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#### Citation Information

Sun, Jianping; Zhuang, Feng Feng; Mullersman, Jerald E.; Chen, Hui; Robertson, Elizabeth J.; Warburton, David; Liu, Yi Hsin; and Shi, Wei. 2006. BMP4 Activation and Secretion Are Negatively Regulated by an Intracellular Gremlin-BMP4 Interaction. *Journal of Biological Chemistry*. Vol.281(39). 29349-29356. <https://doi.org/10.1074/jbc.M603833200> PMID: 16880207 ISSN: 0021-9258

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# BMP4 Activation and Secretion Are Negatively Regulated by an Intracellular Gremlin-BMP4 Interaction\*

Received for publication, April 21, 2006, and in revised form, July 28, 2006 Published, JBC Papers in Press, July 31, 2006, DOI 10.1074/jbc.M603833200

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Bone morphogenetic protein 4 (BMP4) is a potent growth factor that is involved in many important biological processes. Regulation of the level of secreted mature BMP4 determines the biological effects of BMP4 on cells in the local microenvironment. Previous studies suggested that Gremlin, a member of DAN family proteins, antagonizes BMP4 activity by sequestering extracellular BMP4. Herein, we report a novel intracellular regulatory mechanism by which Gremlin interacts with BMP4 precursor, prevents secretion of mature BMP4, and therefore inhibits BMP4 activity more efficiently. Furthermore, we also defined a 30-amino acid peptide sequence within the Gremlin DAN domain that is essential for BMP4 interaction. This novel Gremlin-mediated BMP4 posttranslational regulatory mechanism implies that the level of BMP4 mRNA expression does not truly reflect BMP4 activity when Gremlin and BMP4 are coexpressed within the same cell. Similar regulatory mechanisms may be utilized by other DAN family proteins.

Bone morphogenetic protein 4 (BMP4)<sup>2</sup> is a well studied member of the BMP family (1), which plays an important role in many organ developmental processes including the basic body plan formation, the proximal-distal, left-right, and dorsal-ventral axes (2). BMP4 elicits different biological responses depending upon the concentration of the secreted active form. For example, at early embryonic stages, cells exposed to high concentrations of BMP4 will commit to a ventral fate, while cells exposed to low concentrations of active BMP4 will develop into dorsal neural and muscular tissues (3, 4). Therefore, controlling both expression and activation of BMP4 is critical for BMP4-mediated cell fate decision.

The expression and activity of BMP4 can be regulated at multiple molecular levels, including transcriptional and post-

translational regulation. At the protein level, BMP4 is initially synthesized as an inactive 50-kDa precursor protein within cells. Dimerization of the BMP4 precursor protein occurs by forming an intermolecular disulfide bond. Then, following proteolytic cleavage by members of the subtilisin-like proprotein convertase family, an active carboxyl-terminal mature BMP4 protein dimer (25 kDa for the monomer) is produced, which is then secreted outside the cell to act as a growth factor (5, 6, 7). Furthermore, the secreted active BMP4 can also be inhibited at the extracellular level by interacting with secreted BMP4 antagonists, such as noggin, chordin, Cer1, DAN, and Gremlin (8, 9, 10, 11).

Gremlin was originally identified as a molecule capable of inducing secondary axis formation in the *Xenopus* embryo (12, 13). Recombinant Gremlin protein is known to bind mature BMP2/4 *in vitro* and inhibits these BMP activities. A high level of Gremlin expression is found in nondividing and terminally differentiated cells such as neuron, alveolar epithelial cells, and goblet cells (14). The phenotypes of mice with Gremlin null mutation have shown that Gremlin plays an essential role in limb, lung, and kidney development, possibly due to inappropriate BMP signaling during the respective organ development (15, 16). However, the molecular mechanism of Gremlin-BMP4 interaction remains largely unexplored. Herein, we have found that Gremlin can specifically bind to BMP4 precursor protein inside cells, which prevents the production and secretion of mature BMP4 protein and thus down-regulates BMP4 ligand signaling in a most efficient manner. Thus, Gremlin functions as a highly efficient intracellular BMP4 antagonist, in addition to its classical extracellular antagonistic effect. Moreover, we have also mapped the protein sequences in Gremlin that mediate BMP4-Gremlin interaction.

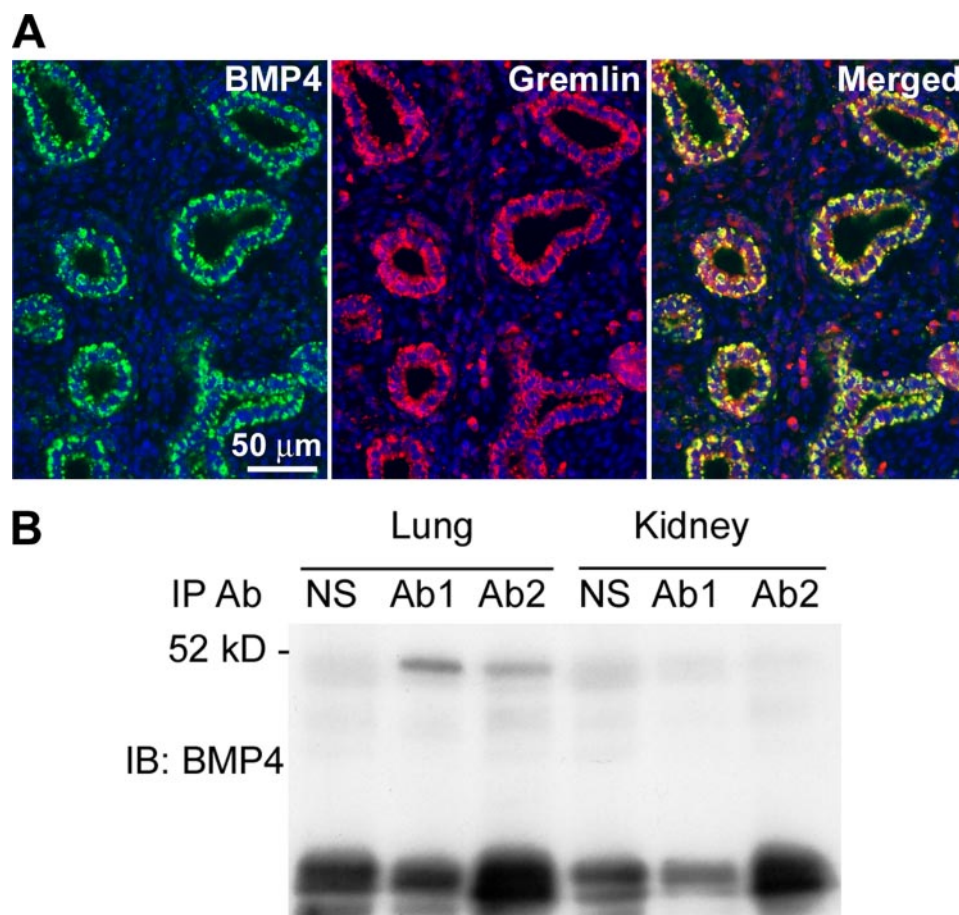
## EXPERIMENTAL PROCEDURES

**Antibodies and Reagents**—The antibodies to BMP4, Gremlin, TGN38, and c-Myc epitope-tag antibodies were purchased from Santa Cruz Biotechnology. Anti-HA epitope-tag antibody was purchased from Covalence. Alexa Fluor 594- or 488-labeled anti-mouse, anti-rabbit, and anti-goat secondary antibodies were purchased from Invitrogen. Human recombinant BMP4 was from R&D Systems. Short peptides corresponding to the amino acid sequence of mouse Gremlin 37–52 (DKAQHNDSEQTQSPPQ) and

\* This work was supported by National Institutes of Health Grants HL68597 and HL61286, American Heart Association grant-in-aid, and Childrens Hospital Los Angeles Research Career Developmental Award. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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<sup>2</sup> The abbreviations used are: BMP, bone morphogenetic protein; HA, hemagglutinin; RT, reverse transcription; CM, conditioned medium; ALP, alkaline phosphatase.



**FIGURE 1. Co-localization and interaction of BMP4 and Gremlin proteins in mouse fetal lung.** *A*, E14.5 mouse fetal lung tissue was co-immunostained by goat anti-BMP4 and rabbit anti-Gremlin antibodies. *B*, endogenous Gremlin protein in tissues lysate was immunoprecipitated (*IP*) by anti-Gremlin antibody (Ab1 from Santa Cruz Biotechnology; Ab2 generated in our laboratory), and the co-precipitated BMP4 precursor protein was detected by immunoblot (*IB*) using anti-BMP4 antibody. NS, normal serum control.

146–175 (PKKFTTMMVTLNCPQLPPTKKKRVRVVKQ) were synthesized by Genscript (Scotch Plains, NJ).

**Plasmid Constructs**—Myc-tagged mouse BMP4 cDNA (Myc-BMP4) and HA-tagged mouse Gremlin cDNA (GRE-HA), as generated previously (7), were subcloned into pcDNA3 expression vector. The Myc epitope was inserted into the carboxyl-terminal immediately adjacent to the cleavage site of mature BMP4, so that both precursor and mature forms of exogenously expressed BMP4 can be detected by Myc epitope. Partial cDNA of mouse Mucin-2, which encodes a Mucin-2 protein fragment of 114 amino acids on the carboxyl terminus (PQNQ . . . LGRK, GenBank<sup>TM</sup> accession number XP\_620590), was generated by high fidelity RT-PCR (Pfu, Stratagene) and verified by DNA sequencing. An HA epitope was also added to the carboxyl terminus of Mucin-2 by inverted PCR. Partial deletions of Gremlin and a variety of Gremlin-Mucin-2 chimeric cDNA constructs were created by inverted PCR in combination with restricted digestion and ligation of PCR products.

**Cell Culture and Transient Transfection**—COS-1 and C2C12 cells (ATCC) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in 5% CO<sub>2</sub>. Plasmid DNAs were transfected into the cells with Lipofectamine Plus (Invitrogen). The transfected cells were first cul-

tured in the medium containing 10% fetal calf serum for 20 h and then cultured in serum-free conditioned medium (CM) for additional 48 h prior to analysis. Experiments were repeated at least three times.

**Western Blot and Protein Co-immunoprecipitation**—Equal amounts of total cell lysate protein or CM from different samples were separated in NuPAGE 4–12% gradient SDS-PAGE gels using a MOP buffering system (Invitrogen). After protein was transferred into polyvinylidene difluoride membrane, proteins of interest were detected by specific antibodies. Proteins in different subcellular compartments were fractionated using the Proteo-Extract subcellular proteome extraction kit (Calbiochem). The fractionation of cytosolic, membrane/organelle, and nucleic proteins has been confirmed as reported (17).

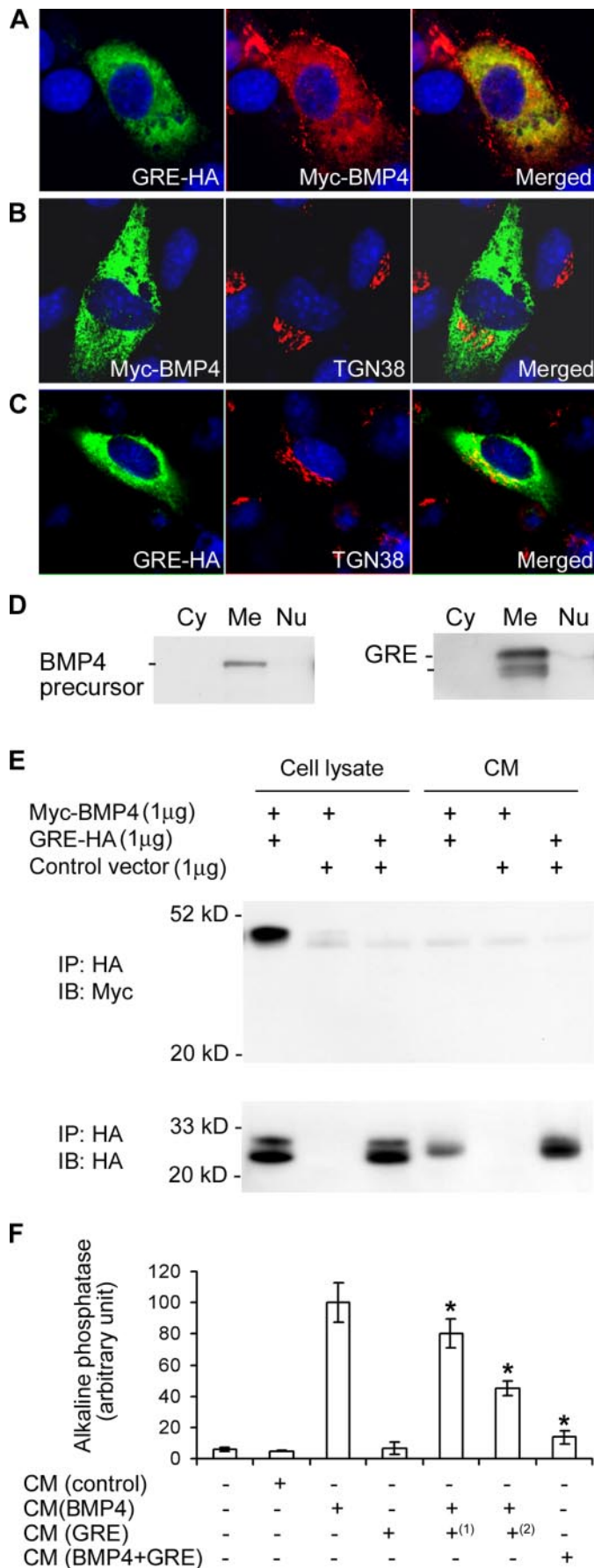
For co-immunoprecipitation experiments, cells were lysed at 4 °C in Nonidet P-40 lysis buffer. Lysates were cleared of insoluble material by centrifugation, followed by incubation with Protein A/G-agarose beads (Santa Cruz Biotechnology). The supernatants of cell lysates were then incubated with HA-specific antibody for 1 h, followed by

further incubation with Protein A/G-agarose beads overnight at 4 °C. The precipitated protein complexes were examined by Western blot.

**Immunostaining**—Co-immunofluorescence staining of endogenous BMP4 and Gremlin was performed in E14.5 embryonic mouse lung using rabbit anti-Gremlin and goat anti-BMP4 antibodies. Co-localization of exogenously expressed Myc-BMP4 and GRE-HA in cultured cells was detected by Myc and HA epitope co-immunofluorescence staining under a Zeiss LSM510 confocal microscope with 400X optical magnification and 4× digital zoom.

**Measurement of BMP4 Activity**—BMP-stimulated mouse myoblast C2C12 cell differentiation into osteoblast cells was used to measure BMP4 activity. Briefly, C2C12 cells were grown in 96-well plate until 90% confluence. Agents (BMP4 or conditioned medium) were then added into the culture medium (Dulbecco's modified Eagle's medium with 5% fetal bovine serum), and the cells were cultured for another 3 days. The cells were then lysed with 0.1% Triton X-100, and the alkaline phosphatase (ALP) activity in the cell lysate was quantified by adding substrate *p*-nitrophenyl phosphate and measuring *A*<sub>405</sub>. Triplicate measurements were always performed for each sample. For BMP4 inhibitory assay by Gremlin peptides, the culture medium containing BMP4 (100 ng/ml) was preincu-





bated with the related peptide at a concentration of 0.5  $\mu\text{M}$  for 3 h at 37  $^{\circ}\text{C}$  before added into C2C12 cells.

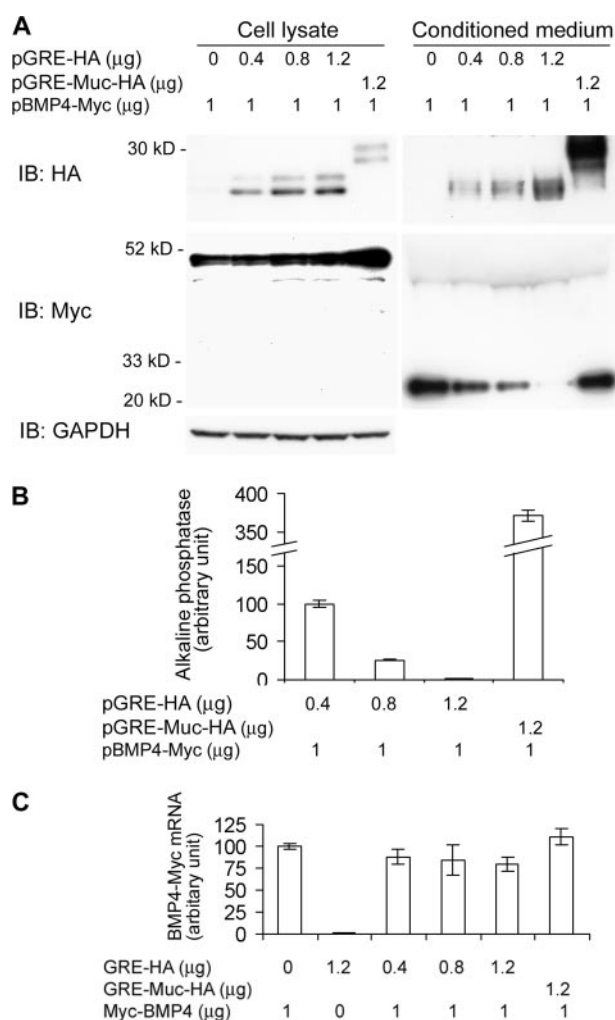
## RESULTS

**BMP4 Precursor Interacts with Gremlin Intracellularly in Mouse Embryonic Lung**—BMP4 gradient formation in tissue is thought to be determined by extracellular interaction between BMP4 and related antagonists. However, for cells that coexpress BMP4 and BMP antagonists, intracellular interaction between BMP4 and BMP antagonists cannot be excluded. To determine the interaction between Gremlin and BMP4, the endogenous protein expression patterns of these two molecules were first compared in developing lung, where both BMP4 and Gremlin play important roles in its organogenesis (15, 16). By co-immunofluorescence staining, Gremlin and BMP4 exhibited a partially overlapping epithelial expression pattern in E14.5 mouse embryonic lung (Fig. 1A), suggesting the possibility that these two proteins could interact intracellularly. Therefore, physical interaction of endogenous Gremlin and BMP4 precursor inside cells was examined by co-immunoprecipitation in mouse fetal tissue lysate. As shown in Fig. 1B, a precursor form of BMP4 (50 kDa) was specifically co-immunoprecipitated with Gremlin protein in fetal mouse lung tissue lysate, but not in kidney tissue, which suggested that tissue specific intracellular interaction between Gremlin and BMP4 precursor can occur *in vivo*. The biological significance of intracellular Gremlin-BMP4 precursor protein interaction and related molecular mechanisms were then further analyzed in cultured cells.

**Intracellular Interaction of Gremlin-BMP4 Precursor Inhibits BMP4 Activity Most Efficiently**—Intracellular interaction of BMP4 and Gremlin was further studied by coexpression of these proteins in cultured C2C12 cells. Both HA epitope-tagged Gremlin (GRE-HA) and Myc-epitope tagged BMP4 (Myc-BMP4) were co-localized in the cytoplasm of co-transfected cells, as shown by co-immunofluorescence staining under a confocal microscope (Fig. 2A). Furthermore, expressed Myc-BMP4 or GRE-HA was also co-localized with TGN38, a marker for the trans-Golgi network (18), suggesting that both proteins are trafficking through the same intracellular compartments (Fig. 2B-2C). The subcellular localizations of Myc-BMP4 and GRE-HA were also verified by Western blot detection in fractionated cell lysates (Fig. 2D). Expression of both Myc-BMP4 and GRE-HA was detected in the membrane/organelle protein

**FIGURE 2. Subcellular localization and protein interaction of coexpressed BMP4 and Gremlin.** A–C, detection of protein expression in co-transfected C2C12 cells under confocal microscope. Expression of Myc-BMP4 and GRE-HA proteins overlaps in cytoplasm (A). Trans-Golgi marker TGN38 was also overlapped with either Myc-BMP4 (B) or GRE-HA (C). Cell nuclei were counterstained by 4',6-diamidino-2-phenylindole (blue color). D, subcellular localization of expressed Myc-BMP4 and GRE-HA was also determined by Western blot detection in different subcellular fractions (Cy, cytosol; Me, membrane/organelle; Nu, nuclei). E, BMP4 precursor protein, but not mature BMP4, was specifically co-immunoprecipitated with Gremlin in co-transfected COS-1 cell lysate. F, BMP4 activities in the conditioned media (CM) of COS-1 cells transfected with the indicated plasmids were evaluated by their stimulatory effects on C2C12 cell differentiation into osteoblast cells, as measured by ALP activity. (1), CM collected separately from the COS-1 cells transfected with either Myc-BMP4 or GRE-HA were mixed and preincubated for 1 h before added into C2C12 cells; (2), COS-1 cells transfected separately with either Myc-BMP4 or GRE-HA were trypsinized and co-cultured during CM collection. \*,  $p < 0.05$  as compared with the CM with BMP4 only.

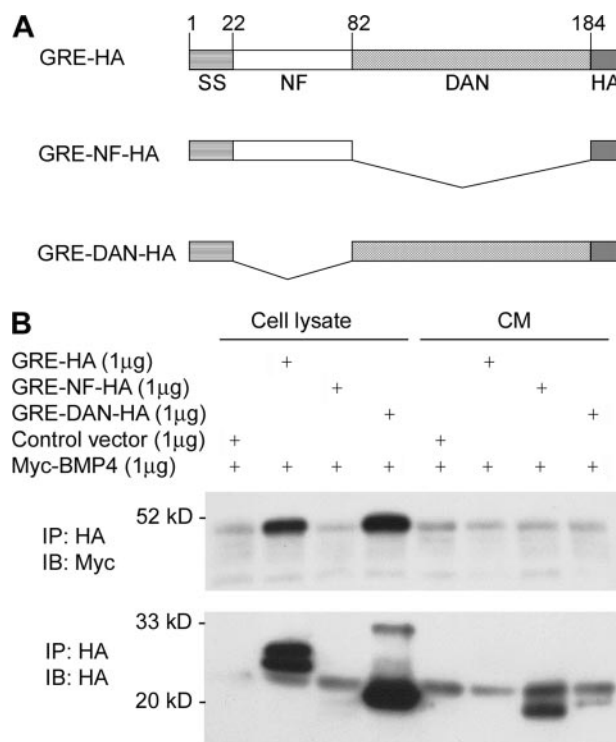
## Gremlin and BMP4 Intracellular Interaction



**FIGURE 3. Expression of Gremlin prevents mature BMP4 secretion from co-transfected COS-1 cells.** *A*, COS-1 cells were co-transfected with the indicated expression vectors, and the expressed GRE-HA and Myc-BMP4 proteins were detected in both cell lysate and concentrated CM with anti-HA and anti-Myc antibodies, respectively. GRE-Muc-HA is a mutated Gremlin that does not bind to BMP4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) detection was used for protein loading control. *B*, the net activities of BMP4 in the CM of the co-transfected cells were measured based on its effect on C2C12 cell differentiation into osteoblast cells by adding equivalent amount of CM into C2C12 cell culture medium. *C*, the same level of exogenous BMP4 mRNA was confirmed by Myc-tag-specific quantitative real-time RT-PCR.

extract fraction (including endoplasmic reticulum and Golgi) but not in the cytosolic and nucleic protein extract fractions. The physical interaction between BMP4 and Gremlin proteins in co-transfected COS-1 cells was then examined by protein co-immunoprecipitation. Myc-BMP4 precursor in cell lysate, but not mature Myc-BMP4 protein in conditioned medium (CM), was co-immunoprecipitated with GRE-HA (Fig. 2E).

The activity of BMP4 secreted into CM was then measured by its effect on myoblast C2C12 cell differentiation into osteoblasts (Fig. 2F), in which characteristic ALP is expressed and the ALP level reflects BMP4 activity. The BMP4 activity in the CM of Myc-BMP4 transfected cells was inhibited by  $20 \pm 9\%$  when an equal amount of CM collected from GRE-HA transfected cells was added. Interestingly, the CM collected from mixed cells that had been transfected separately by either Myc-BMP4



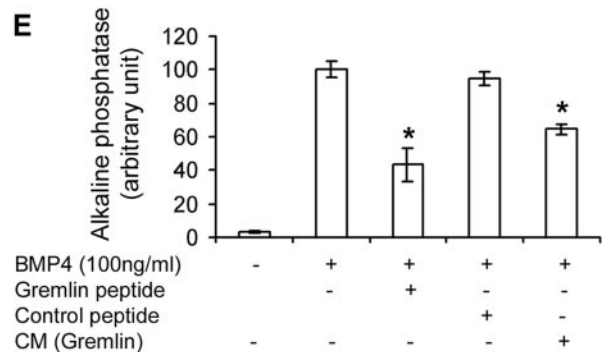
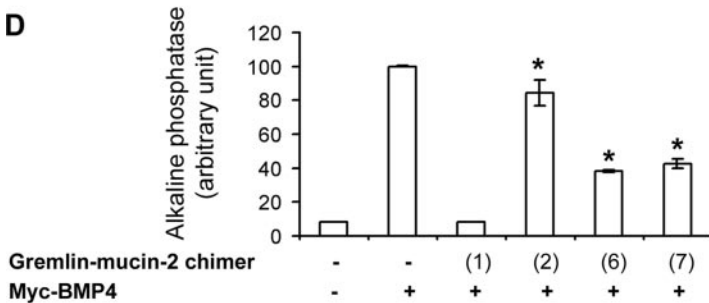
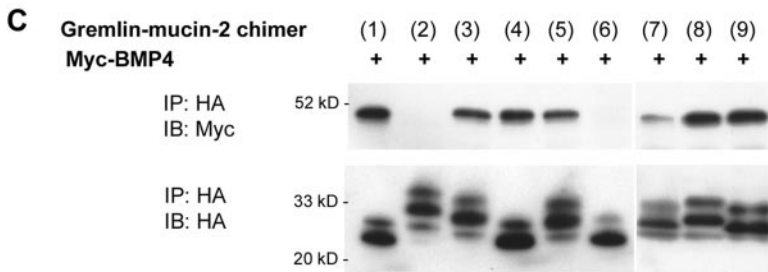
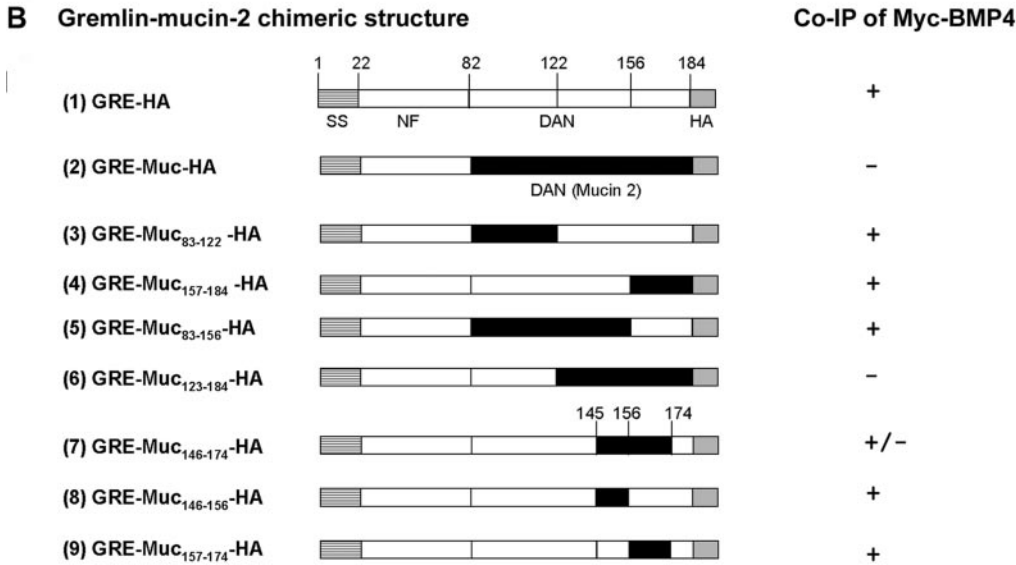
**FIGURE 4. Gremlin DAN domain is essential for its BMP4 binding activity.** *A*, schematic diagram of truncated Gremlin molecules. *B*, co-immunoprecipitation of Myc-BMP4 with different mutant Gremlin molecules from co-transfected COS-1 cell lysate and CM was detected by Western blot.

or GRE-HA exhibited a higher BMP4 inhibitory effect ( $55 \pm 5\%$ ), possibly due to prolonged interaction between BMP4 and Gremlin in the CM. However, BMP4 activity in the CM from cells co-transfected with equal amounts of both GRE-HA and Myc-BMP4 was even more markedly inhibited ( $86 \pm 4\%$ ,  $p < 0.05$ ), suggesting a more efficient inhibitory effect of intracellular Gremlin-BMP4 interaction than extracellular antagonistic effect (Fig. 2F).

**Intracellular Gremlin-BMP4 Precursor Interaction Prevents Mature BMP4 Secretion**—The precursor and mature forms of BMP4 protein in transfected cell lysates and CM were further analyzed. Consistent with previous reports (7), no mature BMP4 was detected in the cell lysate, whereas mature but no precursor BMP4 was detected in concentrated CM (Fig. 3A). Most interestingly, reduced amounts of secreted mature BMP4 protein in CM were detected in inverse proportion to intracellular Gremlin expression (Fig. 3A). Therefore, lack of mature BMP4 in the CM of co-transfected cells may explain that no mature BMP4 was co-immunoprecipitated with Gremlin, as shown above in Fig. 2E. The level of BMP4 precursor in cell lysate was not significantly increased, even though BMP4 secretion was reduced, suggesting that BMP4-Gremlin complex may be subject to facilitated degradation to maintain the same level of BMP4 precursor pool inside cell. Furthermore, both secreted forms of Gremlin and mature Myc-BMP4 in CM were dramatically increased when coexpressed Gremlin was mutated to lose its BMP4 binding activity (Gre-Muc-HA, see below for details), while the level of mutated Gremlin was low in cell lysate. These data suggest that intracellular interaction of Myc-BMP4 precursor and GRE-HA may prevent Myc-BMP4 precursor proc-

**A**

Gremlin	83	VTERKYLKRDWCKTQPLKQT	IHEEGCNSRTIINRF	<b>CYGCNSFY</b>	IPRHIRKEEGSFQSC	<b>SCFCK</b>	145
Mucin-2	825	---PQNQTRVPCSAVSV	MKEISYNGCTKNISMN	-YCFGSGCTFAM	--YSAQVQGLDHR	<b>CSCCK</b>	881
		C <sub>1</sub>	C'	C <sub>2</sub>	C <sub>3</sub>	C <sub>X</sub> C <sub>4</sub>	
Gremlin	146	PKKFTTMMVT	LNCPELQPPTKKKRV	TRVKQ	<b>CRCISIDLD</b>		184
Mucin-2	882	EEKTSVRSVTLE	CPD--GSELSHTY	THIESCLCQD	TVCGLPQAQQV	TRRRSSPRFLGRK	938
		C'		C <sub>5</sub> C <sub>6</sub>			

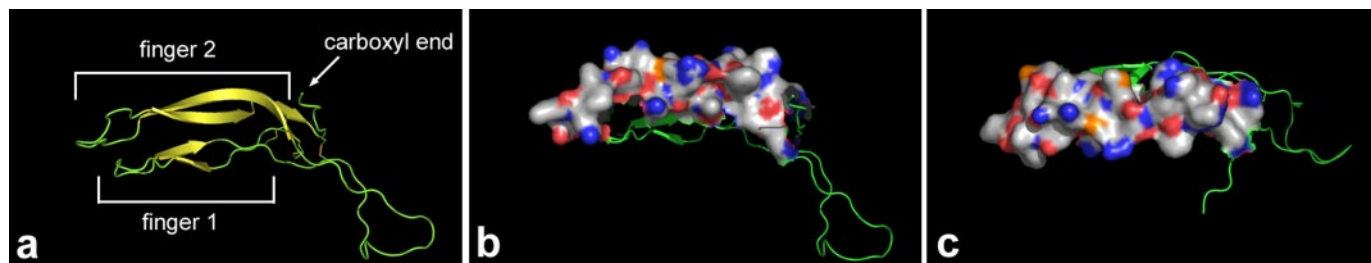


**FIGURE 5. Mapping BMP4-binding motif on Gremlin DAN domain.** *A*, DAN domain protein sequences between Gremlin and Mucin-2 are aligned, and the identical residues are highlighted in **bold**. The Cys residues for the knot structure are also depicted. *B*, a schematic diagram for a variety of Gremlin-Mucin-2 chimeras is illustrated. The result of BMP4-Gremlin co-immunoprecipitation in *C* is also summarized. *C*, co-immunoprecipitated Myc-BMP4 and Gremlin were detected by Western blot using the related epitope antibodies. *D*, net activities of BMP4 in CM from transfected COS-1 cells were analyzed by their stimulatory effects on osteoblast cell differentiation of C2C12 cells, as measured by ALP. The numbered Gremlin-Mucin-2 chimeras are indicated as shown in *B*. \*,  $p < 0.05$  as compared with the inhibitory activity of GRE-HA. *E*, addition of a synthetic peptide (0.5  $\mu$ M) corresponding to the mapped 30-amino acid sequence (145–174) of the Gremlin DAN domain significantly inhibited BMP4 (100 ng/ml)-stimulated osteoblast cell differentiation of C2C12 cell line. Another synthetic peptide with the amino acid sequence from the N-terminal fragment (37–52) of Gremlin was used as a negative control, and the CM of Gremlin-transfected COS-1 cells was used as a positive control. \*,  $p < 0.05$  as compared with BMP4 ligand only.

essing and mature Myc-BMP4 secretion. The net BMP4 activities in the above CM were also quantified by the BMP-stimulated C2C12 cell differentiation assay (Fig. 3*B*). The

alterations of BMP4 activities in the CM were consistent with changes in secreted BMP4 mature protein levels as detected by Western blot in Fig. 3*A*. Moreover, transient Myc-BMP4 gene





**FIGURE 6. Structure modeling of Gremlin DAN domain.** *a*, the ribbon structure of Gremlin DAN domain (amino acids 94–184) is predicted by homology modeling using the program Modeler version 8.1 (28), based on the published structure of human chorionic gonadotropin chain B (27).  $\beta$ -Strand is represented as yellow-colored arrow. *b* and *c*, filled structure of the finger 2 in Gremlin DAN domain is viewed from the side (*b*) and the top (*c*) for the protein interface. Atoms are colored as white for carbon, red for oxygen, and blue for nitrogen. The side chains of lysine and arginine contribute to the positively charged surface by their nitrogen-containing amino groups.

expression at the mRNA level remained similar in all samples with different levels of GRE-HA expression (Fig. 3C), as verified by quantitative real-time PCR using a Myc-tag specific primer and a protocol published previously (19). This excludes the possibility that changes in mature Myc-BMP4 in the CM are due to differences in BMP4 gene expression.

**Gremlin DAN Domain Is Essential to Mediate Gremlin-BMP4 Interaction**—The activity of BMP4 is thought to be precisely regulated extracellularly by many molecules defined as BMP antagonists (9), which have a cystine-knot structure similar to BMPs themselves. DAN family proteins are one subgroup of BMP antagonists with a conserved eight-cystine membered knot structure on their carboxyl terminus, which distinguish them from other BMP antagonists with a nine or 10-membered cystine ring (20). The DAN family includes Gremlin (14, 12), PRDC (21), coco (22), Cer1 (homologue of *Xenopus* Cerberus (10), DAN (23), USAG-1 (24), and sclerostin (25, 26), which are able to directly interact with BMPs and prevent BMPs from binding to their receptors. However, the molecular mechanisms of their protein interaction have never been explored. To further determine the specificity of BMP4-Gremlin binding and the molecular motif in Gremlin that is responsible for this intermolecular interaction, two Gremlin mutant molecules, GRE-DAN-HA and GRE-NF-HA, were generated with truncation of the NH<sub>2</sub>-terminal fragment or the carboxyl-terminal DAN domain, respectively (Fig. 4). Removal of the DAN domain from the Gremlin molecule (GRE-NF-HA) fully abolished its BMP4 binding ability and resulted in rapid and increased secretion of this truncated Gremlin molecule into the CM. In contrast, deletion of the N-terminal fragment of Gremlin did not have any impact on its BMP4 binding ability. Most of the GRE-HA and GRE-DAN-HA proteins with BMP binding activity were retained in the cell lysates. This suggests that the DAN domain in Gremlin is essential for BMP4 binding and that intracellular BMP4 precursor-Gremlin protein complex is not secreted outside the cell.

**Mapping the BMP4 Binding Motif of Gremlin DAN Domain**—Not all the proteins with a highly conserved eight-membered Cys-knot DAN domain homology are able to bind to BMP4 or other BMPs. One of these proteins is Mucin-2 (Fig. 5A). Replacement of the Gremlin DAN domain with the corresponding Mucin-2 DAN domain sequence fully eliminated both its BMP4 binding activity and its BMP4 inhibitory function (GRE-Muc-HA in Figs. 3 & 5, B–D). To further determine

the protein sequence motif of Gremlin DAN domain that is responsible for BMP4 interaction, we made a variety of Gremlin-Mucin-2 chimeras within the DAN domain region without changing its Cys-knot structure (Fig. 5B). These Gremlin-Mucin-2 chimeric proteins were coexpressed with Myc-BMP4 in COS-1 cells by transient transfection. The related BMP4 binding and inhibitory activities were evaluated by co-immunoprecipitation and BMP4-stimulated C2C12 cell differentiation assay. Thus, a 30-amino acid region corresponding to Gremlin amino acid sequence 145–174 (PKKFTTMMVTLNCPQLPPTKKRVTR-VKQ) appears essential to mediate the interaction between Gremlin and BMP4 and its BMP4 inhibitory effect (Fig. 5, B–D). Next, the function of this molecular motif in mediating the interaction between Gremlin and mature BMP4, which is the mechanism of Gremlin antagonistic effect on active BMP4, was tested in the BMP4-induced C2C12 cell differentiation assay. Interestingly, preincubation of active BMP4 (100 ng/ml) with a synthesized linear peptide corresponding to the mapped amino acid sequences (145–174, 0.5  $\mu$ M) significantly inhibited BMP4 activity by  $56 \pm 10\%$  ( $p < 0.05$ , Fig. 5E), similar to the BMP4 inhibitory effect obtained by preincubation with full-length Gremlin in the CM ( $35 \pm 3\%$  inhibition in Fig. 5E). Meanwhile, a control peptide with the amino acid sequence of Gremlin NH<sub>2</sub>-terminal fragment (37–52) at the same concentration (0.5  $\mu$ M) did not display any inhibitory effect on BMP4 signaling activity.

An approximate structure of mouse Gremlin DAN domain (amino acid 94–184; CKTQP . . . SIDLD) was then determined with respect to the published structure of human chorionic gonadotropin chain B by homology modeling using the program Modeler version 8.1 (Fig. 6; Refs. 27 and 28). As previously delineated by Avsian-Kretschmer and Hsueh (20), human chorionic gonadotropin chain B is a good homology modeling template for Gremlin because they both share cystine knots that form eight-membered rings and have “fingers” of similar size. The mapped 30-amino acid region (145–174) spans across a whole finger structure (finger 2), which provides a surface that is independent of the rest of the molecule. In addition, the most dominant feature of finger 2 in Gremlin DAN domain appears to be the positively charged lysine and arginine residue side chains spread across the surface, suggesting that this positively charged surface might bind to the negatively charged patch (residues of aspartic acid and glutamic acid) that has been observed on carboxyl-terminal BMP2/4.



## DISCUSSION

Our studies indicate that inhibition of BMP4 activity can be achieved either by intracellular interaction with Gremlin when coexpressed in the same cells or by an extracellular antagonistic mechanism if Gremlin is expressed in different cells. The intracellular interaction and subsequent inhibition of BMP4 secretion by Gremlin may provide an efficient and fine regulatory mechanism to direct different cellular fates by changing autocrine/paracrine activity in neighboring cells within a small microenvironment or may provide a negative feed back mechanism for certain cells. The intracellular interaction between Gremlin and BMP4 appeared to be tissue-specific, as detected in embryonic lung, but not in embryonic kidney. Thus, Gremlin and BMP4 may not coexpress in the same cells in kidney at this specific stage. Alternatively, BMP family members other than BMP4 may bind to Gremlin in kidney, or BMP antagonists other than DAN family proteins, such as CRIM1 (29), may be the major players in regulating BMP signaling activity in kidney and other tissues.

As reported previously, cleavage of BMP4 precursor into the mature form of BMP4 occurs in the trans-Golgi network (7). Co-immunoprecipitation of Gremlin with the precursor form of BMP4 in cells co-transfected with Gremlin and BMP4 suggests that the intracellular protein interaction between BMP4 and Gremlin occurs in the subcellular compartments before these proteins enter the trans-Golgi network during the secretion process (co-translational or endoplasmic reticulum sites). Binding of Gremlin appears to affect the processing of precursor to mature BMP4 in cells and subsequently inhibit BMP4 secretion. This could be a most efficient and rapid way to down-regulate BMP4-mediated signal activity in cells. However, the exact physiological roles of this intracellular Gremlin regulatory function *versus* extracellular antagonistic function *in vivo* needs to be further investigated.

The intracellular inhibition of BMP4 by Gremlin interaction also implies that measurement of BMP4 ligand mRNA expression level alone by RT-PCR and/or *in situ* hybridization, which are commonly used to evaluate BMP4 activity in developmental biology studies, may not reflect the actual BMP4 activity, since that approach neglects the important posttranslational processing of the BMP4 precursor protein regulated by Gremlin. Whether this intracellular inhibitory effect of Gremlin is a common mechanism to all DAN family proteins remains to be determined. van Bezooijen *et al.* (30) reported that sclerosteosis resulting from a defective sclerostin did not occur via a classical BMP antagonistic mechanism, since addition of exogenous sclerostin into mouse C2C12 cell culture medium did not antagonize BMP-stimulated ALP activity. Based upon our data, an intracellular BMP inhibitory effect may well mediate the biological effect of sclerostin. This novel intracellular Gremlin-BMP4 precursor interaction may therefore help us to more precisely understand the regulation of BMP signaling mechanisms in physiological as well as pathological situations.

By replacing Gremlin DAN domain with the corresponding Mucin-2 DAN domain protein sequences, the BMP4 binding and inhibitory activity of Gremlin is fully abolished, confirming the specificity of intracellular Gremlin-BMP4 interaction. Fur-

thermore, a 30-amino acid peptide motif in Gremlin DAN domain was mapped by introducing a variety of mutations in the protein sequences of Gremlin DAN domain without disruption of the overall Cys-knot structure. From molecular modeling, we speculate that these 30-amino acid residues constitute an independent surface finger structure with multiple positively charged lysine and arginine residues, providing a docking site for negatively charged patch on BMP ligands. Thus, BMP4 inhibition by Gremlin-BMP4 interaction may be mediated by a mechanism that is different from other non-DAN family antagonist-mediated BMP inhibition, such as Noggin (31). Noggin-BMP7 binding is mediated by hydrophobic interaction that masks the hydrophobic interface required for BMP receptor I and II binding (31, 32). Alternatively, Gremlin-BMP4 interaction could result in BMP4 conformational changes, which prevents BMP4 precursor cleavage and maturation within the cell, and reduces BMP4-receptor binding activity outside the cell.

*Acknowledgment*—We thank Sue Buckley for critically reading the manuscript.

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