Bone Morphogenetic Protein-1 Processes Insulin-like Growth Factor-binding Protein 3*

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The bone morphogenetic protein-1 (BMP1)-like metalloproteinases play key roles in extracellular matrix formation, by converting precursors into mature functional proteins involved in forming the extracellular matrix. The BMP1-like proteinases also play roles in activating growth factors, such as BMP2/4, myostatin, growth differentiation factor 11, and transforming growth factor β1, by cleaving extracellular antagonists. The extracellular insulin-like growth factor-binding proteins (IGFBPs) are involved in regulating the effects of insulin-like growth factors (IGFs) on growth, development, and metabolism. Of the six IGFBPs, IGFBP3 has the greatest interaction with the large pool of circulating IGFs. It is also produced locally in tissues and is itself regulated by proteolytic processing. Here, we show that BMP1 cleaves human and mouse IGFBP3 at a single conserved site, resulting in markedly reduced ability of cleaved IGFBP3 to bind IGF-I or to block IGF-I-induced cell signaling. In contrast, such cleavage is shown to result in enhanced IGF-I-independent ability of cleaved IGFBP3 to block FGF-induced proliferation and to induce Smad phosphorylation. Consistent with in vivo roles for such cleavage, it is shown that, whereas wild type mouse embryo fibroblasts (MEFs) produce cleaved IGFBP3, MEFs doubly null for the Bmp1 gene and for the Tlli gene, which encodes the related metalloproteinase mammalian Tolloid-like 1 (mTll1), produce only unprocessed IGFBP3, thus demonstrating endogenous BMP1-related proteinases to be responsible for IGFBP3-processing activity in MEFs. Similarly, in zebrafish embryos, overexpression of Bmp1a is shown to reverse an Igfp3-induced phenotype, consistent with the ability of BMP1-like proteinases to cleave IGFBP3 in vivo.

Bone morphogenetic protein-1 (BMP1)-like proteinases affect morphogenetic events via biosynthetic processing of precursors into mature functional proteins involved in forming the extracellular matrix. Extracellular matrix-related substrates include precursors for collagens I–III, V, VII, and XI; the cross-linking enzyme lysyl oxidase; proteoglycans biglycan and osteoglycin; and basement membrane component laminin-332 (laminin-5) (1). These proteinases are also involved in the biosynthetic processing that produces peptides involved in initiating the mineralization of bones and teeth and in processing the hormone prolactin and the proteoglycan perlecan to produce anti-angiogenic factors (1, 2). In addition, BMP1-like proteinases are involved in activating extracellular latent complexes of some transforming growth factor β (TGFβ) superfamily members, including BMP2, BMP4, growth and differentiation factor 8 (GDF8) (also known as myostatin), GDF11, and TGFβ1 (1, 3).

Insulin-like growth factors, IGF-I and -II, are evolutionarily conserved polypeptide growth factors with important effects on mitogenesis, development, somatic growth, and metabolism (4). In extracellular spaces and in biological fluids, IGFs are normally bound to a family of IGF-binding proteins (IGFBPs), which modulate their function. The six IGFBPs have a shared protein domain structure, consisting of NH2- and COOH-terminal cysteine-rich domains, each with conserved cysteine distribution and some amino acid homology with corresponding domains in other IGFBP family members, separated by linker regions of little sequence similarity between the different IGFBPs (5). IGFBPs have high affinity for binding IGF-I and -II and can modulate bioavailability of IGFs by acting as carriers that prolong their half-life and by inhibiting IGF-stimulated cellular events by sequestering IGFs away from the type I IGF cell surface receptor (5, 6). The functions of IGFBPs can, in turn, be modulated by well documented proteolytic cleavages that occur in the linker regions between NH2- and COOH-terminal cysteine-rich domains, producing cleavage fragments with reduced affinities for binding IGFs (5, 6). IGFBPs also have effects on cellular motility, adhesion, and growth that are independent of their interactions with IGFs and may also be modulated by proteolytic cleavage of IGFBPs into fragments (6).

The proteolytic processing of IGFBPs to release IGFs is reminiscent of the processing of the extracellular antagonists Chordin and Chordin-like 1 by BMP1-like proteinases to release BMP2 and -4 from latent complexes (1, 7). In each case, IGFBPs, Chordin, and Chordin-like are cleaved in linker regions between cysteine-rich domains, thereby freeing ligands to bind cognate cell surface receptors. These similarities, together with the roles of BMP1-like proteinases in activating a growing number of growth factors and the apparent role of proteolytic cleavage in modulating functions of IGFBPs, prompted the current study, designed to determine whether

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2 The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; mTll1, mammalian Tolloid-like 1; mTLD, mammalian Tolloid; MEF, mouse embryo fibroblast; MMP, matrix metalloproteinase; FN, fibronection; NEM, N-ethylmaleimide; PABA, p-aminobenzamidine; SBTI, soybean trypsin inhibitor; NHS-DIG, digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-N-hydroxysuccinimide ester; hpf, hour(s) postfertilization; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldetrazolium bromide.
IGFBPs might be substrates for BMP1-like proteinases. Here we demonstrate that BMP1 cleaves IGFBP3, the most abundant circulating IGFBP and an IGFBP for which much evidence exists of functionally relevant in vivo proteolytic cleavage (6). Human and murine IGFBP3 are shown to be cleaved by BMP1 in vitro, at a single conserved site. This cleavage is shown to negatively impact the ability of IGFBP3 to inhibit IGF-1-induced cell signaling but to enhance some IGF-independent IGFBP3 functions. Wild type mouse embryo fibroblast (MEF) cultures are shown to produce proteolytic fragments of endogenous IGFBP3 identical in size to fragments produced in vitro via cleavage of recombinant IGFBP3 by BMP1, and production of these fragments in such cultures is shown to be inhibited by a metalloproteinase inhibitor that is highly effective against BMP1-like proteinases but ineffective against MMPs. Moreover, these cleavage products are absent from MEFs deficient for BMP1 and for related BMP1-like proteinases mammalian Tolloid (mTLD) and mammalian Tolloid-like 1 (mTLL1).

Thus, BMP1-like proteinases are shown to play a role in IGFBP3 cleavage by at least some cell types. A demonstrated ability of zebrafish Bmp1a to reverse an Igfbp3-induced phenotype in zebrafish embryos is also consistent with ability of zebrafish Bmp1a to reverse an Igfbp3-induced phenotype in zebrafish embryos (14). Furthermore, BMP1-like proteinases but ineffective against MMPs. More-
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Western Ligand Blotting Assay—Western ligand blotting was performed essentially as described by Shimizu et al. (14). To label IGF-I with digoxigenin, 2 μl of NHS-DIG (50 mg/ml in DMSO) was mixed with 250 μl recombinant human IGF-I (200 μg/ml in 0.05 M NaHCO₃) at a molar ratio of 23:1. After incubating for 2 h at room temperature, the mixture was applied to a Sephadex G-25 column (GE Healthcare) to remove non-reacted NHS-DIG. Digoxigenin-labeled IGF-I was stored at 4 °C until use. For ligand-binding Western blotting, 125 ng of recombinant human IGFBP3 was incubated with or without 60 ng of BMP1 in 5 μg/ml dextran sulfate for 6 h at 37 °C. After SDS-PAGE on a 12% acrylamide gel, samples were transferred to a nitrocellulose membrane, which was blocked with 5% nonfat dry milk for 1 h at room temperature. After overnight incubation at 4 °C with goat antibody to human IGFBP3 (1:2500; Roche Applied Science), the membrane was visualized using the ECL Plus kit (GE Healthcare).

Growth Inhibition Assay—MCF-7 cells were plated on 24-well plates (2 × 10⁴/well) and cultured for 48 h. Cells were rinsed with PBS prior to culturing for 2 h in serum-free DMEM, rinsed again, and then cultured another 2 h in serum-free DMEM. After replacing with a fresh 250 μl of serum-free medium, cells were cultured 48 h with recombinant human IGF-I (10 ng/ml) or recombinant human basic FGF (10 ng/ml; R&D Systems) in the presence or absence of intact or BMP1-cleaved recombinant human IGFBP3. For IGF-I inhibition experiments, 250, 125, or 62.5 nm IGFBP3 was incubated with/without 125, 62.5, or 31.25 ng/ml BMP1, respectively, prior to the addition to media. For basic FGF inhibition experiments, 250, 125, or 62.5 ng/ml IGFBP3 was incubated with/without 250, 125, or 62.5 ng/ml BMP1, respectively, prior to the addition to media. Cleavage reactions were 6 h at 37 °C in 50 mM Tris buffer (pH 7.5) with 150 mM NaCl, 5 mM CaCl₂, 5 μg/ml dextran sulfate, 1 mM PABA, 1 mM NEM, 0.2 mM PMSF, and 25 μg/ml SBTL. For each set of proliferation experiments, a culture in which neither IGFBP3 nor BMP1 was added was used as a control to determine the baseline of MCF-7 proliferation. Cell proliferation was measured by a colorimetric assay involving mitochondrial dehydrogenase reduction of tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT). For the assay, MTT reagent (25 μl; ATCC) was added to each well and incubated for 2 h at 37 °C, followed by the addition of detergent reagent (250 μl; ATCC) and further incubation for 2 h at room temperature. MTT was spectrophotometrically measured at 570 nm.

Smad Signaling—MCF-7 Cells were seeded on 6-well plates. At 80–90% confluence, cells were washed with PBS before incubating for 2 h in serum-free DMEM. After replacing with fresh serum-free medium, cells were treated with or without intact or BMP1-cleaved recombinant human IGFBP3 for 30 min. For this, IGFBP3 (125 or 250 ng) was incubated with or without BMP1 (62.5 or 125 ng), in a final reaction volume of 30 μl, for 6 h at 37 °C in 50 mM Tris buffer (pH 7.5) with 150 mM NaCl, 5 mM CaCl₂, 5 μg/ml dextran sulfate, 1 mM PABA, 1 mM NEM, 0.2 mM PMSF, and 25 μg/ml SBTL. Reactions were then diluted to 125 or 250 ng/ml intact or cleaved IGBP3 in serum-free medium, and 250-μl aliquots were distributed into each well of a 6-well plate. Treated cells were washed twice with ice-cold PBS and scraped into and allowed to lyse in 400 μl of 50 mM Tris (pH 7.5) with 150 mM NaCl, 1 mM MgCl₂, 1% Nonidet P-40, 10% glycerol, 1 mM Na₃VO₄, 10 mM NaF, and complete protease inhibitor mixture (Roche Applied Science), for 20 min at 4 °C. Lysates were clarified by centrifuging at 18,000 × g for 20 min, followed by mixing with 5× SDS-PAGE sample buffer and boiling for 5 min. Samples were then separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nonspecific binding sites on the membranes were blocked in 5% nonfat dry milk, followed by probing with rabbit primary antibody to phospho-Smad2 (Ser-465/467, 1:2500; Cell Signaling) or Smad2/3 (1:2000; Cell Signaling) at 4 °C overnight, followed by a 2-h incubation at room temperature with HRP-conjugated anti-digoxigenin antibody (1:2500; Roche Applied Science). For IGF-I Western blotting, a 50 ng of recombinant IGFBP3 was incubated with or without 60 ng of BMP1 in 5 μg/ml dextran sulfate for 6 h at 37 °C. After SDS-PAGE on a 12% acrylamide gel, samples were transferred to a nitrocellulose membrane, which was blocked in 5% nonfat dry milk for 90 min at room temperature, followed by overnight incubation at 4 °C with goat antibody to human IGFBP3 (1:1000; R&D Systems) and then a 1-h incubation at room temperature with secondary donkey anti-goat antibody (1:10,000; Abcam). All blots were visualized using the ECL Plus kit (GE Healthcare).

FN Enhancement of IGBP3 Cleavage by BMP1-like Proteinases—MEFs differentiated from FN-null (FN⁻/⁻) or heterozygous (FN+/⁻) stem cells (15) were plated on 10-mm cell culture dishes. After achieving confluence, cells were washed with PBS and then incubated for 2 h in serum-free DMEM. After replenishing with fresh serum-free DMEM, cells were cultured for 3 days with TGFβ-1 (2 ng/ml; R&D Systems) with/without a 20 μM concentration of the specific hydroxamic acid inhibitor of BMP1-like proteinases, BI-1 (FibroGen) (13). To examine the effects of adding exogenous fibronectin, FN⁻/⁻ MEFs were plated on 6-well plates. After confluence, cells were washed with PBS before being incubated for 2 h in serum-free DMEM. After replenishing with serum-free medium, cells were incubated for 3 days with various concentrations of cellular fibronectin (0–20 μg/ml; Sigma). Conditioned media were harvested and concentrated 40-fold (from 10-mm dishes, 10 ml to 250 μl) or 20-fold (from 6-well plates, 2 ml to 100 μl) using Amicon Ultra-4 10k centrifugal filter units, followed by the addition of 5× Laemmli buffer and 5% β-mercaptoethanol, boiling for 5 min, and subjection to SDS-PAGE on 12% polyacrylamide gels and immunoblotting. For immunoblots, nitrocellulose membranes were blocked in 5% nonfat dry milk, and probed with primary goat anti-mouse IGBP3 antibodies (1:1000) at 4 °C overnight and then with secondary donkey anti-goat antibody (1:10,000; Bio-Rad) for 1 h at room temperature. Blots were visualized using the ECL Plus kit.

Zebrafish—For RNA injections, full-length Igfbp3 sequences were amplified from an adult zebrafish cDNA library (Open Biosystems) using primers 5’-TATGAAATTTTATATGACCGGACTGTTG-3’ (forward) and 5’-GGTCGAGTAGCACCTTGTCTCCCATGTAGC-3’ (reverse), followed by insertion between the EcoRI and XbaI sites of pCS2+, for in
vivo expression of Igfbp3 RNA. The construct for in vitro expression of Bmp1a RNA has been described previously (16). Igfbp3 and Bmp1a plasmids were linearized with BssHII, and capped mRNA was transcribed using the mMessage mMachine SP6 kit (Ambion). Capped mRNAs (1 ng/μl Igfbp3 and/or 200 pg/μl Bmp1a) were injected into one- to two-cell stage zebrafish embryos. After injection, embryos were incubated at 28.5 °C. Morphological changes were imaged at 24 h postfertilization (hpf) with a dissecting microscope (SZ40, Olympus) and imaging software (Rincon). Whole mount in situ hybridizations were performed essentially as described previously (17). Briefly, 90% epiboly embryos were fixed with 4% paraformaldehyde. After dechorionating, embryos were digested with proteinase K (10 μg/ml, 8 min) and hybridized with a previously described digoxigenin-labeled Chordin probe (18). After washing, embryos were blocked for 2 h with blocking reagent (Roche Applied Science) and incubated overnight with alkaline phosphatase-conjugated anti-digoxigenin Fab (1:5000; Roche Applied Science). Embryos were developed with BM purple AP substrate (Roche Applied Science). Reactions were stopped by rinsing with PBS, 0.1% Tween 20. Embryos were fixed with 4% paraformaldehyde and preserved in 70% glycerol for mounting. Images were captured using a Nikon SMZ1500 dissecting microscope and MetaView imaging software (Molecular Devices).

Immunoprecipitation—60 ng of intact or cleaved human IGFBP3 was preincubated with/without 40 ng of recombinant human IGFBP3 (R&D Systems) for 30 min at 37 °C in a 100-μl final volume of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM ZnCl2, 5 mM CaCl2, 1 mM MgCl2, and 1 mg/ml bovine serum albumin. Mouse anti-BMP4 (R&D Systems) or goat anti-IGFBP3 antibodies were then added, and the mix was allowed to rotate for 3 h at 4 °C, followed by the addition of protein G-agarose beads (Roche Applied Science), rotation overnight at 4 °C, and then washing three times in PBS, extraction, SDS-PAGE, and immunoblotting, as previously described (8). For comparing pull-downs employing intact or BMP1-cleaved IGFBP3, IGFBP3 was prepared by incubating 60 ng of IGFBP3 in the presence or absence of 30 ng of BMP1, in a final reaction volume of 30 μl, in the presence of 5 μg/ml dextran sulfate, as described above.

For competition immunoprecipitation assays, 0.025 nM rhIGFBP3 and 0.05 nM BMP-4 were co-incubated with BMP2, TGFβ1, FGF2, or IGF-I, at concentrations noted in Fig. 7. Incubations were for 2 h at room temperature in 200 μl of PBS containing 1 mM CaCl2, 3 mM MgCl2, 0.2% Nonidet P-40, and 1 mg/ml BSA. Subsequently, 5 μl of anti-IGFBP3 or BMP4 antibody and 20 μl of protein G-agarose beads were added to each reaction, and the reaction volume was brought up to 400 μl with PBS. After binding at 4 °C overnight, beads were pelleted and washed three times with PBS containing 0.2% Tween 20 and subjected to extraction, SDS-PAGE, and immunoblotting. Monoclonal anti-BMP2 for immunoblotting was purchased from R&D Systems.

RESULTS

BMP1 Cleaves IGFBP3—To determine whether IGFBP3 might be processed by BMP1, recombinant murine IGFBP3 was co-incubated with BMP1 in an in vitro cleavage assay. As can be seen (Fig. 1A), intact murine IGFBP3 migrates upon SDS-PAGE as a doublet comprising bands of ~42 and ~37 kDa. Upon incubation with BMP1, cleavage products consisting of a doublet comprising bands of ~32 and ~30 kDa and a second doublet comprising bands of ~12 and ~11 kDa appear. NH2-terminal amino acid sequencing of the ~32 and ~30-kDa forms gave an identical sequence, representing the NH2-terminal sequence of mature IGFBP3 resulting from removal of the signal peptide by proteolytic cleavage between amino acids LLRG21 and 22PPVA of pre-IGFBP3. Thus, the ~32- and 30-kDa forms are both BMP1-generated NH2-terminal cleavage products. NH2-terminal amino acid sequencing of both the ~12- and ~11-kDa forms produced the sequence TDTQNFSSS, showing both forms to be BMP1-generated COOH-terminal cleavage products, resulting from proteolytic cleavage between amino acid sequences RYKVDYESQ5195 and 196C-TDTQNFSSS (numbering from the methionine residue at the translation start site), in the IGFBP3 linker region, just 18 residues upstream of the COOH-terminal cysteine-rich...
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The amount of a fibroblasts secrete IGFBP3 that is largely intact but with a small reduced predominant NH2- and COOH-terminal fragments of post-translational modification, potentially consisting of differential glycosylation and/or phosphorylation. Intact human IGFBP3 was found to migrate upon SDS-PAGE as a doublet comprising bands of ~46 and ~41 kDa (Fig. 1B). The ~41-kDa form seemed more sensitive to cleavage, upon incubation with BMP1, than did the ~46-kDa form, suggesting that additional post-translational modification of the ~46-kDa form may interfere with BMP1 processing. Cleavage with BMP1 produced predominant NH2- and COOH-terminal fragments of ~30 and ~16 kDa, respectively (Fig. 1B), and NH2-terminal amino acid sequencing of the ~16-kDa form produced the sequence TDTQFNSSSE, showing BMP1 to cleave human and mouse IGFBP3 at the identical conserved site. In Fig. 1C, it can be seen that on some blots, human IGFBP3 cleavage products occurred as doublets with the ~30 and 16 kDa bands described above accompanied by faint bands of somewhat slower electrophoretic mobilities. It can also be seen that the SDS-PAGE electrophoretic mobilities of murine and human IGFBP3 are quite different, although mature intact murine and human IGFBP3 comprise similar numbers of amino acids (271 and 270 amino acids, respectively), with similar predicted molecular weights of 29,474 and 29,341, respectively, and 81% amino acid identity. Nevertheless, the differences in post-translational modification that contribute to these different mobilities do not effect a change in the site of BMP1 cleavage in IGFBP3 from the two species.

Endogenous BMP1-like Proteases Play a Role in the Proteolytic Cleavage of Endogenous IGFBP3 in MEF Cultures—It has previously been demonstrated that IGFBP3 is secreted in MEF cultures (19). As can be seen (Fig. 2A), normal mouse embryo fibroblasts secrete IGFBP3 that is largely intact but with a small amount of a ~30-kDa cleavage product, similar in size to the NH2-terminal cleavage product produced by cleavage of recombinant murine IGFBP3 by BMP1 in vitro (e.g. Fig. 1A). We previously showed that TGFβ1 induces increased levels of BMP1 in cultures of fibrogenic cells, such as fibroblasts (20). In that regard, it is of interest that when TGFβ1 was added to normal MEF cultures, cleavage of IGFBP3 to the ~30-kDa fragment was enhanced (Fig. 2A). To determine whether the induced cleavage was indeed due to endogenous BMP1-like proteases, MEFs were incubated in the presence of a 20 μM concentration of the previously described hydroxamic acid-based inhibitor BI-1, which is highly effective for BMP1-like proteases and has an IC50 value of 6, 2, and 4 nM for BMP1-like proteases BMP1, mTLL1, and mTLL2, respectively, but which is ineffective against MMPs (13, 21). As can be seen (Fig. 2A), the BI-1 inhibitor was efficient in inhibiting cleavage of endogenous IGFBP3 by endogenous MEF proteases, consistent with the conclusion that cleavage of IGFBP3 in MEF cultures is via endogenous BMP1-like proteases, although this result, in and of itself, does not exclude possible roles by meprin or ADAM/ADAMTS metalloproteases, which have not been tested for sensitivity to inhibition by BI-1.

Previously, comparison of cleavage patterns of various proteins in cultures of wild-type MEFs with cleavage patterns of the same proteins in cultures of MEFs derived from embryos homozygous null for both the Bmp1 gene, which encodes alternatively spliced mRNAs for the two proteases BMP1 and mTLD, and the Tll1 gene, which encodes the BMP1-like protease mTLL1, has led to identification and verification of a number of in vivo substrates of the mammalian BMP1-like proteases (11, 22). Thus, in a genetic approach toward ascertaining whether products of the Bmp1 and/or Tll1 genes are involved in the processing of endogenous IGFBP3 in MEF cultures, we compared processing of IGFBP3 in the conditioned media of wild type and Bmp1/Tll1 doubly null MEFs. Processing of IGFBP3 to the 30-kDa fragment was found to be essentially blocked in both untreated Bmp1/Tll1 doubly null MEFs and Bmp1/Tll1 doubly null MEFs treated with TGFβ1 (Fig. 2B). Thus, a BMP1-like protease(s) encoded by the Bmp1 and Tll1 genes appears to be responsible for a preponderance of detectable IGFBP3 processing in MEF cultures.

Cleavage by BMP1 Counteracts the Ability of IGFBP3 to Bind IGF-I or to Block IGF-I-induced Signaling—Toward determining the functional consequences of IGFBP3 cleavage by BMP1-like proteases, we sought to determine whether cleavage by BMP1 counteracts the ability of IGFBP3 to block IGF-I-in-
Thus, to drive BMP1 cleavage of IGFBP3 nearer to completion, in vitro cleavage reactions were conducted in the presence of dextran sulfate because the use of such polymers has been ascertained via monitoring levels of phospho-Akt, a key intermediate in IGF-I-induced signaling (25), showed BMP1-cleaved IGFBP3 to have a markedly reduced ability to block IGF-I-induced signaling compared with intact IGFBP3 (Fig. 3A). Subsequently, a Western ligand blotting assay, employing digoxigenin-labeled IGF-I, directly demonstrated that cleavage by BMP1 destroys the ability of IGFBP3 to bind IGF-I (Fig. 3B). Fig. 3C is a control immunoblot, which demonstrates that similar amounts of intact and BMP1-processed IGFBP3 were employed in the Western ligand blotting assay of Fig. 3B.

BMP1-processed IGFBP3 Has an Enhanced Ability to Block FGF-induced Proliferation—Although IGFBP3 cleavage by BMP1 and other proteases yields fragments with reduced affinity for IGF-1, previous studies have demonstrated instances in which IGFBP3 fragments with decreased IGF-1 affinity can exhibit IGF-independent biological activities that exceed or differ from activities exhibited by intact IGFBP3 (6).

To determine whether BMP1 cleavage fragments of IGFBP3 might exhibit such activity, we compared the ability of intact and BMP1-cleaved IGFBP3 to inhibit the mitogenic effects of FGF because an NH2-terminal fragment produced by plasmin cleavage of IGFBP3 has been shown to exceed intact IGFBP3 in such activity (26). As expected, intact IGFBP3 was markedly more effective at inhibiting IGF-1-induced proliferation of MCF-7 cells than was BMP1-cleaved IGFBP3 (Fig. 4A). However, BMP1-cleaved IGFBP3 was significantly more effective at inhibiting FGF-induced proliferation in the same cells (Fig. 4B). BMP1-processed IGFBP3 Has an Enhanced Ability to Induce Smad Phosphorylation—IGFBP3 has also been reported to be capable of inhibiting breast cancer cell proliferation, in an IGF-independent manner, via stimulating Smad2/Smad3 phosphorylation, by enhancing TGF-β1 signaling and/or by stimulating Smad2/Smad3 phosphorylation in an apparently TGF-β1-independent manner (27, 28). Because BMP1-processed IGFBP3 has an increased ability to block FGF-induced breast cancer cell proliferation compared with intact IGFBP3 (see above), we sought to determine whether BMP1-processed IGFBP3 might also have an increased ability in this other IGF-independent effect, stimulation of Smad phosphorylation. As can be seen (Fig. 4C), BMP1-processed IGFBP3 had a markedly increased ability to stimulate Smad2 phosphorylation compared with intact IGFBP3.

Zebrafish Bmp1a Can Reverse the Phenotypic Effects of Igfbp3 Overexpression in Zebrafish Embryos—Previously, zebrafish Igfbp3 expression was shown to first be detectable during embryogenesis as early as the blastula stage, via PCR (29), with ready detection of Igfbp3 transcripts via whole mount in situ hybridization by 14 hpf (30). Moreover, morpholino knock-down experiments have shown Igfbp3 to play roles in development of pharyngeal cartilage and the inner ear (30). To determine whether BMP1 can counteract Igfbp3 function in an in vivo environment, we overexpressed Igfbp3 in zebrafish embryos in the presence or absence of overexpressed Bmp1a, the product of one of two zebrafish BMP1 genes (16). Surprisingly, Igfbp3 overexpression resulted in a variety of apparently dorsaled phenotypes, ranging from mild to severe (Fig. 5, B–D and K) compared with uninjected embryos (Fig. 5A). As expected, due to the known roles of Bmp1a in dorsoventral patterning (7), Bmp1a overexpression resulted in embryo ventralization (Fig. 5, E–G and K). Importantly, co-overexpression...
FIGURE 4. BMP1-cleaved IGFBP3 has a reduced ability to inhibit the mitogenic action of IGF-1 but an increased ability to inhibit the mitogenic action of FGF. Serum-starved MCF-7 cells were induced to proliferate with IGF-I (A) or FGF (B) in the absence of IGFBP3 (control (Ct)) or in the presence of increasing amounts of intact (I) or BMP1-processed (P) IGFBP3. For each set of proliferation experiments, a culture in which neither IGFBP3 nor BMP1 was added was used as a control (Blank) to determine the base line of MCF-7 proliferation. After 48 h, the levels of cell proliferation were assessed via a colorimetric assay of mitochondrial dehydrogenase reduction of MTT. The numbers on the ordinate axis represent OD at 570 nm. Assays were performed in quadruplicate and repeated twice with similar results. Data are presented as mean ± S.E. (error bars). In the presence of IGF-I (A), the levels of proliferation were not significantly different between control and BMP1-processed IGFBP3-treated cells (A) but were significantly reduced between control and intact IGFBP3-treated cells. In contrast, in the presence of FGF (B), levels of proliferation were not significantly different between control and intact IGFBP3-treated cells but were significantly reduced between BMP1-processed IGFBP3-treated cells compared with controls in a dose-dependent way. In A and B, levels of proliferation were not significantly different between cultures in which growth factors were added together with the highest concentrations of either intact (A) or cleaved (B) IGFBP3 and the control cultures, to which no growth factors had been added. Thus, although levels of proliferation inhibited by 250 ng/ml intact IGFBP3 were not markedly less than those inhibited by 125 ng/ml, it can be seen that levels of proliferation with the 125 ng/ml intact IGFBP3 culture are already close to proliferation levels of the “Blank” culture, *. * p < 0.05; **, ** p < 0.01. C, immunoblots of levels of phosphorylated Smad2 (pSmad2) and of Smad2/3 protein levels, in the same samples, in MCF-7 cells treated with varying amounts of intact IGFBP3 or IGFBP3 processed by BMP1.

of Igfbp3 with Bmp1a resulted in reversal of the Igfbp3-induced phenotype, with a majority of embryos displaying ventralized phenotypes (Fig. 5, H–J and K), similar to those seen in embryos injected with RNA for Bmp1a alone. A straightforward explanation of these results is that Bmp1a is able to cleave Igfbp3 in the in vivo setting of the zebrafish embryos, thereby reversing the ability of Igfbp3 to induce the observed dorsalized-like phenotype.

Consistent with the conclusion that Igfbp3 overexpression dorsalizes zebrafish embryos, whole mount in situ hybridization showed expansion of the dorsal marker Chordin domain at 90% epiboly in 35.3% of embryos in which Igfbp3 was overexpressed (Fig. 6, A and B). These embryos, with expansion of the Chordin domain at 90% epiboly, probably correspond to some portion of the ~50% of embryos with intermediate to strongly dorsalized morphology observed at 24 hpf (Fig. 5). Consistent with the conclusion that Bmpla overexpression is able to reverse the Igfbp3-induced phenotype, in situ hybridization showed reduction of the Chordin domain at 90% epiboly in the majority of embryos in which both Bmp1a and Igfbp3 had been overexpressed (82.1%; Fig. 6D), similar to the kind of reduction of the Chordin domain seen at 90% epiboly in embryos in which Bmp1a alone had been overexpressed (86.1%; Fig. 6C).

IGFBP3 binds BMP4 in a specific manner that is competed by binding to IGF-I—BMP1-like proteinases are known to act in vertebrate dorsoventral patterning via cleavage of the extracellular proteins Chordin and Chordin-like, which otherwise antagonize bone morphogenetic protein signaling by binding ligands BMP2/4 in latent complexes (7). Thus, co-immunoprecipitation experiments were performed, to determine whether a role in dorsoventral patterning might derive from an ability of IGFBP3 to bind BMP2/4. As can be seen (Fig. 6E), immunoprecipitation of BMP4 was found to readily co-precipitate IGFBP3. Similarly, in the converse experiment, immunoprecipitation of IGFBP3 was found to readily co-precipitate BMP4. Comparison of results of attempts to immunoprecipitate BMP4 with intact or BMP1-processed IGFBP3 supported the conclusion that cleavage by BMP1 very much reduces the ability of IGFBP3 to bind BMP4 (Fig. 6F).

To assess the degree of specificity with which IGFBP3 binds BMP4, we attempted to compete IGFBP3-BMP4 binding with 4-fold molar excesses of the unrelated growth factor FGF2, the distantly related growth factor TGFβ1, or the closely related growth factor BMP2. As can be seen (Fig. 7A), although a 4-fold molar excess of either FGF2 or TGFβ1 showed no ability to compete against the binding of IGFBP3 to BMP4, BMP2, which is highly homologous to BMP4, competed with BMP4 in a dose-dependent way for binding to IGFBP3. Because BMP2 and BMP4 migrate with somewhat different mobilities on SDS-PAGE (and because the monoclonal antibodies used for the two factors did not cross-react), we were able to probe the same blot for BMP2, thus showing that the BMP2, which had displaced BMP4 from IGFBP3, had replaced it as the IGFBP3-bound ligand (Fig. 7A). Because the above results support the conclusion that IGFBP3 binding to BMP2/4 is highly specific, we next sought to determine whether such binding competes with the well-established ability of IGFBP3 to bind IGF-I. As can be seen (Fig. 7B), IGF-1 was able to compete away binding of BMP4 to IGFBP3 in a dose-dependent manner. In addition, IGF-1 appeared to compete away binding of BMP4 to IGFBP3 at considerably lower concentrations than did BMP2 (Fig. 7, compare A and B), thus suggesting that IGF-1 might bind IGFBP3 with greater affinity than does BMP2/4.
FN Enhances the Cleavage of IGFBP3 by BMP1-like Proteinases—IGFBP3 binds the extracellular matrix protein FN (9, 10), and we have previously shown FN to be capable of enhancing the ability of BMP1-like proteinases to cleave three tested substrates (8). It has also been shown that whereas exogenous IGFBP5 remains intact when added to FN/H11002/H11002 mouse embryo cells, the addition of exogenous FN induces proteolytic cleavage of the added IGFBP5 by the same cells (31). To determine whether the presence of FN can have an effect on the cleavage of IGFBP3, we compared the electrophoretic mobility of IGFBP3 in the conditioned media of cultures of MEFs differentiated from stem cells either FN-null (FN/H11002/H11002) or heterozygous for the FN gene (FN/H11001/H11002). As can be seen (Fig. 8A), MEFs heterozygous for the FN gene show markedly more IGFBP3 proteolytic processing than do FN−/− MEFs to produce fragments similar in size (~32 and ~34 kDa) to those seen upon BMP1 processing of murine IGFBP3 in vitro (~30 and ~32 kDa; Fig. 1A). Similarly, the addition of exogenous FN to cultures of FN−/− MEFs was sufficient to induce IGFBP3 proteolytic processing (Fig. 8B). To determine whether FN-induced cleavage of IGFBP3 in MEFs was due to BMP1 and/or related proteinases, MEFs were cultured in the presence of the previously described hydroxamic acid-based inhibitor BI-1 (Fig. 8A), which is highly specific for BMP1-like proteinases (13). FN-induced proteolysis responsible for producing the ~32-kDa fragment was very much reduced in the presence of the inhibitor, supporting the conclusion that such proteolytic activity is provided by BMP1 and/or BMP1-related proteinases.

DISCUSSION

In addition to serving as the major IGF carrier protein in the circulation, IGFBP3 is produced locally in various adult and developing tissues (30, 32–39), suggestive of paracrine and/or autocrine functions at such sites. Such local functions in tissues
probably involve important roles in cell proliferation and differentiation and in metabolic function, based on documented IGF-dependent and -independent effects of IGFBPs (4–6). It has also been documented that both IGF-dependent and -independent effects of IGFBPs are modulated by limited proteolytic cleavage of IGFBPs into identifiable fragments (6). In fact, it has previously been shown that IGFBP3 can be cleaved by various serine and cysteine proteinases, by some MMPs, and by two members of the ADAM family of metalloproteinases (6, 40–43), although the physiological significance of some of these cleavage reactions remains to be determined. BMP1-like proteinases have been shown to play roles in regulating cellular...
BMP1 is shown here to cleave human and mouse IGFBP3 at a single conserved site. As in Chordin and Chordin-like, two extracellular antagonists of BMP2 and -4, the IGFBP3 cleavage site lies within a linker region between cysteine-rich domains. Just as cleavage of Chordin by BMP1-like proteinases produces fragments with reduced affinity for cognate ligands (50), cleavage of IGFBP3 is shown here to produce fragments with reduced affinity for IGF-I, as evidenced by reduced IGF-I binding in a Western ligand blotting assay and by the diminished ability of BMP1-cleaved IGFBP3 to block IGF-I-induced signaling in MCF-7 human breast cancer cells. However, both intact IGFBP3 and proteolytic fragments of IGFBP3, with little or no demonstrable affinity for IGFs, have been reported in various studies to have IGF-independent functions, and in some cases, IGFBP proteolytic fragments have been shown to have increased IGF-I-independent activity compared with the intact molecule (6). Consistent with such reports, BMP1-cleaved IGFBP3 is shown here to have an increased ability to inhibit the mitogenic activity of FGF and to stimulate Smad2/3 phosphorylation, compared with intact IGFBP3. Such increased activity of the cleaved material in inhibiting FGF mitogenic activity may be due to NH2-terminal fragments of BMP1-cleaved IGFBP3 because an NH2-terminal fragment produced by plasmin cleavage of IGFBP3 has previously been shown to exceed intact IGFBP3 in such activity (26). The finding here that proteolyzed IGFBP3 has an increased ability to stimulate Smad2/Smad3 phosphorylation, compared with the intact molecule, is a novel finding and augments previous findings that intact IGFBP3 inhibits the growth of some epithelial cancer cells, via potentiating TGF-β1-stimulated Smad phosphorylation and by stimulating Smad2/Smad3 phosphorylation in an apparently TGF-β1-independent manner (27, 28). Interestingly, we have shown previously that BMP1 can contribute to TGFβ1 activation via cleavage of latent TGFβ-binding protein 1 (LTBP1) (3). Thus, BMP1-like proteinases may affect levels of Smad2/3 signaling via more than one mechanism.

Most cells express more than one IGFBP, such that regulation of IGF-dependent and -independent IGFBP functions within cells by proteinases must take into account the ability to cleave multiple IGFBPs. In this regard, it is of interest that proteolysis of IGFBP2 through -6 has been reported in various studies of biological fluids (5, 6). Thus, we have begun studies of the ability of BMP1-like proteinases to cleave additional IGFBPs and have thus far found IGFBP5 to be cleaved in vitro by BMP1 at what appears to be a single site (data not shown). Delineation of the profile of which IGFBPs are cleaved or not cleaved by BMP1-like proteinases awaits future studies.

In an approach designed to explore the ability of BMP1-like proteinases to affect IGFBP3 function in an in vivo setting, we overexpressed Igfbp3 in zebrafish embryos in the presence or absence of overexpressed Bmp1a. Surprisingly, Igfbp3 overexpression resulted in apparent dorsalization of zebrafish embryos. Importantly, however, co-overexpression of Bmp1a with Igfbp3 resulted in embryo ventralization, showing the ability of Bmp1a to reverse the dorsalizing activity of Igfbp3 and thus demonstrating the ability of BMP1-like proteinases to impact Igfbp3 function in vivo. Consistent with the results of these overexpression studies, evidence is also presented here.
that IGFBP3 is capable of binding BMP2/4 in a specific manner and that cleavage of IGFBP3 by BMP1-like proteinases decreases the ability of IGFBP3 to bind BMP2/4. However, the ability of zebrafish Igfbp3 to dorsralize and the ability of IGFBP3 to bind BMP2/4 were unexpected because previous loss-of-function, morpholino knockdown experiments had not revealed an obvious role for Igfbp3 in dorsoventral patterning (30). In regard to this apparent contradiction between the results of our Igfbp3 overexpression experiments and previous morpholino knockdown experiments in zebrafish embryos (30), it should be noted that the roles of gene products involved in dorsoventral patterning of zebrafish embryos can be masked in morpholino loss-of-function experiments if such roles overlap and are redundant with the dorsoventral patterning activity of other gene products (7). Thus, although further definition of possible roles of Igfbp3 in dorsoventral patterning is clearly beyond the scope of the current study, future studies into such roles could include simultaneous morpholino knockdown of Igfbp3 and other proteins known to dorsralize embryos via extracellular binding of bone morphogenetic proteins in an approach designed to identify other gene products whose roles in dorsoventral patterning overlap/are redundant with those of Igfbp3. Alternatively, the indication from competition assays, performed herein, that IGF-I may bind IGFBP3 with greater affinity than does BMP2/4 suggests the possibility that at sufficient physiological concentrations, IGF-I could block BMP2/4 binding by endogenous Igfbp3, whereas excess levels of Igfbp3, produced by overexpression, would be free to bind extracellular BMP2/4, thus affecting dorsoventral patterning. Clearly, the biological importance of possible in vivo IGFBP3-BMP2/4 interactions, during development and/or in adult tissues, remains to be determined, as do the intriguing possibilities that such interactions could provide a means of cross-talk between IGF and bone morphogenetic protein signaling pathways and that cleavage of IGFBP3 and Chordin by the same class of proteinases could serve to orchestrate the two pathways.

We have previously shown that FN binds BMP1 and is capable of enhancing the ability of BMP1-like proteinases to cleave the substrates Chordin, probiglycan, and type I procollagen (8). All three of the preceding substrates also bind FN, suggesting a mechanism whereby FN might facilitate interactions between substrates and BMP1-like proteinases. Like the preceding substrates, FN is known to bind IGFBP3 (9, 10). In addition, it has been shown that the addition of FN to FN−/− MEF cultures induces the proteolytic cleavage of otherwise intact IGFBP5 (31). Here we extend such findings by showing that FN enhances the cleavage of endogenous IGFBP3 by endogenous BMP1-like proteinases in MEF cultures. However, the mechanism whereby FN induces BMP1-like proteinases to process IGFBP3 in cell cultures is unclear. We previously showed that BMP1 cleavage of Chordin, probiglycan, and type I procollagen is enhanced by FN in vitro as well as in cell cultures. In contrast, although we sometimes observed FN enhancement of BMP1 cleavage of IGFBP3 in vitro (data not shown), such enhancement was modest and not consistently reproducible. Thus, although FN binds both IGFBP3 (9, 10) and BMP1 (8), suggestive of a direct effect in facilitating cleavage, other factors found in MEF cultures but absent from in vitro cleavage assays seem important to full FN enhancement of IGFBP3 processing by BMP1-like proteinases. Indeed, the incomplete processing of recombinant IGFBP3 by BMP1 under standard in vitro reaction conditions (Fig. 1) seems consistent with the probability that other factors are involved in the efficient cleavage of IGFBP3 by BMP1-like proteinases under physiological conditions. Relevant to this possibility is the growing number of endogenous modulators of the activities of BMP1-like proteinases reported in the literature (1, 8, 51, 52).

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