. BMP-7 propeptide (+) and growth factor dimer (●) eluted together from the chelating column (lanes 3 and 6) and sieved together through the Superose 12 column (lane 7). The position of the reduced growth factor monomer is indicated (●).

FIG. 1. Construction and purification of recombinant human BMP-7. A, schematic representation of the recombinantly expressed BMP-7 molecule: unprocessed monomer (■), processed N-terminal propeptide (+), processed growth factor dimer (●), and monomer (○). The propeptide (gray) contains a His<sub>6</sub> tag prior to the predicted N terminus of BMP-7 and following the BM-40 signal peptide. The sequence of the recombinant construct is given below the schematic drawings. The sequence APLA is contributed by BM-40, after the BM-40 signal peptide is cleaved. The furin-type consensus cleavage site (RSIR) is underlined in the sequence and indicated in the drawings. The vertical line represents the interchain disulfide bond in the processed growth factor dimer (white). B, SDS-PAGE analysis (12.5% acrylamide) of serum-free medium from untransfected and BMP-7 transfected 293 cells. Proteins were stained with Coomassie Brilliant Blue. Samples were run with (+) or without (−) disulfide-bond reducing agent (20 mM dithiothreitol). Positions of molecular weight markers are shown. Following transfection, major bands were found that were consistent with the calculated sizes of the processed propeptide (+) (31,641 Da), the processed growth factor dimer (●) (31,362 Da), and the reduced growth factor monomer (○) (15,681 Da). C, purification of BMP-7 using chelating chromatography and molecular sieve chromatography. Concentrated serum-free medium from BMP-7 transfected 293 cells was applied to a cobalt chelating column, and bound proteins were eluted. SDS-PAGE (12.5% acrylamide), followed by Coomassie Brilliant Blue staining, showed proteins present in the starting material (lanes 1 and 4), proteins in the fraction that flowed through the column (lanes 2 and 5), and bound proteins (lanes 3 and 6). The presence (+) or absence (−) of dithiothreitol (DTT) is indicated. Contaminants of the bound and eluted fractions were then removed by Superose 12 gel filtration chromatography. BMP-7 propeptide (+) and growth factor dimer (●) eluted together from the chelating column (lanes 3 and 6) and sieved together through the Superose 12 column (lane 7). The position of the reduced growth factor monomer is indicated (●).

1B). Bands were clearly present in the medium of BMP-7 transfected cells that corresponded to the predicted electrophoretic mobilities of the prodomain (~31,600 Da in samples both with and without disulfide bond reducing agent) and the processed growth factor dimer (~31,400 Da without reducing agent; ~15,700 Da with reducing agent). N-terminal sequencing of the growth factor band confirmed that processing had occurred at the R<sub>XX</sub>Y consensus cleavage site. Potent growth factor activity of recombinant BMP-7 was confirmed using a cell differentiation assay (induction of alkaline phosphatase in ATDC5 cells) (24) (data not shown).

**Purification and Stability of the BMP-7 Complex—6-Histidine-tagged recombinant BMP-7 was purified from serum-free medium by cobalt chelating chromatography. Aliquots of the starting sample, flow-through, and elution fractions were analyzed on 12.5% SDS-PAGE after Coomassie Blue staining. As expected, histidine-tagged propeptides bound to the chelating column and were eluted. Co-elution of the untagged processed growth factor dimer with the tagged propeptide suggested a noncovalent association of prodomain and processed growth factor. Molecular sieve chromatography was used to purify further the recombinant BMP-7. Representative fractions from these purification steps are shown in Fig. 1C. Free propeptide chains or free growth factor molecules were not detected by Western blotting at any stage during the purification. Approximately 0.5 mg of BMP-7 complex was purified from 1 liter of serum-free medium.

To determine the molecular mass of the purified recombinant BMP-7, molecular sieve chromatography in combination with laser light scattering was used (Fig. 2). The mass of the eluting BMP-7 was 95,000 Da (±5,000 Da). The predicted molecular mass of a complex composed of two propeptides and a growth factor dimer is ~94,600 Da. There are four sites of potential N-linked glycosylation, and one of these (N372) has been shown to be glycosylated, contributing between 3,000 and 9,000 Da to the total molecular mass of the processed growth factor dimer (2).

In addition, equilibrium ultracentrifugation was used to determine the molecular weight of the recombinant BMP-7. Results yielded an average molecular mass of 89,500 Da ± 5,000 Da (data not shown). Based on co-elution during chelating chromatography and calculation of molecular mass by laser light scattering and by equilibrium ultracentrifugation, we concluded that recombinant BMP-7 forms a complex consisting of two propeptides noncovalently associated with a processed growth factor dimer.
A pulse-chase assay was performed in order to investigate the stability of the recombinant BMP-7 complex (Fig. 3). BMP-7 transfected cells were metabolically labeled with [35S]cysteine-methionine for 1 h and then chased for periods of time up to 24 h with non-radioactive medium. The BMP-7 growth factor domain contains 7 cysteines and 3 methionines, whereas the prodomain contains 7 methionines and no cysteine residues. By 2 h of chase, pulse-labeled BMP-7 was fully secreted. The apparent total amounts and relative ratio of prodomain to growth factor domain between 2 and 24 h of chase remained constant, indicating that the BMP-7 complex is stable over this time period. A band corresponding to the full-length unprocessed monomer was maximally secreted by 2 h and then was either degraded or processed extracellularly.

**Dissociation of the BMP-7 Complex—**Complete dissociation of the BMP-7 complex was accomplished using phosphate buffer containing 1 M NaCl, 8 M urea, and 20 mM octylglucopyranoside (Fig. 4). When BMP-7 complex was applied to a chelating column in this buffer, the processed growth factor dimer was present in the unbound flow-through fractions (Fig. 4, *lanes 2 and 5*), whereas the histidine-tagged propeptide chains were bound to the column (*lane 3*). Unprocessed BMP-7 monomers containing the histidine tag were also bound to the column (Fig. 4, *lane 6*). When either 8 M urea in chelating column buffer or 20 mM octylglucopyranoside in column buffer was used separately, small amounts of growth factor were still detected in the bound material by Western blotting (Fig. 4, *lanes 7 and 8*). Approximately 200 ng of separated propeptide chains and 100 ng of processed growth factor dimer were retained from 1 liter of serum-free medium.

**Treatment with acidic pH also dissociated the BMP-7 complex.** Samples of purified BMP-7 were dialyzed into 0.2 M NH4HCO3, pH 8.0, or 0.1 M acetic acid, pH 2.5, and then analyzed by rotary shadowing electron microscopy. In ammonium bicarbonate, the BMP-7 complex was visualized as a globular structure with a mean circumference of ~46 nm (Fig. 5A). The BMP-7 complex treated with acetic acid revealed globules with a significantly smaller mean circumference of ~37 nm (Fig. 5B). In addition, the distribution of measurements of the complex treated with acid was more heterogeneous.
ous (Fig. 5B) than the distribution of measurements of the complex in ammonium bicarbonate (Fig. 5A). Further comparison of the distribution of measurements of the complex treated with acid with measurements obtained from rotary-shadowed images of the separated growth factor dimer (mean circumference $= 28$ nm, Fig. 5C) and separated propeptide chains (mean circumference $= 34$ nm, Fig. 5D) suggested that in acetic acid the BMP-7 complex was dissociated into its constituent components. However, these results cannot exclude a conformational change of an associated complex in acid.

The distributions of measurements of the rotary-shadowed BMP-7 complex (Fig. 5A) and of the separated growth factor (Fig. 5C) were predominantly unimodal. In contrast, the rotary-shadowing data for the separated propeptide chains revealed a more heterogeneous distribution of sizes (Fig. 5D), suggesting that the dissociation of the propeptide chains from the growth factor dimer results in conformational changes in the propeptide. Therefore, secondary structures of the BMP-7 complex, the separated propeptide chains, and the separated growth factor dimer were determined by far-UV CD spectroscopy (Table I).

The propeptide contained 21% $\alpha$-helix, 31% $\beta$-sheet, 20% $\beta$-turn, and 28% other structure. The secondary structure of the propeptide domain as a component of the complex was also determined theoretically by subtracting the normalized spectra for the growth factor from that of the complex (Fig. 6B). The difference spectra showed an increase in the amount of $\beta$-sheet (from 31 to 35%) and a decrease in $\beta$-turn structure (from 20 to 17%) (Table I). All together, these data suggest that the propeptide chain undergoes a conformational change when the BMP-7 complex is dissociated.

The BMP-7 Prodomain Targets BMP-7 to Fibrillin Microfibrils—In order to determine whether the BMP-7 complex is targeted to the extracellular matrix through interactions between the prodomain and extracellular matrix components, we tested whether BMP-7 binds to fibrillins and/or LTBP by using ELISA binding assays. Recombinant fibrillin-1 and LTBP-1 polypeptides used in these assays are depicted in Fig. 7A. A specific region of fibrillin-1, contained in the recombinant N-terminal half of fibrillin-1 (rF11), was found to bind to the BMP-7 complex and to the separated propeptide chains but not to the separated growth factor dimer (Fig. 7B). In these assays, the recombinant C-terminal half of fibrillin-1 (rF6) as well as smaller recombinant peptides like rF18 did not bind to BMP-7 (Fig. 7C and data not shown). When subdomains of rF11 were tested, rF23 and rF18 interacted with BMP-7 propeptide chains but not with the same high affinity displayed by rF11 (Fig. 7C). LTBP-1 recombinant peptides spanning the whole molecule were tested, and these did not bind to BMP-7 with high affinity (Fig. 7D).

Next, we investigated whether the BMP-7 complex is tar-
spectra shown were smoothed, and the base lines were subtracted.

analyzed by CD spectroscopy in the far-UV range (260 to 180 nm). The

the separated propeptide chains (Fig. 8A). mAb 2 and mAb 33 reacted specifically with propeptide chains and with the uncleaved precursor molecules (Fig. 8B). mAb 6 reacted specifically with the growth factor dimer and monomer and with uncleaved precursor molecules (Fig. 8B).

Unprocessed BMP-7 monomers (with a predicted molecular mass of ~47,300 Da) could be visualized by immunoblotting, migrating with an estimated molecular mass of ~55 kDa. These constituted only a small percentage of the total recombinant protein and were not visible by Coomassie Blue staining of purified samples (see Fig. 1B). mAb 6 was also tested for reactivity with other BMPs. No cross-reactivity was detected in ELISA with BMP-2 or BMP-4 (Fig. 8C) or with BMP-5 or BMP-6 (Fig. 8D).

In human tissues, both the BMP-7 prodomain and the growth factor domain were detected on matrix structures that resembled fibrillin microfibrils. Although tissue distribution patterns of fibrillin microfibrils were ubiquitous and abundant, BMP-7 distribution was less ubiquitous and less abundant. However, antibodies to BMP-7 demonstrated fibrillar patterns similar to the signature fibrillar pattern of immunolocalization displayed by fibrillin. Representative immunolocalization results are shown in Fig. 9 and Fig. 10. In the 20-week fetal kidney capsule and subcapsular connective tissue, fibrillin fibrils are abundant and fibrillar in appearance (Fig. 9A). Both the prodomain (Fig. 9C) and growth factor domain (Fig. 9E) were localized to fibrils in this area of the kidney. In addition, in the connective tissue between the adrenal gland and the kidney, fibrillin (Fig. 9B) as well as BMP-7 prodomain (Fig. 9D) and growth factor domain (Fig. 9F) immunolocalization patterns were similarly fibrillar in appearance.

Double labeling experiments demonstrated that the BMP-7 growth factor domain Fig. 10A) and prodomain (Fig. 10D) were present on fibrillin-1 fibrils (Fig. 10, B and E) in fetal human skin. In Fig. 10, arrows in A–C point to examples of fibrils that were stained with mAb 6, specific for BMP-7 growth factor domain, and with pAb 9543, specific for fibrillin-1. Similarly, in Fig. 10, arrows in D–F point to examples of fibrils that were stained with mAb 2, specific for BMP-7 prodomain, and with pAb 8162, specific for fibrillin-1.

**DISCUSSION**

Whether or not all members of the TGFβ superfamily are secreted as stable complexes of prodomains noncovalently associated with processed growth factor dimers has not been established. Opposite conclusions were reached in two previously published studies, one on BMP-7 (25) and one on BMP-2 (26). A soluble recombinant BMP-7 complex was described based upon co-immunoprecipitation of propeptides with the growth factor dimer and gel filtration chromatography (25). However, a similar study concluded that the majority of recombinant BMP-2 mature growth factor was not associated with its propeptides, which appeared to be much more abundant in the medium of the transfected cells than the mature growth factor dimer (26). Studies of recombinant myostatin (growth and differentiation factor-8) indicated the noncovalent association of propeptides with the processed growth factor dimer (27).

Our study of recombinant BMP-7 confirms and extends the previously published report (25). Similar to published co-immunoprecipitation data indicating noncovalent association of propeptides and growth factor dimers, we found co-elution of growth factor with histidine-tagged propeptide chains after chelating chromatography. We extended these findings, using laser light scattering as well as equilibrium sedimentation, and we demonstrated that the complex is composed of two prodomain chains and one growth factor dimer. In contrast to the previous report, we showed that the recombinant complex was stable over a 24-h pulse-chase experiment. Consistent with

**TABLE I**

Percent secondary structure of BMP-7 complex, propeptide, and growth factor dimer

| Sample name    | α-Helix | β-Sheet | β-Turn | Other |
|----------------|---------|---------|--------|-------|
| BMP7 complex   | 18      | 31      | 19     | 31    |
| Propeptide     | 21      | 31      | 20     | 28    |
| Propeptide (calculated) | 20      | 35      | 17     | 29    |
| Growth factor  | 12      | 29      | 22     | 37    |
| Growth factor* | 14      | 31      |        |       |

* Data were recalculated from the crystal structure for 139 residues, Protein Data Bank code 1bmp (7).

geted to fibrillin microfibrils in vivo. In order to perform these investigations, monoclonal antibodies were generated, using the purified BMP-7 complex as immunogen. Cloned hybridomas were selected that recognized either the BMP-7 prodomain (mAb 2 and mAb 33) or the BMP-7 growth factor domain (mAb 6). Immunoblotting demonstrated that each monoclonal antibody reacts only with molecules containing either the prodomain or the growth factor domain and with none of the other multiple proteins contained in the medium of the transfected 293 cells (shown in the Coomassie Blue-stained lanes in Fig.

**FIG. 6. Secondary structure of BMP-7 measured by CD spectroscopy.** A, the BMP-7 complex (blue line), separated growth factor dimer (green line), and separated propeptide chains (red line) were analyzed by CD spectroscopy in the far-UV range (260 to 180 nm). The spectra shown were smoothed, and the base lines were subtracted. Δε = ε -1 cm. B, the structure of the propeptide within the complex was determined by subtracting the normalized spectra of the growth factor from that of the BMP-7 complex. The resultant spectra were calculated (black line) and are shown in comparison with the measured spectra of the separated propeptide chains (red line).
FIG. 7. Binding of recombinant fibrillin-1 to BMP-7. A, drawings of recombinant fibrillin-1 and LTBP-1 polypeptides used in these assays. B, high affinity binding was found between rF11 (soluble ligand) and the BMP-7 complex (black) and separated propeptide chains (red) as coated substrates. No binding was detected when rF11 was tested with the separated BMP-7 growth factor domain (dark blue) or with bovine serum albumin (light blue) as the coated substrate. C, various fibrillin-1 recombinant polypeptides were used as soluble ligands with BMP-7 complex as the coated substrate. rF11, rF23, rF31, rF45, and rF18 were each detected with pAb 9543. rF6 was detected by pAb 8162. D, recombinant LTBP-1 polypeptides were tested as soluble ligands with BMP-7 propeptide chains as the coated substrate. rF11 binding was also determined at the same time.

BMP-7 Prodomain Targets the Complex to Fibrillin
other previous results (2), we found that the complex is fully
dissociated with 8 M urea in buffer containing 20 mM octylglu-
copyranoside. In addition, we showed that acidic pH either
dissociates the BMP-7 complex or subjects it to gross confor-
mational changes. Refolding experiments of growth factor do-
mains of other TGFβ superfamily members have demonstrated
the need for harsh denaturants and aggregation suppressors
(28) and have suggested that a principal function of the prodo-
main may be to solubilize the growth factor dimer by shielding
the pronounced hydrophobic surfaces present on the mature

Fig. 8. Characterization of BMP-7 mAbs. A, Coomassie Blue-stained SDS-PAGE of medium proteins from BMP-7 transfected 293 cells
(rhBMP-7). Samples were run with (+) or without (−) disulfide bond reducing agent. Positions of molecular weight markers are designated. B,
immunoblotting of medium proteins from BMP-7 transfected 293 cells. Medium samples shown in A (rhBMP-7) were separated by SDS-PAGE with
(+) or without (−) disulfide bond reducing agent and transferred to nitrocellulose paper. Anti-histidine (anti-HIS), mAb 2, and mAb 33 immunoblot
the prodomain of BMP-7 (+). mAb 6 immunoblots the processed growth factor dimer (▲) and monomer (●). Small amounts of unprocessed monomer
(■) are also recognized by mAb 2, mAb 33, and mAb 6. C, ELISA demonstrating specificity of mAb 6. BMP-2, BMP-4, and BMP-7 were used to coat
wells, and dilutions of mAb 6 were tested. D, ELISA demonstrating specificity of mAb 6. BMP-5, BMP-6, and BMP-7 were used to coat wells, and
dilutions of mAb 6 were tested.
growth factor (29). Our preparations of recombinant BMP-7 complex were more soluble than the separated mature growth factor. However, we were not able to maintain concentrations of the BMP-7 complex greater than 250 µg/ml (2.6 µM) in physiological buffers, indicating that the solubility of the complex is not high. Comparison of the secondary structures of the separated propeptide and growth factor components and of the complex suggested that dissociation of the complex resulted in structural changes in the propeptides. Taken all together, these biochemical studies clearly show that the BMP-7 prodomain contributes to the structure of the BMP-7 complex and therefore to its biochemical properties like solubility and stability, and the data demonstrate that the BMP-7 complex is structurally similar to the small latent complex of TGFβ.

Further investigations were performed to compare the function of the prodomain of BMP-7 with the prodomain of TGFβ. In contrast to TGFβ LAP, the prodomain of BMP-7 does not contain any cysteine residues. Therefore, the prodomain of BMP-7 cannot be disulfide-linked to a larger binding protein, as is the case for TGFβ LAP. Nevertheless, we found that the BMP-7 prodomain binds to fibrillin-1, an extracellular matrix architectural macromolecule similar in structure to the TGFβ-binding protein, LTBP-1.

Fibrillin-1 is an extracellular matrix macromolecule that is the major component of connective tissue architectural elements called microfibrils (20). In the human genome, there are
three fibrillins (30) and four LTBPs (18) that share similar modular structures composed primarily of calcium-binding epidermal growth factor like repeats and of signature repeats with 8 cysteine residues, which we called 8-Cys repeats when we first sequenced fibrillin-1 (31). Because the function of certain 8-Cys domains in LTBPs is to interact with LAP, we speculate that certain other 8-Cys domains, present in the fibrillins as well as in the LTBPs, may interact with the proregions of other members of the TGFβ superfamily. However, data generated using fibrillin-1 recombinant polypeptides (Fig. 7C) indicate that the interaction between fibrillin-1 and the prodomain of BMP-7 may not be fully attributed to a single 8-Cys domain.

Instead, our current data suggest that this interaction requires regions that flank 8-Cys-1, because rF23 binds but rF31 does not bind, and may also require the contribution of 8-Cys-3 and flanking regions in rF18. Furthermore, the tissue form of fibrillin-1 is the complex polymeric aggregate called the microfibril, and this form of fibrillin-1 may be the more relevant substrate for testing binding interactions with BMP-7 than recombinant monomeric peptides of fibrillin-1. Ongoing studies are addressing these issues.

To test whether the prodomain targets the BMP-7 complex to the extracellular matrix, and specifically to fibrillin microfibrils, we generated monoclonal antibodies that recognize either the prodomain or the processed growth factor domain, and we performed immunohistochemical analyses of tissues. We found that both the prodomain and the growth factor domain of BMP-7 are present in certain tissues and that immunolocalization of these domains yielded extracellular fibrillar patterns similar to fibrillin staining patterns. Double labeling demonstrated co-localization of BMP-7 and fibrillin-1. These results suggest that an important function of the prodomain of BMP-7 may be to specifically target the growth factor complex to fibrillins in the extracellular matrix.

Mice lacking BMP-7 demonstrate that BMP-7 is required during the later but not early stages of kidney development (4, 5). BMP-7 is expressed in the mesenchyme and epithelium of the developing kidney (32). However, our immunolocalization studies failed to identify BMP-7 in the kidney cortex, except for faint and probably intracellular staining (data not shown). Instead, clear immunolocalization of BMP-7 was found in the kidney capsule and in adjacent adrenal connective tissues. Similarly, although BMP-7 is expressed in the interdigital space of the developing autopod, BMP-7 immunolocalization in the interdigital space was not detected (data not shown). Fibrillins are present in the developing kidney (20) and in the interdigital space of the developing autopod (33). In the dersms at all stages of chick development (33), BMP-7 was easily detected and co-localized with fibrillin microfibrils. We interpret these data to suggest that immunolocalization reveals BMP-7 when it is stored in the extracellular matrix, but immunolocalization fails to detect BMP-7 in tissues where BMP-7 is actively utilized.

Is fibrillin required for BMP activity? Complete loss of fibrillin-2 in mice resulted in a defect in limb patterning (syndactyly), and mice doubly heterozygous for loss of BMP-7, even in those tissues where BMP-7 is expressed in the interdigital space of the developing autopod, whereas fibrillin-2 is well expressed (33), indicating that fibrillin-1 cannot compensate in this tissue in fibrillin-2 null mice. According to this reasoning, it is likely that the prodomain of BMP-7 binds to both fibrillins. Ongoing analyses of fibrillin-1 null mice demonstrate loss of BMP-7 immunolocalization in mutant mice compared with wild-type mice, but only in postnatal tissues when fibrillin-2 is no longer available (data not shown). These studies indicate that fibrillin-2 also binds to BMP-7. We are currently investigating whether loss of BMP-7 immunolocalization in postnatal tissues in fibrillin-1 null mice is associated with specific pathologies. Because mutations in fibrillin-1 result in the multiple manifestations of the Marfan syndrome, a heritable disorder of connective tissue affecting the skeleton, the eye, and the cardiovascular system (35), and because fibrillin-1 mutant mice demonstrate similar cardiovascular phenotypes (36), these analyses of fibrillin-1 null mice may suggest which tissue pathologies are because of dysregulation of BMP-7 signaling.

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