Identification and Characterization of a Novel Follistatin-like Protein as a Binding Protein for the TGF-β Family*

Follistatin is an activin-binding protein that prevents activin from binding to its receptors and neutralizes its activity. Follistatin also binds bone morphogenetic proteins (BMPs). In this study, we report the identification of a novel follistatin-like protein from mouse. The mouse cDNA encodes a 256-residue precursor and most likely a mouse homologue of human FLRG, which was found at the breakpoint of the chromosomal rearrangement in a B-cell line. Whereas follistatin has three follistatin domains, which are presumed to be growth factor binding motifs, FLRG possesses only two follistatin domains. Northern blotting revealed that mRNAs for FLRG were abundantly expressed in heart, lung, kidney, and testis in mouse. The recombinant mouse FLRG proteins were found to have binding activity for both activin and bone morphogenetic protein-2. Like follistatin, FLRG has higher affinity for activin than for BMP-2. The FLRG protein inhibited activin-induced and BMP-2-induced transcriptional responses in a dose-dependent manner. Glutathione S-transferase fusion proteins encoding various regions of FLRG were produced and studied. Ligand blotting using 125I-activin revealed that the COOH-terminal region containing the second follistatin domain was able to bind activin. Our finding implies that cellular signaling by activin and BMPs is tightly regulated by multiple members of the follistatin family.

The members of the transforming growth factor-β (TGF-β) superfamily includes TGF-βs, activin, inhibin, and bone mor-

phogenetic proteins (BMPs) (1). They regulate diverse biological cellular functions such as cellular growth, differentiation, and development.

The activities and cellular signaling of the TGF-β superfamily are regulated through multiple mechanisms. Intracellularly, for example, inhibitory Smad proteins, Smad6 and Smad7, act as ligand-inducible inhibitors of signal transduction (2). Extracellularly, multiple binding proteins for the TGF-β family have been characterized as regulators of TGF-β signaling. Follistatin regulates all aspects of the biological activities of activin (3, 4). Signaling by TGF-β is regulated by decorin (5). The activities of bone morphogenetic protein (BMP) are regulated by BMP-binding proteins such as chordin, noggin, the cerberus/Dan families, and follistatin (6). Among the binding proteins, follistatin is biochemically the best characterized. Follistatin binds activin with high affinity (K\text{d} = 50–500 pM) and prevents activin from binding to its own receptors (4, 7), thereby neutralizing the biological activity of activin. Follistatins undergo multiple levels of modifications such as glycosylation and proteolytic processing, and can be purified as proteins with multiple molecular heterogeneity (8). The major species of follistatins are follistatin 315 (FS-315) and follistatin 288 (FS-288), which are generated by alternative splicing events from the common precursor gene (8, 9). We have recently shown that the splicing isoform FS-288, which has stronger activin inhibitory functions than FS-315, triggers the endocytotic degradations of activin, thereby completely blocking its biological activities (10). Follistatin also acts as a BMP-binding protein, although the affinity of follistatins for BMPs is lower than that of follistatins for activins (11). The binding proteins for activin and BMPs play significant roles in the process of neural induction in early development and in the regulation of hormonal homeostasis in endocrine organs (12). For example, overexpression of chordin, noggin, or follistatins in Xenopus embryos induces neuronal tissues via a default pathway (11, 12).

Structurally, follistatins are composed of a signal peptide, followed by the NH2-terminal domain and three follistatin domains (FS domains) (13). FS domains consist of ~70 amino acids, have 10 conserved cysteine residues, and are presumed to serve as growth factor binding motifs (14). These domains are found in various secreted proteins, including follistatins, follistatin-related protein (FRP, also known as TGF-β-stimulatable clone, TSC-36) (15, 16), SPARC (for secreted protein acidic and rich in cysteine, also known as osteonectin or BM-40) (17), agrin, and matrix glycoprotein SC1(18). Among the proteins that contain FS domains, only follistatins are reported to bind TGF-β family members (3). Interestingly, SPARC binds the B chain of platelet-derived growth factor and vascular endothelial growth factor and regulates their activities (19, 20).
Since the TGF-β family and the platelet-derived growth factor family are dimeric proteins and belong to the family of proteins containing cystine knot motifs (21), proteins containing FS domains are likely to recognize the common binding pockets found in the TGF-β family and the platelet-derived growth factor family.

In this paper, we report the identification of a mouse follistatin-like protein, which is most likely a mouse homologue of human follistatin-related gene (FLRG). The FLRG protein contains two FS domains and acts as a binding protein for activin and BMP-2.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning of Human and Mouse Follistatin-like Protein**—A human cDNA called FLRG, which is closely related to follistatin cDNA, has recently been reported (22). On the basis of the sequence of human FLRG, we performed polymerase chain reaction (PCR) using the primer pair 5'-GCTGCAGCAGAGTCTCTGTGAG-3' (sense) and 5'-GACGTTCGGAGCCGAGCT-3' (antisense) on the mRNA isolated from the human cervical cancer cell line HeLa. The PCR product covers the 290-base pair COOH-terminal region and 220 base pairs of the 3' non-coding region of human FLRG. Using the PCR product as a hybridization probe, two cDNA libraries from human and mouse cDNA clones. Both human and mouse clones were sequenced, and amino acid sequences were deduced.

**DNA Construct**—An expression construct of mFLRG for transcriptional assay was made by subcloning the full-length coding region of mFLRG into EcoRI-XhoI-digested pcDNA3 (Invitrogen). For mammalian expression of cDNA libraries were used. The PCR product covers the 5'-GCCGCG-3' and 3'-TCTCTTCCTCCTC-3' of the 5'- and 3'-flanking regions, respectively. The PCR-amplified product was digested with EcoRI and ApoI-digested mammalian expression vector pcDNA3.1/Myc-His (+) (Invitrogen). For bacterial expression of full-length mFLRG and its various regions, the following PCR primers were used. For full-length FLRG, a primer pair of sense primer P5 (5'-GGCCTCGAGGTATGTGCAGATGCCTGG-3') and antisense primer P6 (5'-GGCCTCGAGGTATGTGCAGATGCCTGG-3'). For the first FS domain of FLRG (GST-A), a primer pair of sense primer P3 (5'-GGCCTCGAGATTCGCGTGTCGTCGCTG-3') and antisense primer P4 (5'-GGGCTCGAGATGGGATCCAGCGGCGGCGG-3'). For the COOH-terminal domain containing the second FS domain of FLRG (GST-B), a primer pair of sense primer P5 (5'-GGCCTCGAGATTCGCGTGTCGTCGCTG-3') and antisense primer P5 (5'-GGGCTCGAGATGGGATCCAGCGGCGGCGG-3'). For bacteriophage T7 RNA polymerase expression, 500 ng of mFLRG protein tagged with Myc and 6 histidine residues was incubated with 200 ng of bovine activin A (for K562 cells and CHO cells) in the absence and presence of various concentrations of mFLRG and conditioned medium was purified by passing it through a Protein A (Invitrogen) column (Invitrogen) according to the manufacturer's protocol.

**RESULTS**

**Cloning of Human and Mouse Follistatin-like Protein**—Mouse FLRG was cloned by hybridization with a 510-base pair fragment of human FLRG. The alignment of the deduced amino acid sequences of mouse FLRG and human FLRG is shown in Fig. 1A. Human FLRG encodes 263 amino acid residues, and mFLRG encodes 256 amino acid residues. Although human and mouse FLRGs show marked sequence similarities, they are rather diverse compared with the follistatins. FLRGs show only 83% identity at the amino acid level. By contrast, human and mouse follistatins showed 98% identity at the amino acid level, and even mouse and Xenopus laevis follistatins show 83% identity at the amino acid level. Although mouse and human FLRGs show some divergence compared with follistatins structurally they are conserved; domain organization and putative Asn-linked glycosylation sites are completely conserved (Fig. 1A). Both mouse and human FLRG have an NH2-terminal domain, two FS domains, and a COOH-terminal acidic domain (Fig. 1, B and D). The amino acid sequences of mFLRG and
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A

| mammalian FLRG | human FLRG |
|---------------|------------|
| mFLRG | hFLRG |
| mFLRG | hFLRG |
| mFLRG | hFLRG |

B

| mammalian FLRG | human FLRG |
|---------------|------------|
| mFLRG | hFLRG |
| mFLRG | hFLRG |
| mFLRG | hFLRG |

C

| mammalian FLRG | human FLRG |
|---------------|------------|
| mFLRG | hFLRG |
| mFLRG | hFLRG |
| mFLRG | hFLRG |

D

| mammalian FLRG | human FLRG |
|---------------|------------|
| mFLRG | hFLRG |
| mFLRG | hFLRG |
| mFLRG | hFLRG |

Fig. 1. Structure of mouse FLRG. A, comparison of amino acid sequences of mFLRG and human FLRG (hFLRG). Numbers in the left and right columns represent amino acid positions. In the consensus alignment, identical amino acids in mFLRG and human FLRG are shown. + denotes conservative amino acid replacements. Asterisks denote potential Asn-linked glycosylation sites. B, comparison of amino acid sequences of mFLRG and mouse follistatin (mFS). Numbers in the left and right columns represent amino acid positions. In the consensus alignment, identical amino acids in mFLRG and mouse follistatin are shown. + denotes conservative amino acid replacements. FS domains I and II in FLRG and follistatin, and FS domain III in follistatin, are shown by bent arrows below the alignment. Putative cleavage sites of signal peptides are shown by arrows. C, alignment of FS domains found in mFLRG and mouse follistatin (mFS). Numbers in parentheses in the left and right columns represent amino acid positions.
mouse follistatins are aligned in Fig. 1B. Whereas follistatin has three FS domains, FLRG contains only two FS domains. Of the two FS domains of FLRG, the first FS domain of FLRG most resembles the first FS domain of follistatin, and the second FS domain of FLRG most resembles the second FS domain of follistatin. Thus, structurally, FLRG lacks the third FS domain found in follistatin (Fig. 1C). Additionally, FLRG lacks the heparin binding sites found in the first FS domain of follistatin (underlined in Fig. 1C) (29). Follistatins are known to bind heparin and heparan sulfate proteoglycan (7). The finding that FLRG lacks heparin binding sites suggests that FLRG and follistatins associate with heparin and activin in different ways (see “Discussion”).

Structural Organization of FLRG and Comparison with Other Proteins Containing Follistatin Domains—The FS domains of FLRG are conserved regions found in various secreted proteins (Fig. 1D). As shown in Fig. 1D, FS domains can be found not only in follistatins and FLRG, but also in FRP (TSC-36) and SPARC. FS domains have 10 conserved cysteine residues and are presumed to serve as growth factor binding motifs. Although the growth factor(s) that FRP binds and modulates is yet to be determined, FRP modulates the growth of cancer cells and has also been characterized as an autoantigen involved in systemic rheumatic diseases (15, 30), which suggests that FRP may regulate growth factor activities in the same way as follistatin and FLRG. SPARC is reported to bind and modulate the activities of vascular endothelial growth factor via its disulfide-bonded EF-hand sequence and follistatin-like domain (20).

Production of Mouse FLRG Protein and Characterization as a Binding Protein for Activin and BMP—in order to characterize mFLRG protein, we transfected mFLRG cDNA tagged with Myc and 6 histidine residues at the COOH terminus into COS-7 cells, as described under “Experimental Procedures.” Conditioned medium was partially purified by passing it through a nickel resin column that binds the histidine tag.
FIG. 4. Effects of mouse FLRG protein on activin A-induced transcription. A, inhibition of activin signaling by mFLRG protein in K562 cells. K562 cells were transfected with 3TP-lux and CMV-β-gal, and incubated without or with 15 ng/ml activin A in the absence (lanes 1 and 2) or increasing amounts of mFLRG protein (100 ng/ml in lane 3, 1 μg/ml in lane 4, and 3 μg/ml in lane 5) for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations.

B, inhibition of activin signaling by mFLRG protein in K562 cells. K562 cells were transfected with CAGA-lux and CMV-β-gal and incubated without or with 15 ng/ml activin A in the absence (lanes 1 and 2) or increasing amounts of mFLRG protein (100 ng/ml in lane 3, 1 μg/ml in lane 4, and 3 μg/ml in lane 5) for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations.

C, inhibition of activin signaling by mFLRG protein in CHO cells. CHO cells were transfected with 3TP-lux and CMV-β-gal, and incubated without or with 15 ng/ml activin A in the absence (lanes 1 and 2) or increasing amounts of mFLRG protein (100 ng/ml in lane 3, 1 μg/ml in lane 4, and 3 μg/ml in lane 5) for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations.
Purified protein was run on SDS-PAGE and probed with an antibody that recognizes the Myc epitope. As shown in Fig. 2A, a distinct signal with an apparent molecular mass of 37 kDa was detected by immunoblot analysis. The molecular mass is consistent with the predicted molecular mass of Myc/His-tagged and glycosylated FLRG. Next, we performed pull-down analysis. Activin A was incubated with the purified mFLRG protein and pulled down with nickel resin. Activin A associated with mFLRG was detected by anti-inhibin βA monoclonal antibody. As shown in lane 1 of Fig. 2B, 14-kDa monomeric activin A was detected by Western blotting, indicating that activin A was clearly pulled down with mFLRG. This association was inhibited by coinubcation with FS-288, which is a high affinity activin-binding protein (Fig. 2B, lane 2). To compare the mode of association of follistatin and FLRG with activin A, GST fusion proteins that contained the entire mature regions of rat FS-288 and mouse FLRG were prepared, and binding with activin A was studied by solid phase binding as described under “Experimental Procedures.” As shown in Fig. 2C, GST-rat FS-288 and GST-mouse FLRG show distinct activin binding. We estimate that the relative affinity of FLRG for activin A is lower (estimated $K_d$ is 850 pM) than that of FS-288 for activin A (estimated $K_d$ is approximately 47 pM), and is closer to that of FS-315 for activin A (estimated $K_d$ is approximately 430 pM) (31).

Elucidation of the Binding Domain for Activin by Ligand Blotting—In order to elucidate the domain of mFLRG that is involved in activin binding, GST fusion proteins encoding various regions of mFLRG were produced. Fig. 3A shows the schematic structure of the mFLRG protein. Since FS domains are presumed to be growth factor binding domains, we have focused on the two FS domains found in FLRG. GST-A contains the first FS domain, whereas GST-B contains the COOH-terminal domain including the second FS domain and the short acidic COOH-terminal region. GST-C contains both of the FS domains and the short acidic COOH-terminal region. GST-D contains the second FS domain without the short acidic COOH-terminal region. $^{125}$I-Activin A was prepared, and ligand blotting was performed using the GST fusion proteins. As shown in Fig. 3B, GST protein and GST-A protein did not show detectable activin binding, whereas GST-B and GST-C clearly show activin binding. Thus, the COOH-terminal domain including the second FS domain is capable of associating with activin A. Similar to mFLRG, the COOH-terminal region of human FLRG is capable of activin binding (data not shown). Interestingly, the GST-D, having the second FS domain without the short acidic COOH-terminal region, did not bind activin as well as did GST-B, suggesting the important role of the COOH-terminal acidic region of FLRG in activin binding.

Mouse FLRG Protein Inhibits Activin-induced Transcriptional Response in a Dose-dependent Manner—Human K562 erythroleukemia cells, whose proliferation and differentiation are regulated by activin, were transfected with a reporter plasmid, βTP-lux or CAGA-lux, and the inhibitory effect of mFLRG on activin A-induced signaling was studied (Fig. 4, A and B). FLRG decreased the activin A-induced βTP transcriptional activity in a dose-dependent manner (Fig. 4A). Similar inhibitory effects on activin signaling were observed when another reporter construct, CAGA-lux, was used instead of βTP-lux (Fig. 4B). Thus, the inhibitory effect of FLRG on activin signaling is authentic. A fairly high concentration of FLRG protein is needed for the complete inhibition of activin signaling, since our FLRG preparation is not as pure as the recombinant follistatin protein produced in CHO cells (data not shown). We have studied the effect of mFLRG protein in another activin-responsive cell line, CHO cells. In CHO cells, mFLRG inhibited activin A-induced 3TP-lux induction (Fig. 4C) and CAGA-lux induction (Fig. 4D). By contrast, TGF-β1-induced signaling is not affected by FLRG protein in CHO cells (Fig. 4E), suggesting the specific association of FLRG with particular TGF-β family members.

Mouse FLRG cDNA and Protein Control BMP-2-induced Signaling—Since FLRG is capable of binding BMP-2 as well as follistatins, we next investigated the effect of FLRG overexpression on BMP-2-induced signaling. We have chosen rat C6 glioma cells, since this cell line responds to BMP-2 to induce SBE4-lux (Fig. 5A). Overexpression of mFLRG cDNA inhibited BMP-2-induced signaling in C6 cells. Thus, FLRG is capable of binding to BMP-2 to inhibit its signaling. mFLRG cDNA overexpression neither affects BMP receptor expression or alters cellular responses to other peptide growth factor such as epidermal growth factor (data not shown). Similarly, FLRG protein also inhibited BMP-2-induced signaling in the rat astroglial cell line RNB (Fig. 5B). The effect is specific since the proliferative response to retinoic acid was not altered by FLRG stimulation. These data indicate that exogenously added FLRG protein blocks activin A-induced and BMP-2-induced signaling.

Tissue Distribution of Mouse and Human FLRG mRNAs—Northern blots of poly(A)+ RNA extracted from several mouse tissues showed the expression of FLRG mRNA of approximately 2.2 kb. Mouse FLRG mRNA is abundantly expressed in heart, lung, kidney, and testis (Fig. 6A). Expression levels of human FLRG mRNA (Fig. 6B) and human follistatin mRNA (Fig. 6C) in various tissues were quantitated by measuring the hybridization signal on multiple human RNA dot blots as described under “Experimental Procedures.” In human, FLRG is abundant in placenta, heart, aorta, testis, adrenal gland, lung, and ovary (Fig. 6B). In human, follistatin messages are rather ubiquitously expressed compared with FLRG, and abundant in adult and fetal liver, heart, ovary, and testis (Fig. 6C).

DISCUSSION

In this report, we describe the identification of mouse follistatin-like protein, which is likely to be a mouse homologue of human FLRG. FLRG contains two FS domains and is a binding protein for activin and BMP-2. We have previously shown that follistatins are binding proteins for activin (3, 10). Follistatin binds activin with high affinity and regulates all aspects of activin bioactivity. Other than follistatins, α2-macroglobulin and a member of the serine protease inhibitor (serpin) superfamily bind activin (32–34). However, their affinities for activin are low and they are unable to neutralize the bioactivity of activin. They are likely to act as molecules that deliver activins.

cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations. D, inhibition of activin signaling by mFLRG protein in CHO cells. CHO cells were transfected with CAGA-lux and CMV-β-gal, and incubated without or with 15 ng/ml activin A in the absence (lanes 1 and 2) or increasing amounts of mFLRG protein (100 ng/ml in lane 3, 1 μg/ml in lane 4, and 3 μg/ml in lane 5) for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations.
to the site of action or to be involved in clearance of activin. Like follistatins, FLRG binds activin with high affinity and neutralizes the biological activity of activin. Thus, FLRG is the second activin-binding protein shown to inhibit the activity of activin. In addition to the domain containing the COOH-terminal Glu-rich acidic region, clearly shows binding to activin A. Interestingly, the COOH-terminal region of FLRG lacks the heparin binding site (Fig. 1C). In accordance with this finding, we could not detect any apparent association of FLRG protein with heparin-Sepharose (data not shown). Since heparin or sulfate cellulofine column was used in our previous procedure to purify follistatins from porcine and human follicular fluids, it is unlikely that our previous follistatin preparation contained FLRG protein. It remains to be determined whether FLRG attaches to the cell surface via proteoglycans, in a similar way to FS-288. However, since the acidic Glu-rich domain of the COOH terminus of FS-315 prevents the binding of heparan sulfate to FS-315 (29, 36), and FLRG also contains an acidic Glu-rich domain at the COOH terminus, it is unlikely that FLRG associates with heparan sulfate proteoglycan. The affinity of FS-288 for activin is higher than that of FS-315 for activin, explaining the strong inhibitory activity of FS-288 on activins (31). The affinity of FLRG for activin A is lower than that of FS-288 for activin A, and close to that of FS-315 for activin A. Furthermore, FLRG resembles FS-315 in that both contain COOH-terminal Glu-rich acidic regions. In that sense, FLRG is more similar to FS-315 than to FS-288. However, the proposed heparin binding region found in the first FS domains of follistatins (FS-315 and FS-288) is not found in FLRG (Fig. 1C). Thus, even FLRG and FS-315 differ in their mode of association with heparin, and also possibly with activin (29).

We have identified the region of FLRG that is involved in the association with activin A. Interestingly, the COOH-terminal domain, which includes the second FS domain and the short acidic COOH-terminal region, clearly showed binding to activin, whereas the first FS domain did not show detectable binding. Thus, although structurally similar, the two FS domains of FLRG have different functions. Identification of the factor(s) that may be capable of binding to the first FS domain will help to clarify the additional functions of FLRG. Our preliminary studies indicate that the COOH-terminal region of FS-288, which includes the third FS domain, is not involved in activin binding. Thus, the modes of association of FS-288 and FLRG with activin are likely to be different. A previous report suggested the involvement of both the NH2-terminal and COOH-terminal regions of follistatin in activin binding (37). Furthermore, one report suggests that the COOH-terminal acidic region of FS-315 may be responsible for different modes of interaction of FS-315 and FS-288 with activins (38). Since the NH2-terminal domain of FLRG preceding the first FS domain is structurally similar to that of follistatins, this domain may also have a functional role in FLRG activity like follistatin (37). In accordance with this speculation, we found that the whole molecular structure is important for the complete inhibitory effect of FLRG (data not shown). The finding that a truncated FLRG protein that lacks the NH2-terminal region clearly shows activin binding on ligand blotting (Fig. 3) suggests an auxiliary role for the NH2-terminal region of FLRG in the activity of the protein. A similar observation was recently reported for the BMP antagonist chordin. The cysteine-rich domains (CRs) are the BMP-binding modules in chordin. Two CRs are found to bind BMPs, but the activity of individual CRs is lower than that of full-length chordin. Thus, the cysteine-rich regions of follistatins and chordin are likely to have common roles in activin and/or BMP binding.

Recently, the binding site on the type II activin receptor has been identified through alanine scanning mutation of the extracellular ligand-binding domain of the receptor (39). A cluster

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**Characterization of a Novel Binding Protein for TGF-β Family**

![Graph](image)

**Fig. 5. Effects of mouse FLRG cDNA expression and protein on BMP-2-induced signaling.** A, inhibition of BMP-2 signaling by mFLRG cDNA expression in C6 glioma cells. C6 cells were transfected with SBE4-lux, CMV-β-gal, and increasing amounts of mFLRG cDNA, and incubated without or with 10 ng/ml BMP-2 for 24 h as indicated. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations. B, inhibition of BMP-2 signaling by mFLRG protein in RNB astrocytoma cells. RNB cells were transfected with SBE4-lux and CMV-β-gal, and incubated without or with 10 ng/ml BMP-2 in the absence (lanes 1 and 2) or increasing amounts of mFLRG protein (100 ng/ml in lane 3, 1 μg/ml in lane 4, and 3 μg/ml in lane 5) for 24 h. The luciferase activity of each cell lysate was measured and normalized to the β-galactosidase activity. The values in the figure represent the means and S.E. of triplicate determinations.
of three hydrophobic residues (Phe-42, Trp-60, and Phe-83) that are important for activin binding to its receptors was identified. FSs (FS and FLRG) and activin receptors do not show any significant homology to each other. However, FS domains contain cysteine-rich regions, as do the extracellular domains of activin receptors. Thus, it is likely that the spacing of cysteines and the tertiary structures found in FS domains and activin receptors play an important role in growth factor association.

Recombinant mouse FLRG protein produced in mammalian cells inhibited activin A-induced signaling in human K562 cells and CHO cells (Fig. 4). We have used two reporters, 3TP-lux and CAGA-lux. The effects of FLRG on both reporters are similar: FLRG inhibits activin signaling. Thus, FLRG is the member of the activin-binding protein family that neutralizes activin biological activities extracellularly.

More recently, we and others have shown that follistatin is capable of binding to BMPs (11, 40). Although the affinity of follistatin for BMPs is lower than that of follistatin for activins (the estimated $K_d$ is approximately 23 nM), follistatin neutralizes all the functions of BMPs when overexpressed in Xenopus embryos (11). Recent reports also suggest that follistatin is likely to act as a binding protein not only for activins but also for other members of the TGF-β family, including BMP subfamily members such as BMP-7 and growth and differentiation factor 9 (41). In this study, we also analyzed the effects of FLRG on BMP binding and BMP-mediated signaling. Interestingly, FLRG shows binding to BMP-2 similar to that of follistatins (Fig. 2C). The affinity of FLRG for BMP-2 is close to that of follistatin for BMP-2 (Fig. 2C). Although the affinity of FLRG to BMP-2 is low, we can detect the association of BMP-2 with GST-C, which have both of the two FS domains of FLRG by ligand blotting (data not shown). In order to study the effect of FLRG on BMP-2 responsiveness, we have used the luciferase construct SBE4-lux in astrocyte cell lines. The reporter construct contains 4 repeats of Smad binding elements and responds not only to activin/TGF-β but also to BMPs (26). The elements are presumed to serve as binding sites for Smad4, which is a common mediator Smad for all the members of the TGF-β family (26, 27). In accordance with this finding, the SBE4 reporter is activatable not only by activin/TGF-β but also by BMPs. FLRG protein inhibited BMP-2-induced transcriptional response in BMP-responsive RNB cells (Fig. 5). Recently, the role of BMPs in the generation of glial fibrillary acidic protein-positive astrocytes from embryonic neural progenitor cells has been documented (42). Thus, it is an attractive hypothesis that follistatins (follistatin and FLRG) might be involved in the process by regulating the activities of BMPs.

The mode of interaction of follistatins with BMP is different from that of follistatins with activins (11). Whereas complexes containing follistatin and activin dissociate from activin receptors, tertiary complexes containing follistatin, BMP, and BMP receptor exist (4, 11). Whether the modes of interaction of FLRG with activin and with BMPs are different is a subject for future study.

We have shown that FLRG mRNA is highly expressed in heart, lung, kidney, and testis in mouse (Fig. 6A) and abundantly expressed in placenta, heart, testis, ovary, adrenal gland, and lung in human (Fig. 6B). Recent investigations using follistatin-overexpressing transgenic mice suggest that
follistatin exerts its functions mainly in a paracrine and/or autocrine manner (41). FLRG is also likely to act as an autocrine/paracrine factor to modulate the activities of activins and BMPs in these organs. The expression of follistatin mRNA is rather ubiquitous when compared with that of FLRG mRNA (Fig. 6C). The temporal and spatial expression of follistatin and FLRG is likely to regulate the activities of activins and BMPs differently. Since follistatins and FLRG are secreted proteins that act as endocrine regulators, they may act at sites remote from the site of mRNA production. Future studies should determine the expression site(s) of FLRG protein by the use of specific antibodies raised against the protein.

Activin and BMPs are multifunctional growth and differentiation factors that belong to the TGF-β superfamily. Activin has numerous biological activities: regulation of secretion of follicle-stimulating hormone from pituitary cells, stimulation of differentiation of erythroblasts, and mesoderm induction in Xenopus embryos. BMPs were originally discovered by virtue of their ability to induce ectopic bone formation. Subsequent investigations indicate that the action of BMPs is not limited to bone formation. BMPs act as mesoderm inducers both in amphibians and in mammals. BMPs are also involved in apoptosis in rhombomere formation and digit formation and pattern formation in early development. BMPs, in collaboration with leukemia inhibitory factor, induce the generation of glial fibrillary acidic protein-positive astrocytes from embryonic neural progenitor cells and specify the generation of glial cell lineages (41). The diverse biological activities of BMPs are regulated by multiple BMP-binding proteins.

Our findings that multiple members of the follistatin family (follistatin and FLRG) act as binding proteins for activin and BMPs not only shed new light on the molecular diversity of proteins containing FS domains, but also suggest that the regulatory mechanisms of activin/BMP biological signaling are more dynamic and complex than thought previously.

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