Splicing Factor 3b Subunit 4 Binds BMPR-IA and Inhibits Osteochondral Cell Differentiation*

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Bone morphogenetic protein (BMP)-2/4 play critical roles in early embryogenesis and skeletal development. BMP-2/4 signals conduct into cells via two types of serine/threonine kinase receptors, known as BMPR-I (IA and IB) and BMPR-II. Here we identified splicing factor 3b subunit 4 (SF3b4) as a molecule that interacts with BMPR-IA, using a yeast two-hybrid screening with a human fetal brain cDNA library. Co-immunoprecipitation/immunoblotting analysis confirmed their interaction in mammalian cells. By separation of the cell components, SF3b4 was present in the cell membrane fraction with BMPR-IA as well as in the nucleus. Overexpression of SF3b4 inhibited BMP-2-mediated osteogenic and chondrocytic differentiation of C2C12 cells, and phosphatidylinositol 3-kinase (4) are activated following BMP-mediated signals conduct into the cell via two types of serine/threonine kinase receptors known as BMP receptor type I (IA and IB) and type II receptors (8). With regard to BMP-2/4 signaling, three structurally related type I receptors, BMPR-IA (also known as ALK3), BMPR-IB (also known as ALK6), and ActRRIA (also known as ALK2) and one type II receptor, BMPR-II, have been identified. When the ligand interacts with the receptor complex, BMPR-II transphosphorylates BMPR-I at the GS box to activate its kinase. BMPR-I activation induces the phosphorylation of transiently associated Smad1/5/8. These Smads then form heteromorphic complexes with Smad4, which is then translocated into the nucleus. In the nucleus, they regulate the transcription of specific target genes in concert with various DNA-binding proteins, co-activators, or corepressor proteins. Although the Smad pathway is widely represented in most of the cell types and tissues, additional pathways, including those of MAPKs (ERK1/2, p38 MAPK, SAPK/JNK) and phosphatidylinositol 3-kinase (4) are activated following treatment with BMPs.

Recently, a variety of molecules that modulate TGFβ-mediated signal transduction pathways have been identified, using yeast two-hybrid systems with Smads and the TGFβ receptor as bait proteins. These include NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) (9), Tiu1 (TGF-interacting ubiquitin ligase 1) (10), WW1P1 (WW domain-containing protein) (11), and PIASy (12) that bind Smad7; Dvl-1, Erbin, and Par-3 (13) that bind Smad3; and Daxx (14), TRAP-1 (TGFβR-I associated protein-1) (15), and STRAP (serine-threonine kinase receptor-associated protein) (16) that bind TGFβ receptors. In contrast, only a few molecules, such as BRAM1 (BMP-receptor associated molecule 1) (17) have been identified that bind BMP receptors. Since BMP transduces MAPKs as well as Smad1/5/8, there may be specific molecules that mediate those signaling pathways.

In this study, we attempted to identify these intervening molecules specific to BMP-mediated signal transduction pathways, using a yeast two-hybrid system with a BMPR-IA intracellular domain (ICD) as a bait protein, and found that a molecule pre-
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Cell Culture, Immunoprecipitation, and Immunoblot—
COS7 and 293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and C2C12 cells in DMEM containing 15% FBS. ATDC5 cells were maintained in DMEM/F-12 containing 5% FBS. For the induction of chondrocyte differentiation, ITS PREMIX (insulin (5 μg/ml), transferrin (5 μg/ml), and selenous acid (5 ng/ml)) (BD Biosciences) was added to the confluent cells. All medium contained 100 μg/ml penicillin and 100 μg/ml streptomycin.

Mouse monoclonal anti-FLAG M2 antibody was purchased from Sigma, rat monoclonal anti-HA antibody (3F10) from Roche Applied Science, anti-cadherin from LAB VISION, and anti-histone H1 (clone AE-4) from Upstate Biotechnology. Rabbit antibodies against p-ERK1/2, p-SAPK/JNK, p-p38 MAPK, ERK1/2, SAPK/JNK, p38 MAPK, and phospho-Smad1/5/8 were from Cell Signaling Technology. The secondary antibodies conjugated with peroxidase were from Cappel.

Expression plasmids of SF3b4 and its truncation forms were prepared by inserting the cDNAs into pBFX (18) using Xhol and BamHI sites, or p3xFlag-CMV14 (Sigma) using the restriction sites as indicated in Table 1. 293 cells were transfected with the expression plasmids as indicated, using FuGENE6 (Roche Applied Science) according to the manufacturer’s instructions. After 48 h, the cells were lysed in 50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 5 mM CaCl2, 1 mM phenylmethanesulfonyl fluoride, and 10 mM N-ethylmaleimide. Lysates were gently stirred and then clarified by centrifugation (15,000 × g, 20 min). An aliquot of the lysate was applied to immunoblotting, and the remainder was immunoprecipitated for 1 h with an anti-HA antibody (3F10; Roche Applied Science) and 50 μl of protein G-Sepharose (Amersham Biosciences). Immunoprecipitates were washed four times in the above lysis buffer, applied to 10% SDS-PAGE under a reducing condition, and transferred to a polyvinylidine fluoride membrane. The membrane was incubated with 5% skim milk in TBS (50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl), containing 0.05% Tween 20 (TBS-T). Anti-FLAG M2 and anti-HA antibodies were used as the primary antibody diluted in TBS-T containing 1% skim milk. The signal was visualized using peroxidase-conjugated secondary antibodies. Detection was performed with Western Lightning Plus (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

To immunoblot MAPks and Smad1/5/8, following treatment with BMP-2 (500 ng/ml; R&D Systems) for the indicated times at 37 °C, cells were lysed in 10 mM Tris-HCl, pH 7.4, 140 mM NaCl, 1% Triton X-100, 1.5 mM EDTA, 1 mM Na2PO4, 25 mM NaF, 1 mM Na3VO4, and 1 tablet/10 ml of Protease Inhibitor Mixture Tablets (Roche Applied Science). Immunoblot was performed as described above. All of the primary antibodies were diluted to 1:1000 with TBS-T containing 1% skim milk. Each experiment was repeated at least three times with essentially similar results.

To examine the effects of SF3b4 on the BMPR-1A levels on the cell surface, COS7-stable transfectants overexpressing HA-tagged BMPR-1A were prepared by transfecting pcDNA3-HASL-ALK3. Then these cells were transfected with SF3b4/p3xFlag-CMV14 or an empty vector as the mock. Two identical cell plates were prepared: one for bio-

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening—To screen the molecules that bind the BMPR-1A ICD, a ProQuest™ two-hybrid system with Gateway™ technology (Invitrogen) and a ProQuest™ premade human fetal brain cDNA library (Invitrogen) were used. To construct bait plasmids, cDNA fragments encoding BMPR-1A ICD and the BMPR-1A constitutively active form (Q223D) ICD were prepared using the PCR technique and subcloned into an entry vector DONR201 (Invitrogen) by Gateway™ technology, with forward primer 5′-GGGGACAAAGTTGAAAAACGAGCTACAAACATTATTCGAGATCATGTCAGC-3′ and reverse primer 5′-GGGGACACTTTGTAACAGAAGCTGGTTCATCAAGATTTACATGTCAGC-3′. Expression plasmids of BMPR-1A ICD (pcDNA-HASL-ALK3 (WT)) and its constitutively active Q223D form (pcDNA3-HASL-ALK3 (QD)) with a HA tag at the C terminus (a kind gift from Dr. T. Imamura) were used as templates. Primers to generate full-length cDNA of SF3b4 and its mutants are summarized in Table 1. The fragments were subcloned into DE3T2 yeast expression vector (Invitrogen). The bait plasmid and cDNA library were co-transformed into a MaV203 host yeast strain according to the manufacturer’s instructions. Yeast transformants were plated and screened on the synthetic complete (SC) medium lacking leucine (L), tryptophan (W), and histidine (H) containing 10 mM 3-amino-1,2,4-triazole (3AT) (i.e. SC – L – W + 3AT). After 60–72 h, robust colonies over 2 mm in diameter were replica-cleaned and incubated for the following 2 days. Colonies that recovered after replica-cleaning were restreaked onto the SC – L – W and grown for 2 days. They were then further screened for the expression of three reporter genes (HIS3, URA3, and lacZ) by growth on SC – L – W + 3AT and SC – L – W – uracil (U), no growth on SC – L – W + 0.2% 5-fluoroorotic acid, and change of color to blue in the presence of X-gal. Plasmids were extracted from positive colonies and transformed into Escherichia coli strain ElectroMAX™ DH10B cells (Invitrogen), using the electroporation technique. Clones harboring the target cDNA were isolated, and their cDNA sequence was determined using an ABI-310 DNA sequencer.

Previously reported as splicing factor 3b subunit 4 (SF3b4) strongly binds the ICD but not its constitutively active form. In mammalian cells, a proportion of SF3b4 was present together with BMPR-IA in the cell membrane fraction, and co-immunoprecipitation/immunoblot analysis demonstrated their direct interaction. Overexpression of SF3b4 inhibited osteogenic and chondrocytic differentiation of C2C12 and ATDC5 cells, respectively. A reporter gene assay demonstrated that SF3b4 inhibits the Smad1/5/8-specific Id reporter gene activity. The levels of cell surface BMPR-IA in SF3b4-overexpressing cells were substantially decreased. We further performed molecular modeling of BMPR-IA, which provided insight into the sites of the interaction, although it was not determined. These results suggest that SF3b4 regulates osteochondral differentiation by interacting with BMPR-IA and down-regulating its cell surface levels.
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TABLE 1
Primer sequences for deletion mutants of SF3b4

| Primer                        | Sequence                                      |
|-------------------------------|-----------------------------------------------|
| Deletion mutants of SF3b4 for yeast two-hybrid assay using with Gateway™ technology | Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGCCATGGCTGCCGGGCCGATCTCCGAGCGGAATC |
|                                | Reverse: GGGGACAAGTTTGTACAAAAAAGCAGGCTCTTATGCCTTCAAGAAGGACTCCAAGGGTGAG |
| R-R-PG                        | Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATTTCATGGAACTGACCTCGGAAG |
| R-PG                          | Reverse: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATTTCATGGAACTGACCTCGGAAG |
| PG                            | Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATTTCATGGAACTGACCTCGGAAG |
| R1                            | Reverse: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATTTCATGGAACTGACCTCGGAAG |
| R2                            | Reverse: GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACATTTCATGGAACTGACCTCGGAAG |
| pBFX deletion mutants for immunoprecipitation in mammalian cells | Forward: ACCTACCTCCTAGAAGTATGCAGGCTGCCACCTCTCTAGAG |
|                                | Reverse: GGATTCCTTGCGAGGGGCGCTGGCATGAGTACG |
| CMV14 deletion mutants for localization in mammalian cells | Forward: CGGGATCCGGCGCGGGGGCTCTTTCGCCATGGCTGCCGGGCCGATCTCCGAGCGGAATC |
|                                | Reverse: CGGGATCCGGCGCGGGGGCTCTTTCGCCATGGCTGCCGGGCCGATCTCCGAGCGGAATC |
| PG-less                       | Reverse: CGGGATCCGGCGCGGGGGCTCTTTCGCCATGGCTGCCGGGCCGATCTCCGAGCGGAATC |

tin-labeling and the other for direct immunoblot analysis. After 48 h, the cell surface proteins were labeled with biotin. Briefly, the cells were washed twice with phosphate-buffered saline (PBS) containing 1 mM MgCl2 and 0.1 mM CaCl2 (PBS2+) and incubated for two 20-min periods at 4 °C with 1.0 mg/ml sulfo-NHS-LC-biotin (Pierce) diluted in PBS2+.

Nuclear and membrane fractions were subjected to SDS-PAGE and immunoblotting. Anti-histone H1 and anti-cadherin were used to detect these markers of the nucleus and cell membrane, respectively.

**Assay for Alkaline Phosphatase (ALP) Activity**—For the ALP assay, C2C12 cells were plated onto a 96-well plate at a density of 3 × 10^4 cells/well in growth medium. After 24 h, the medium was replaced with DMEM containing 2% FBS and BMP-2. At the days indicated after plating, the test medium was removed, and cells were washed with PBS. Then 200 μL of ALP buffer (1 mM MgCl2, 1 mM ZnCl2, and 1% Nonidet P-40 in 0.1 M glycine, pH 9.6) was added to each well, and cells were incubated for 1 h at 37 °C. Ten microliters of the cell lysate was diluted 10 times by adding 90 μL of ALP buffer, and then 100 μL of 10 mM p-nitrophenyl phosphate as substrate was added. The sample was mixed well and incubated in a 96-well plate at 37 °C for 30 min, followed by measuring the absorbance at 405 nm. Histochemical analysis of ALP activity was performed as described previously (19). Briefly, cells were plated at 7 × 10^4/well in 12-well plates. After 14 h (designated as day 1), the medium was replaced with medium containing BMP-2 (300 ng/ml). At the time points indicated, cells were fixed for 10 min with 10% buffered formalin at room temperature. After washing with PBS, the cells were incubated in 0.1 M Tris-HCl, pH 8.5, containing 0.1 mg/ml naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide, 2 mM MgCl2, and 0.6 mg/ml fast blue BB salt (Sigma) at room temperature.

**Alcian Blue Staining**—ATDC5 cells were plated at 4.0 × 10^4 cells/well in 12-well plates. At each time point, cells were washed with PBS, fixed with 10% buffered formalin for 30 min, and stained with 0.1% Alcian blue in 0.1 M HCl overnight. Excess stain was washed off with distilled water, and pictures were taken. Staining levels were quantified by solubilizing the sample in 6 M guanidine hydrochloride for 8 h at room temperature. The absorbance at 630 nm was measured using a spectrophotometer.
Real Time RT-PCR—At the time points indicated, mRNA was isolated from ATDC5 cells using a Micro-FastTrack™ 2.0 kit (Invitrogen), and cDNA was synthesized with the Super-Script™ premptamplification system (Invitrogen). The mouse aggrecan primers, mouse SF3b4 primers, and their TaqMan probes were designed using Primer Express software (Applied Biosystems). The TaqMan probe contained a reporter dye at the 5’-end and a quencher dye at the 3’-end. The sequences of the primers and probe are as follows: forward primer (5’-GATACTT-TCAGCCGCCTTGGGA-3’), reverse primer (5’-AGGCCAATA-TAATGAGCCGTAGC-3’), and probe (5’-CAGGACCCCCA-AGATCATGCG-3’); forward primer (5’-CTGCTCTTGGCCCCGTAA-3’), reverse primer (5’-GACAGGTCA-AAGATGGGCTTTC-3’), and probe (5’-CCCTGGGCGAGGG- TAATCTCCAC-3’) for Agc; and forward primer (5’-CAGCA- AGAGCAAGGAAAAGAAA-3’), reverse primer (5’-ATGCCTCATGCTGAAGGTTGGA-3’), and probe (5’-GAGGACCCAATGAAGGAAAAGAAA-3’) for Agc.

Identification of a Molecule That Interacts with BMPR-IA—To search for molecules that interact with BMPR-IA, we screened ~700,000 yeast transformants from a human fetal brain cDNA library with BMPR-IA ICD and its constitutively active form (Q223D) as bait. We obtained 22 colonies by screening with BMPR-IA ICD, and of them, 17 were identified as splicing factor 3b subunit 4 (SF3b4). Another colony was identified as Exportin 7, and the other four colonies turned out to be false positive. Colonies expressing SF3b4 fulfilled all of the screening conditions, including the blue color on the X-gal plate, growth on SC − L − W + His plates, and colony color on the SC − L − W − U plates, and stability on the SC − L − W + 0.2% 5-fluoroorotic acid plate, indicating strong interaction. The interaction with BMPR-IA was further confirmed using a colony expressing full-length SF3b4 (Fig. 1B). The colony expressing Exportin 7 became slightly blue on the X-gal plate after 24 h but did not fulfill either of the other conditions (data not shown). The inability to bind to BMPR-IA ICD was confirmed using a colony expressing full-length Exportin 7. With the BMPR-IA constitutively active form (Q223D) ICD as bait, no colonies grew on the screening plates. Thus, the following experiments focused on SF3b4.

SF3b4 is a 49-kDa protein involved in splicing of a region immediately upstream of the branch point sequence in the pre-sprecleosomal complex (26). This molecule contains two well conserved RNA recognition motifs (RRMs) at the N terminus and a proline- and glycine-rich motif (Pro-Gly motif) at the C terminus (Fig. 1A). To characterize the interaction between BMPR-IA and SF3b4, SF3b4 truncation mutants were generated as described under “Experimental Procedures” and tested for their ability to interact with the BMPR-IA ICD in a yeast two-hybrid assay. Full-length, R-R-PG, and R-PG bound BMPR-IA, but PG, R1, and R2 did not, suggesting that a stretch of RRM2 and the Pro-Gly motif is sufficient for the interaction (Fig. 1B). We further examined whether SF3b4 binds BMPR-IA...
and found that full-length SF3b4 did not bind the ICD of BMPR-IB in the yeast two-hybrid system (data not shown).

**SF3b4 Interacts with BMPR-IA in Mammalian Cells**—Next, we tested whether a similar association occurs in mammalian cells, using a full-length SF3b4 and several deletion mutants with Flag tag at the N terminus (Fig. 2A). 293 cells were transiently transfected with both an expression plasmid of FLAG-tagged SF3b4 (FLAG-SF3b4) and pcDNA3-HASL-ALK3 (WT), and co-immunoprecipitation/immunoblot analysis was performed. When BMPR-IA-HA was immunoprecipitated with an anti-HA antibody, full-length SF3b4 was co-precipitated, but R-PG and PG were not (Fig. 2B). These results indicate that SF3b4 actually binds BMPR-IA in the cell, but its full-length is required for adequate interaction, contrasting with the interaction in the yeast two-hybrid system.

Since BMPR-IA exhibits high sequence similarity to TβR-I, we examined whether SF3b4 interacts with TβR-I or not. By co-immunoprecipitation/immunoblot analysis, SF3b4 was not co-precipitated with TβR-I (Fig. 2C).

SF3b4 is a member of the spliceosome and is thought to be localized in the nucleus, whereas BMPR-IA is on the cell membrane. We examined whether these molecules are colocalized, using subcellular fractionation and immunoblotting. After co-transfection of pcDNA3-HASL-ALK3 (WT) and SF3b4/pBFX or its deletion mutants, cell lysates were immunoprecipitated (IP) with anti-HA antibody and then immunoblotted using the anti-FLAG antibody. SF3b4 expression was measured by anti-FLAG immunoblotting of aliquots from cell lysates. C, failure of interaction of SF3b4 with TGFβ-receptor I (TβR-I). 293 cells were transiently transfected with pcDNA3-HASL-TβR-I and SF3b4/pBFX, followed by immunoprecipitation/immunoblot analysis. Note that TβR-I does not interact with SF3b4. D, SF3b4 localized not only in the nucleus but also on the cell membrane, co-localized with BMPR-IA. COS7 cells were transiently transfected with pcDNA3-HASL-ALK3 (WT) and a full-length SF3b4/p3xFlag-CMV14, and fractionated as described under “Experimental Procedures.” Histone H1 and caderin were used as markers of nuclear and cell membrane fractions, respectively.

**SF3b4 Inhibits Osteochondral Differentiation**—Since BMPs induce osteogenesis, we tested whether SF3b4 inhibits BMP-2-induced osteogenesis of C2C12 cells. After transfection of SF3b4, BMP-2 induced osteogenesis, whereas BMPR-IA was not (Fig. 2D). SF3b4 was observed in the cell membrane fraction as well as in the nuclear fraction, whereas BMPR-IA was in the cell membrane fraction (Fig. 2D), indicating the colocalization of SF3b4 and BMPR-IA on the cell membrane.

**FIGURE 1.** SF3b4 interacts with BMPR-IA at RRM2 and the Pro-Gly motif. A, the full-length SF3b4 and its deletion mutants used in this study. Two RNA recognition motifs (RRM1 and RRM2) and C-terminal proline- and glycine-rich motif (Pro-Gly motif) are shown. B, interaction of a full-length SF3b4 and its deletion mutants with BMPR-IA. Note the interaction of the full-length SF3b4, R-R-PG, and R-PG with BMPR-IA.

**FIGURE 2.** SF3b4 interacts with BMPR-IA in mammalian cells. A, structures of SF3b4 deletion mutants. B, interaction between full-length SF3b4 and BMPR-IA. 293 cells were transiently transfected with pcDNA3-HASL-ALK3 (WT) and SF3b4/pBFX or its deletion mutants. Cell lysates were immunoprecipitated (IP) with anti-HA antibody and then immunoblotted using the anti-FLAG antibody. SF3b4 expression was measured by anti-FLAG immunoblotting of aliquots from cell lysates. C, failure of interaction of SF3b4 with TGFβ-receptor I (TβR-I). 293 cells were transiently transfected with pcDNA3-HASL-TβR-I and SF3b4/pBFX, followed by immunoprecipitation/immunoblot analysis. Note that TβR-I does not interact with SF3b4. D, SF3b4 localized not only in the nucleus but also on the cell membrane, co-localized with BMPR-IA. COS7 cells were transiently transfected with pcDNA3-HASL-ALK3 (WT) and a full-length SF3b4/p3xFlag-CMV14, and fractionated as described under “Experimental Procedures.” Histone H1 and cadherin were used as markers of nuclear and cell membrane fractions, respectively.
affects BMP-2/4-mediated osteogenesis in C2C12 cells (19). Stably transfected cell lines expressing SF3b4 and control mock-transfected lines were established. Twenty-four hours after plating, they were treated with BMP-2 at a concentration of 300 ng/ml (day 0), and ALP activity was measured. By histochemical analysis, mock transfectants showed a gradual increase of ALP activity by BMP-2 treatment, but SF3b4-expressing cell lines did not (Fig. 3A). Without BMP-2 treatment, ALP activity was unaltered during the culture period examined in both mock and SF3b4 transfectants (data not shown). By the ALP assay, the activity in SF3b4-expressing cell lines was unaltered after BMP-2 treatment (Fig. 3B). These results indicate that SF3b4 inhibits the osteogenic differentiation of C2C12 cells.

**SF3b4 Inhibits Chondrocytic Differentiation**—BMP-mediated signaling is involved in chondrocytic differentiation. We examined whether SF3b4 affects differentiation, using ATDC5 chondrocytic cells. These cells grow in a monolayer up to confluence. Several days after confluence, they start to grow again, piling up to form cartilaginous nodules. We established both SF3b4-expressing cell lines and mock transfectants of ATDC5 cells. Similar to the original ATDC5 cells, mock transfectants formed cartilaginous nodules stained by Alcian blue, whereas SF3b4-expressing cell lines did not (Fig. 4A). When measured after extraction with 6 M guanidine-HCl, the staining intensity gradually increased in mock transfectants, but that of SF3b4 transfectant remained unaltered during culture (Fig. 4B).

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*FIGURE 3. Overexpression of SF3b4 inhibits the differentiation of C2C12 cells into osteoblastic cells.* A, histochemical analysis of ALP activity. Original (data not shown) and mock-transfected C2C12 cells (upper panels) exhibit ALP activity when treated with BMP-2 (300 ng/ml), whereas stable transfectants overexpressing SF3b4 show little activity (lower panels). B, ALP assay. Note that ALP activity of SF3b4 transfectants (open circle) is much less than the mock transfectant (solid circle). The asterisks indicate a significant difference between SF3b4- and mock transfectants (Student’s t test; *, p < 0.01; **, p < 0.05).

*FIGURE 4. SF3b4 delays chondrocytic differentiation.* A, Alcian blue staining. To investigate the role of SF3b4 in chondrocyte differentiation using ATDC5 cells, we established a stable cell line that expressed SF3b4 and compared the chondrocyte differentiation with ATDC5 mock transfectant. Mock cells began to produce glycosaminoglycans at day 15, as shown by the formation of nodular structures and the Alcian blue-positive area. However, SF3b4 cells were negatively stained (i.e. they hardly ever generated glycosaminoglycans). B, quantification of the staining intensity by Alcian blue. The intensity of Alcian blue staining of the SF3b4-stable line (open circle) extracted with 6 M guanidine-HCl solution was also not increased at A630, whereas that of the mock transfectant was increased (solid circle). C, real time RT-PCR analysis of aggregcan gene (Agc) and type II collagen gene (Col2a1). The levels are indicated as fold that of day 2. D, real time RT-PCR analysis of SF3b4 during chondrocyte differentiation of ATDC5 cells. The asterisks indicate significance in B and C (Student’s t test; *, p < 0.01).
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![Table 1: SF3b4 has no significant effect on Smad1/5/8 phosphorylation.](image)

We further performed real time RT-PCR to make a quantitative comparison of the transcription levels of aggrecan (Agc) and α1 chain of type II collagen (Col2a1) genes. Whereas the level of Agc expression in mock transfectants increased to 45- and 42-fold that of day 2 at day 15 and day 20, the level in SF3b4 transfectant increased to 5- and 13-fold at day 15 and day 20. The level of Col2a1 expression in mock transfectants increased to 30- and 64-fold at day 15 and day 20, but that in SF3b4 transfectant increased to only 7- and 18-fold at day 15 and 20, respectively (Fig. 4C).

Since the overexpression of SF3b4 inhibited chondrocyte differentiation, we hypothesized that the endogenous level of SF3b4 is down-regulated during the differentiation. By real-time RT-PCR, its expression level declined to ~30% that at day 2. This reciprocal expression pattern of SF3b4 strongly suggests that chondrocyte differentiation proceeds by down-regulating the expression of SF3b4 (Fig. 4D).

**SF3b4 Inhibits the Smad1/5/8 Pathway but Not the Smad2/3 Pathway**—To determine the nature of the inhibitory effect of SF3b4 on osteochondrogenic differentiation, we examined a number of pathways known to be activated by BMP-2/4.

Initially, we investigated the effects of SF3b4 on Smad1/5/8 phosphorylation in ATDC5 and C2C12 cells. By immunoblot for phospho-Smad1/5/8, the phosphorylation was observed as early as 10 min after BMP-2 treatment, peaked at 30 min, sustained up to 60 min, and rapidly attenuated in the SF3b4-expressing and the control cells (Fig. 5). There were no significant differences of the Smad1/5/8 phosphorylation patterns up to 180 min between the two cell types.

As the bands of phospho-Smad1/5/8 become rapidly attenuated after 120 min, effects of SF3b4 on Smad1/5/8 in the following time periods could not be evaluated by immunoblot analysis. Thus, we attempted to investigate the effects, using a reporter gene assay. When a reporter plasmid pGL3-Id985WT, known to be specific to the Smad1/5/8 pathway (20), was used, reporter gene activity up to 9-fold. The co-expression of SF3b4 together with them inhibited this activity in a dose-dependent manner (Fig. 6A). When a truncated mutant of SF3b4 that lacks the Pro-Gly motif and therefore does not bind BMPR-IA was expressed in place of the full-length SF3b4, no inhibition of the reporter gene activity was observed (Fig. 6B). The effects of SF3b4 on TGFβ1 signaling were examined using the pGL3ti-(SBE)4 reporter gene assay (21). The expression of Smad4 in the presence of TGFβ1 (10 ng/ml) activated the reporter gene ~3.3-fold, but co-expression of SF3b4 did not affect activity (Fig. 6C). These results indicate that SF3b4 specifically inhibits the BMP-2/4-mediated Smad1/5/8 pathway without affecting the initial phosphorylation of Smad1/5/8, by interacting with BMPR-IA.

**SF3b4 Has No Significant Effects on MAPK Pathways**—Since MAPK pathways play critical roles in chondrocyte proliferation and differentiation (27–29), we examined the effects of SF3b4 on p38 MAPK, ERK1/2, and SAPK/JNK in confluent ATDC5 cells, using both ELISA (data not shown) and immunoblot analysis (Fig. 7). BMP-2 increased the phosphorylation of p38 MAPK with a peak at 30–60 min in both control and SF3b4-overexpressing cell lines, with the same phosphorylation patterns (Fig. 7A). BMP-2 phosphorylated ERK1/2 with the peak at 15–30 min in both cell types. ELISA and immunoblot analysis exhibited no significant differences of phosphorylation patterns between the two (Fig. 7B). BMP-2 did not phosphorylate SAPK/JNK in both cell types (Fig. 7C).

**SF3b4 Decreases Cell Surface BMPR-IA Levels**—Although SF3b4 down-regulated the osteochondral differentiation, it had no effects on phosphorylation of Smad1/5/8 and MAPKs. We speculated that SF3b4 might regulate the cell surface levels of BMPR-IA. When different levels of SF3b4 were expressed in HA-tagged BMPRI-A-expressing stable COS7 cells, the levels of the receptor on the cell surface decreased in accordance with the expression levels of SF3b4 (Fig. 8), suggesting that SF3b4 may down-regulate the BMP-mediated signal transduction by decreasing BMPR-IA on the cell surface.

**Molecular Modeling of BMPR-IA**—Based on the findings that SF3b4 did not bind BMPR-IB ICD, we searched for a clue to the specific binding of SF3b4 with BMPR-IA using the difference in amino acid sequences between BMPR-IA ICD and BMPR-IB ICD and the three-dimensional structure model of BMPR-IA ICD. Comparison of the ICDs in human BMPR-IA and -IB revealed 12 positions where amino acid groups were different between the two. These positions were conserved in either the BMPR-IA or -IB subfamily. All of these positions were located in the C-terminal lobe (Figs. 9 and 10), and two were in the activation segment common to kinases of the T/R family.

In the three-dimensional structure model, all of the positions except for Ala339 were observed on the protein surface (Fig. 10, B–D), and Tyr458, Ile482, Val483, Asn488, Ala495, and Leu497 were spatially close to each other (Fig. 10D). The surface area formed by Tyr458, Ile482, Val483, Asn488, Ala495, and Leu497 was about 380 Å². Although this area appeared smaller than the average interface area of the heterocomplex (30), it could be the interaction surface if the surrounding amino acid residues were also involved in the interaction. Thus, we investigated whether this surface is involved in the interaction using several point mutations. SF3b4 bound to the ICD of BMPR-IA when even five of the six amino acid residues were replaced with the corresponding amino acid residues in BMPR-IB (data not shown).

The inability of SF3b4 to bind the constitutively active Q223D form suggested that the conformational change of the ICD by phosphorylation might abrogate the binding. Thus, we tested whether the activation segment is responsible for the interaction by mutation of Asn388 and Leu398. These amino acid residues were replaced with the amino acid residues in BMPR-
IB. SF3b4 bound the mutant, excluding direct involvement of the activation segment in the interaction with SF3b4.

DISCUSSION

In this study, we have found that SF3b4 interacts with the intracellular domain of BMPR-IA, using a yeast two-hybrid system. There is no significant difference between ATDC5 control and SF3b4-stable lines. BMP-2 treatment in both control and stable lines. There is no significant difference between the two. Co-expression of SF3b4 on SAPK/JNK pathway were examined. Immunoblotting does not detect phospho-SAPK/JNK after BMP-2 treatment in both cell types.

**FIGURE 7. SF3b4 has no effect on MAPK phosphorylation.** A, on immunoblotting, p38 phosphorylation is increased at 30 min after BMP-2 treatment. There is no significant difference between ATDC5 control and SF3b4-stable lines. B, in ATDC5 cells, ERK1/2 phosphorylation is observed at 30 min after BMP-2 treatment in both control and stable lines. There is no significant difference between the two. C, effects of SF3b4 on the Smad2/3 pathway mediated by TGFβ were examined. Immunoblotting does not detect phospho-SAPK/JNK after BMP-2 treatment in both cell types.

**FIGURE 8. SF3b4 decreases cell surface levels of BMPR-IA.** COS7 cells stably expressing HA-tagged BMPR-IA were transfected with different amounts of a SF3b4 expression vector (0, 0.5, 1, and 2 μg) and a mock plasmid. After 48 h, the cell surface proteins were labeled with biotin and precipitated with streptavidin-agarose CL4B and applied to the immunoblot. SF3b4 is expressed at levels in a dose-dependent manner (top). Immunoblot results of the cell surface BMPR-IA (middle) and total BMPR-IA (bottom) by anti-HA (12CA5) are shown.

IB. SF3b4 bound the mutant, excluding direct involvement of the activation segment in the interaction with SF3b4.
tem. SF3b4, originally identified as a member of the spliceosome complex U2 small nuclear ribonucleoprotein, contributes to the recognition of the intron’s branch point (31). Although known to be localized in the nucleus, SF3b4 is also fractionated into the cell membrane together with BMPR-IA, and they interact with each other in the cell. Overexpression of SF3b4 inhibits the BMP-mediated Smad1/5/8 pathway but not the TGFβ-mediated Smad2/3 pathway as assessed by reporter gene assays. Furthermore, SF3b4 substantially delays the osteogenic and chondrocytic differentiation of C2C12 and ATDC5 cells, respectively. Although SF3b4 has no significant effects on phosphorylation of Smads and MAPKs, it down-regulates the cell surface levels of BMPR-IA. Our findings clearly demonstrate a novel role of SF3b4 to regulate BMP-mediated osteochondral cell differentiation by binding BMPR-IA.

SF3b4 was the only molecule that bound the ICD of BMPR-IA of 700,000 clones screened in the yeast two-hybrid system. Using a similar system, Nishanian and Waldman (32) identified the same molecule that binds the ICD of BMPR-IA, which confirms our results. Since no other molecules, such as BRAM1...
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(17), were found, SF3b4 appears to bind BMPR-IA more strongly than other molecules previously identified. When the constitutively active form (Q223D) of BMPR-IA was used as bait, no molecule was cloned. These results suggest that SF3b4 interacts with nonphosphorylated BMPR-IA or that the ternary structure of the Q223D form is distinct from the original BMPR-IA, although it conducts BMP signaling. Functional analysis with various truncation forms in the yeast two-hybrid system indicated that both the RRM2 and Pro-Gly motif are required for the interaction. Similar domain functional analysis by Nishanian and Waldman (32) demonstrated that sequential truncation of the Pro-Gly motif from the C terminus substantially attenuates the interaction, concluding that the N-terminal GS domain interacts with BMPR-IA, which contrasts to our data. When computer analysis using the GlobPlot program (33) was performed, the Pro-Gly motif was identified as a domain that lacks a definite three-dimensional structure under an isolated native condition (data not shown). Furthermore, the full-length SF3b4 is required for adequate interaction in mammalian cells. Taken together, the structural studies of the complex would be required to precisely identify the interacting sites.

We have shown that BMP-2 transduces p38 MAPK and ERK1/2 pathways in ATDC5 cells but that SF3b4 does not affect their phosphorylation. In contrast, SF3b4 substantially inhibits Smad1/5/8-specific Id reporter gene activity, whereas SF3b4 did not appear to affect the phosphorylation of Smad1/5/8 in the initial 2 h after BMP-2 treatment, as assessed by immunoblot analysis. There may be a slight difference in the phosphorylation levels that cannot be detected by the immunoblot, or the difference may occur in the following periods, when the phosphorylation is no more observed by immunoblot. Alternatively, the inhibitory effect may be independent of Smad1/5/8 phosphorylation. Actually, the region in the BMPR-IA that interacts with SF3b4 appeared to be independent of the Smad phosphorylation site. Our finding that SF3b4 decreases cell surface levels of BMPR-IA suggests that SF3b4 facilitates internalization of the receptor. The Pro-Gly motif of SF3b4 contains the PPXY motif, which binds the WW domain (34). A number of molecules containing the PPXY motif, including SF3b4, have been presented to bind the WW domain (35). For instance, Smurfl1, containing the WW domain, binds Smad7 at its PPXY motif and facilitates the internalization of TβR-I (36). Although we have not observed the direct interaction of SF3b4 with Smurfl1, SF3b4 may interact with molecules containing the WW domain and exert a specific function.

The accuracy of a three-dimensional structure model largely depends on the alignment of target and template (23). We aligned the sequence of the ICD of BMPR-IA and that of TβR-I without ambiguity, which enabled the generation of a reliable three-dimensional structure model. Since point mutations in the activation segment did not abrogate the interaction, SF3b4 appears to interact with sites indifferent to phosphorylation. Although we did not determine the binding region, we could exclude putative candidate regions. The mechanisms underlying the interaction of these receptors with Smads appear complicated. Indeed, the interaction of TβR-I with Smad2 has been demonstrated by co-immunoprecipitation under a condition in which phosphate transfer is blocked either by the kinase-negative mutation of TβR-I or by modifications of the C-terminal phosphorylation sites of Smads (37, 38). Direct binding of BMPR-I with Smad1/5/8 has not been reported even under nonphysiological conditions. The model of BMPR-IA generated in this study provides a useful tool toward understanding the specific function of these type I receptors.

We have demonstrated that SF3b4 inhibits BMP-mediated osteogenic and chondrocytic differentiation in C2C12 and ATDC5 cell types, respectively. Down-regulated SF3b4 expression during chondrocytic differentiation supports this inhibitory function. BMPs have various roles, whose specificity is probably determined by distinct intracellular machinery. The inhibitory effect of SF3b4 on cell differentiation may therefore be peculiar to osteochondrogenic cell lineages. Indeed, although SF3b4 directly binds BMPR-IA, its function has not been determined in other cell types (32). By in situ hybridization, SF3b4 is expressed at a high level in limbs and somites at embryonic day 11 (39), suggesting its involvement in skeletal development. Recent studies have suggested that spliceosomal proteins participate in cellular functions besides the original splicing function. Heterozygote mice of SF3b3 subunit 1 (another U2 small nuclear ribonucleoprotein component) knock-out exhibited various skeletal alterations along the anterior-posterior axis, with altered expression of Hox genes, and heterozygotes of SF3b3 subunit 2 display similar skeletal abnormality (40). Although these studies support our findings, the detailed mechanisms by which SF3b4 regulates the cell surface BMPRI-A remain to be elucidated.

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