

GDF11 Forms a Bone Morphogenetic Protein 1-Activated Latent Complex That Can Modulate Nerve Growth Factor-Induced Differentiation of PC12 Cells

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All transforming growth factor β (TGF- β) superfamily members are synthesized as precursors with prodomain sequences that are proteolytically removed by subtilisin-like proprotein convertases (SPCs). For most superfamily members, this is believed sufficient for activation. Exceptions are TGF- β s 1 to 3 and growth differentiation factor 8 (GDF8), also known as myostatin, which form noncovalent, latent complexes with their SPC-cleaved prodomains. Sequence similarities between TGF- β s 1 to 3, myostatin, and superfamily member GDF11, also known as bone morphogenetic protein 11 (BMP11), prompted us to examine whether GDF11 might be capable of forming a latent complex with its cleaved prodomain. Here we demonstrate that GDF11 forms a noncovalent latent complex with its SPC-cleaved prodomain and that this latent complex is activated via cleavage at a single specific site by members of the developmentally important BMP1/Tolloid family of metalloproteinases. Evidence is provided for a molecular model whereby formation and activation of this complex may play a general role in modulating neural differentiation. In particular, mutant GDF11 prodomains impervious to cleavage by BMP1/Tolloid proteinases are shown to be potent stimulators of neurodifferentiation, with potential for therapeutic applications.

Although the prodomains of transforming growth factor β 1 (TGF- β 1), -2, and -3 are cleaved by furin-like convertases, the severed prodomains remain noncovalently associated with the mature regions of these molecules in latent complexes (2). It is generally accepted that the cleaved prodomains of other TGF- β -like molecules do not remain associated with, or modulate the activities of, the mature portions of these proteins (9, 14). One exception is the cleaved prodomain of the TGF- β -like molecule myostatin, also known as growth differentiation factor 8 (GDF8), which was recently shown to form a noncovalent latent complex with mature myostatin both in vitro and in vivo (13, 16, 39, 42, 45). Modes of inhibition and activation of myostatin are of considerable interest, as this molecule is responsible for negative regulation of skeletal muscle growth and mass during development and in the adult (13, 16, 23, 25, 45).

It is still not entirely clear which processes activate TGF- β 1 to -3 latent complexes in vivo, although there is evidence that cleavage within prodomain sequences by plasmin, matrix metalloproteinases (MMPs), and/or other proteases (5, 19, 20, 31, 44) and/or interactions with thrombospondin (4, 32) and integrins (1) may be involved. In contrast, we previously demonstrated that myostatin can be released from noncovalent, latent association with its prodomain and, thus activated, by cleavage within prodomain sequences by bone morphogenetic protein 1 (BMP1)/Tolloid (TLD)-like metalloproteinases (42). Moreover, myostatin prodomain that had been rendered impervious to cleavage by BMP1/TLD-like proteinases, by a single amino

acid substitution, was capable of inducing marked increases in muscle mass when administered to adult mice, whereas wild-type prodomain was not (42). The latter results thus demonstrated cleavage at the prodomain site employed by BMP1/TLD-like proteinases to be necessary to in vivo activation of myostatin. Myostatin is not the only TGF- β superfamily member to be activated by BMP1/TLD-like proteinases, as these proteinases activate BMPs 2 and 4 via cleavage of the extracellular protein antagonist chordin (27–29, 33). Aside from roles in activating BMP2/4 and myostatin, BMP1/TLD-like proteinases have been demonstrated to biosynthetically process the propeptides of various precursors to yield the mature, active forms of numerous proteins involved in formation of the extracellular matrix (11). Thus, these proteinases may serve to regulate both signaling by a subset of TGF- β -like factors and matrix formation and to orchestrate the two processes in morphogenetic events.

TGF- β -like proteins are divided into subfamilies on the basis of sequence similarities (6). By this criterion, myostatin forms a subfamily with the protein GDF11, also known as BMP11, as the two share 90% identity in their mature domains, although there is only 49% identity between their prodomains (7, 26). GDF11 is believed to play roles in inducing mesoderm in early development (7) and in anterior-posterior patterning of the axial skeleton (24). Interestingly, GDF11 was recently shown to act as a negative feedback inhibitory signal in neurogenesis of the olfactory epithelium (43), in a fashion reminiscent of the inhibition of muscle mass by myostatin. Thus, myostatin and GDF11 share structural and functional features.

Here, we demonstrate that GDF11 forms a latent, noncovalent complex with its cleaved prodomain and that the GDF11

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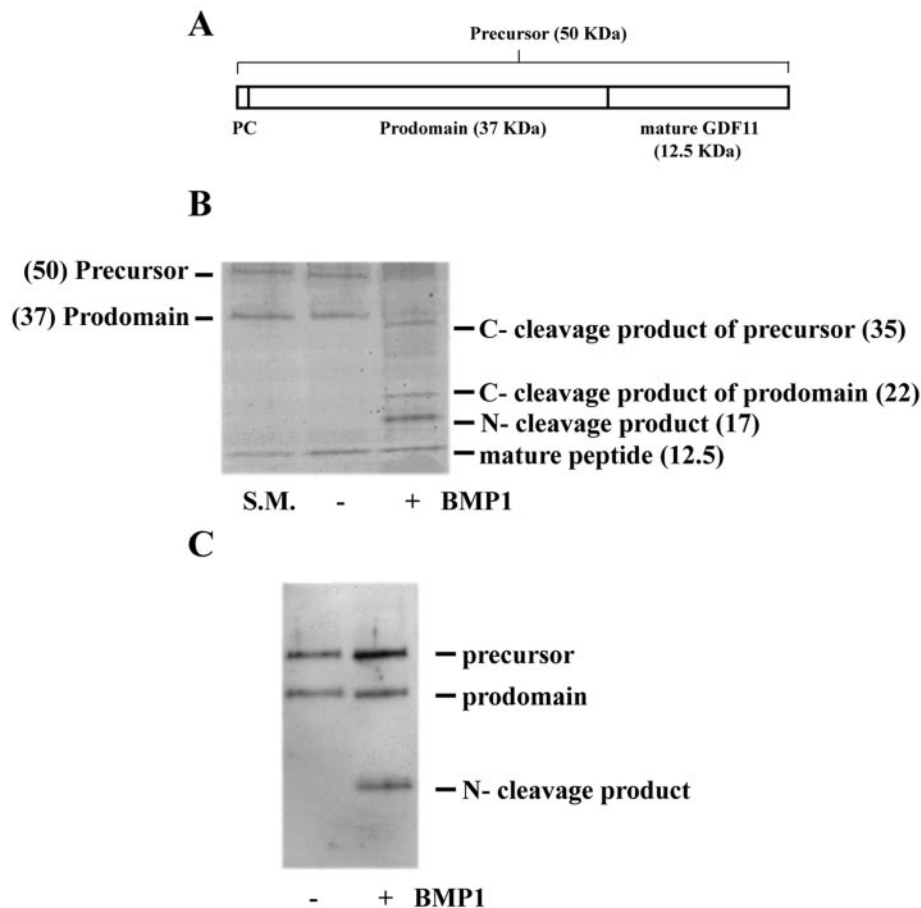


FIG. 1. Cleaved propeptide forms a noncovalent association with GDF11 and is cleaved by BMP1. (A) Schematic of the precursor molecule of GDF11. A small box on the NH₂ terminus represents the protein C epitope, while a vertical line represents the site at which propeptide and mature GDF11 sequences are separated by a recognition site for cleavage by furin-like proprotein convertases. (B) Electrophoretic patterns are shown on a Coomassie blue-stained 4-to-15% acrylamide gradient SDS-PAGE gel for GDF11 starting material (S.M.), affinity purified by binding to an anti-protein C column, and for the same material subsequent to overnight incubation in the absence (–) or presence (+) of BMP1. Approximate molecular masses (in kilodaltons) are shown (in parentheses) for the various bands. (C) Western blot analysis of GDF11 processing by BMP1 was performed, using an antibody directed against the protein C epitope on the NH₂ terminus on the GDF11 prodomain. SDS-PAGE separation of proteins prior to Western blotting was on a 4-to-15% gradient gel.

labeled with 10 μ M BrdU for the final 5 h and then washed with PBS, fixed with ethanol, and stained with anti-BrdU–fluorescein antibody (Roche) according to the manufacturer's manual.

For the p27^{Kip1} Western blot assay, PC12 cells, treated with NGF and GDF11 as described above, were scraped into hot SDS-sample buffer and 50 μ g of protein/sample was separated by SDS-PAGE on a 12% gel. Samples were transferred to a nitrocellulose membrane and probed with anti-p27^{Kip1} antibody (Cell Signaling). The same blot was reprobed with anti- α -tubulin antibody (Oncogene) as a control for loading.

For reverse transcription-PCR (RT-PCR) analyses, the RNA of PC12 cells treated with/without 50 ng/ml NGF, as described above, was isolated with TRIzol reagent (Invitrogen), and cDNA was synthesized using 1 μ g RNA, random primers, and SuperScript II reverse transcriptase (Invitrogen). PCR was performed using 20 pmol of each primer in 50- μ l reaction volumes at 95°C for 3 min, followed by 25, 30, or 35 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 10 min. Primers were the following: GDF-11, 5'-ACCACCGAGACGGTCATAAG-3' (forward) and 5'-CAAAGGCGTTGATCTCGATT-3' (reverse); GDF8, 5'-ACGCTACCACGGAAACAA TC-3' (forward) and 5'-TGGTCCTGGGAAGGTTACAG-3' (reverse); BMP1, 5'-CGCCTGTGCTGGTATGACTA-3' (forward) and 5'-ACGTGGAAACCCT CAGACAC-3' (reverse); mTLL1, 5'-GATGAGTGTGCCAAGCTGA-3' (forward) and 5'-GTGCTTTGAAGCCCTTCTTG-3' (reverse); HPRT, 5'-GCAG ACTTTGCTTTCCTTGG-3' (forward) and 5'-TCCACTTTCGCTGATGACA

C-3' (reverse). Products were resolved on 1.5% agarose gels and visualized with ethidium bromide.

RNAi. A total of 1×10^5 PC12 cells/well were plated on a rat tail type I collagen-coated six-well plate. Cells were transfected with/without 100 pmol Stealth synthetic RNA interference (RNAi) duplexes for GDF11 (sense, 5'-GC CCUCUGUGUUGCCAUUCCACUU-3') or a scrambled sequence (sense, 5'-GCCUCUGUGGUCCUAUCCAUCUU-3') (Invitrogen) using Lipofectamine 2000. DMEM containing 1% HS, 1% FBS, with/without 100 ng/ml NGF was added to cells after 6 h. Cells were changed into fresh medium after 16 h and were then allowed to differentiate for an additional 32 h. Neurite outgrowth/differentiation of PC12 cells was analyzed as described above. RNA isolation and RT-PCR for determination of GDF11 RNA levels were also performed as described above.

RESULTS

Excised GDF11 prodomain forms a noncovalent association with mature GDF11 and is cleaved by BMP1. Purification of recombinant GDF11 on an anti-protein C antibody column yielded three bands of ~50, ~37, and ~12.5 kDa, observable upon SDS-PAGE and staining with Coomassie blue (Fig. 1B).

NH₂-terminal amino acid sequencing showed the top two bands to begin with protein C epitope sequences, thus identifying them as the NH₂ terminus of the prodomain, from which signal peptide sequences had been removed. This, taken together with their mobilities on SDS-PAGE gels, is consistent with the identity of the 50-kDa band as a precursor form, comprising the prodomain still covalently bound to mature GDF11, and the 37-kDa band as cleaved prodomain (Fig. 1A). Thus, furin-like proprotein convertase activity provided by 293 T-REx cells apparently processes some (~60%), but not all, GDF11 prodomains at the furin recognition site that divides prodomain and mature portions of the GDF11 precursor. NH₂-terminal sequencing identified the 12.5-kDa form as mature GDF11. This latter result indicates that mature GDF11 was retained on the protein C epitope affinity column. The most straightforward interpretation of the latter result is that mature GDF11 was retained on the protein C affinity matrix via formation of a noncovalent complex with its cleaved, protein C epitope-tagged propeptide.

It can be seen in Fig. 1B that the ~50-, ~37-, and ~12.5-kDa forms are stable to overnight incubation at 37°C in the absence of additional proteins (Fig. 1B, -BMP1). In contrast, incubation of the same material in the presence of BMP1 (Fig. 1B, +BMP1) results in virtual disappearance of the 50-kDa precursor form and the 37-kDa prodomain, with the concomitant appearance of 35-, 22-, and 17-kDa forms. A Western blot of the same material, probed with anti-protein C antibody (Fig. 1C), demonstrates that the 17-kDa form corresponds to the protein C-tagged NH₂-terminal fragment of the BMP1-cleaved prodomain. NH₂-terminal amino acid sequencing of purified 17-kDa material confirmed this conclusion. Moreover, the NH₂-terminal sequence of the 22-kDa form was DALQPED, which shows this species to correspond to the COOH-terminal fragment of BMP1-cleaved prodomain and which locates the BMP1 cleavage site to between residues Gly119 and Asp120. This cleavage site shows similarities to the majority of previously characterized BMP1 cleavage sites, particularly in having an Asp in the P1' position (Fig. 2) (11). In experiments similar to those of Fig. 1, the BMP1-related mammalian proteinase mTLL1 was found to cleave GDF11 with efficiency similar to that of BMP1, whereas the other two BMP1-related mammalian proteinases, mTLD and mTLL2, showed markedly less activity (data not shown). The observation that GDF11, unlike myostatin (42), is not efficiently cleaved by mTLL2 is consistent with the possibility that this muscle-specific proteinase (33) may have evolved with cleavage of myostatin as a central function.

BMP1 cleavage within propeptide sequences activates GDF11. To determine possible effects of BMP1 cleavage of prodomain sequences on GDF11 activity, levels of signaling were compared for affinity-purified GDF11 and for the same material incubated 6 h in the presence or absence of BMP1. Signaling was monitored via a reporter gene assay composed of A204 rhabdomyosarcoma cells transfected with a reporter vector in which the SMAD-responsive element (CAGA)₁₂ is fused to the luciferase gene. This assay has previously been shown to be efficient in detecting the signaling activities of myostatin, GDF11, and activin (39, 42). As can be seen in Fig. 3, both recombinant GDF11 starting material and GDF11 incubated alone for 6 h at 37°C had undetectable signaling activity in the

GDF11	LDLHDFQG.....DALQPEDF
Myostatin	IDQYDVQR.....DDSSDGSL
Procollagens	
α1 (I)	DGGRYRA.....DDANVVRD
α2 (I)	YDGFYRA.....DQPRSAPS
α1 (II)	DPLQYRA.....DQAAGGLR
α1 (III)	GGFAPYYG.....DEPMDFKI
α2 (V)	DPLPEFTE.....DQAAPDDK
α1 (VII)	RPLPSYAA.....DTAGSQLH
Laminin 5 γ2 chain	DTGDCYSG.....DENPDIEC
Probiglycan	DDGPFMMN.....DEEASGAD
<u>Chordin</u>	
N-terminal site	DPEHRSYS.....DRGEPGVG
C-terminal site	KLGDPMQA.....DGPRGRCF
Prolysyl oxidase	SHVDRMVG.....DDPYNPYK
DMP1	FDDEGMQS.....DDPESTRS

FIG. 2. BMP1 cleavage site within the GDF11 prodomain. Shown is the alignment of the site at which BMP1 cleaves the GDF11 prodomain with sites at which BMP1 has previously been shown to cleave other substrates (11). Aspartate residues conserved at the P1' positions of the various scissile bonds are in boldface, as are methionines and residues with aromatic side chains, noted as being NH₂ terminal to scissile bonds in the majority of previously identified substrates of BMP1-like proteinases.

assay. In contrast, incubation of GDF11 with BMP1 for 6 h prior to addition to the reporter assay resulted in a dramatic increase in levels of signaling, denoting BMP1-mediated activation of GDF11.

We have previously demonstrated that substitution of the cleavage site P1' Asp with Ala inhibits processing of the myostatin prodomain and procollagen C propeptides by BMP1-like proteinases (17, 42). Thus, a P1' Asp is necessary for cleavage of at least some sites by BMP1-like proteinases. To determine whether the same is true for the GDF11 site, Asp120 was replaced with Ala and the recombinant mutant GDF11 was expressed, purified, and incubated for 6 h with/without BMP1. As can be seen in Fig. 3, GDF11 with the D120A mutation is not activated by BMP1. Consistent with the results of Fig. 3, Western blot assays similar to that of Fig. 1C showed D120A GDF11 precursor and prodomain to be impervious to cleavage by BMP1 (not shown). Thus, the GDF11 latent complex can be activated via cleavage of prodomain sequences by BMP1-like proteinases, and blockage of such cleavage blocks GDF11 activation.

Addition of exogenous prodomain sequences antagonizes signaling by activated GDF11. We next sought to determine whether free GDF11 prodomain is able to antagonize signaling when exogenously added in vitro to mature, previously activated GDF11. Towards this end, recombinant prodomain was produced, free of other GDF11 sequences, but fused to the Fc portion of murine IgG2a. Prodomain was produced as an Fc fusion protein to aid in purification and because the Fc sequences stabilize proteins and can also prolong in vivo half-lives of proteins, which may be of use for possible in vivo studies of the GDF11 prodomain in the future. A similar

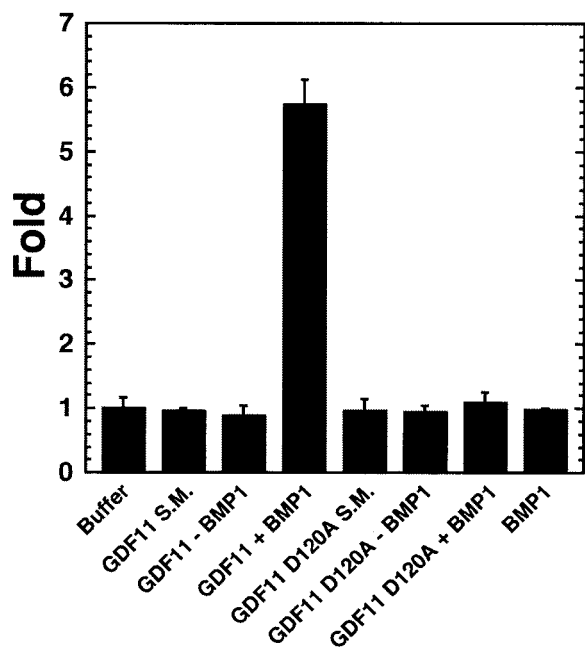


FIG. 3. BMP1 cleavage within prodomain sequences activates GDF11. Purified GDF11 latent complex starting material (GDF11 S.M.) is shown to have negligible levels of GDF11 signaling activity, similar to those of buffer containing no protein, and the same is true for GDF11 latent complex incubated in the absence of BMP1 (GDF11-BMP1), in an assay employing A204 cells transfected with the pGL3(CAGA)₁₂ reporter vector in which luciferase expression is under the control of multiple SMAD-responsive elements. In contrast, GDF11 latent complex incubated in the presence of BMP1 (GDF11 + BMP1) shows an almost sixfold induction of GDF11 signaling activity levels. GDF11 starting material in which the Asp in the P1' position of the BMP1 cleavage site has been substituted for by Ala (GDF11 D120A S.M.) has no signaling activity above that of buffer, nor does the same material incubated either in the absence (GDF11 D120A-BMP1) or presence (GDF11+BMP1) of BMP1. As a control, BMP1 alone (BMP1) is shown to have no signaling activity in the assay. Numbers on the ordinate axis represent fold increases in signaling compared to buffer alone.

prodomain-Fc fusion protein has previously worked well in studies of the myostatin prodomain (42). As can be seen in Fig. 4A, active GDF11 was markedly inhibited, in a dose-dependent way, by preincubation with the prodomain-Fc fusion protein. Closely related myostatin was similarly inhibited by the GDF11 prodomain-Fc fusion protein, with somewhat slower kinetics, whereas the more distantly related TGF- β -like molecule activin A was not appreciably inhibited. A recombinant IgG2a Fc domain control, not fused to GDF11 prodomain sequences, did not inhibit any of the TGF- β -like molecules. Thus, the GDF11 prodomain can specifically inactivate mature GDF11 in *trans*, even when the two molecules have not been cosynthesized or cosecreted by the same cell but have been added together in vitro.

The data in Fig. 4B demonstrate that mutant prodomain-Fc fusion protein with the D120A substitution can bind and inactivate mature GDF11 as effectively as wild-type prodomain-Fc fusion protein. However, Fig. 4C demonstrates that whereas preincubation (6 h) of wild-type prodomain-Fc fusion protein with BMP1 destroys its ability to inhibit signaling by mature

GDF11, preincubation with BMP1 has no effect on the ability of the D120A-substituted prodomain-Fc fusion protein to inhibit signaling. The D120A-substituted prodomain-Fc fusion protein was thus shown to be a specific inhibitor of GDF11, impervious to cleavage by BMP1-like proteinases. As such, the D120A-substituted prodomain-Fc fusion protein shows promise as a reagent for the specific inhibition of GDF11 in cell culture, organ culture, and in vivo environments in which BMP1-like proteinases are produced.

MEFs doubly homozygous null for the *Bmp1* and *Tll1* genes are deficient in processing/activating GDF11. We have previously demonstrated in vivo roles for BMP1-related proteinases in the processing of various candidate substrates by demonstrating differences in the proteolytic processing of these substrates in wild-type MEF cultures compared with cultures of MEFs doubly homozygous null for the *Bmp1* gene, which encodes alternatively spliced mRNAs for BMP1 and mTLD, and the *Tll1* gene, which encodes the related proteinase mTLL1 (8, 27, 35, 36, 40). We have been unable to detect endogenous GDF11 in MEF cultures. Thus, to compare processing of GDF11 prodomain sequences in wild-type and *Bmp1/Tll1* doubly null MEFs, both cell types were transfected with a vector for expression of recombinant GDF11 containing a protein C epitope tag between the signal peptide and prodomain and a COOH-terminal Flag tag (Fig. 5A). As can be seen, blots using anti-protein C antibodies (Fig. 5B) and anti-Flag antibodies (Fig. 5C) both showed conditioned medium of the double-null, but not wild-type, MEFs to contain full-length GDF11 precursor, in which no proteolytic cleavages, beyond removal of the signal peptide, had occurred. This included lack of cleavage by furin-like convertase activities (Fig. 5A). Western blot analysis with anti-PC antibody (Fig. 5C) also demonstrated medium of doubly null, but not wild-type, MEFs to contain free GDF11 propeptide of a size consistent with its having been cleaved from the precursor by furin-like convertases, but in which no additional cleavages have occurred. In addition, probing with anti-Flag antibody (Fig. 5C) showed wild-type MEF medium to contain markedly higher levels of a COOH-terminal cleavage product of the precursor than did the double-null medium. The size of this fragment is consistent with cleavage of the precursor having occurred within propeptide sequences at the BMP1 cleavage site, without cleavage having occurred at the furin cleavage site.

The most straightforward explanation of the above results is that endogenous *Bmp1* and/or *Tll1* gene products are involved in the processing of GDF11 propeptide sequences by cells. Relatively low levels of the COOH-terminal cleavage product observed in the medium of doubly null cells (Fig. 5C) indicate that reduced processing within propeptide sequences takes place, at or near where we've demonstrated BMP1-like proteinases to cleave, even in the absence of *Bmp1/Tll1* products. It remains to be determined whether such activity is provided by mTLL2, which is expressed in MEFs (35), or by some other, as-yet-unidentified proteinase(s). It is presently unclear why mature GDF11 was not detected by anti-Flag antibody in wild-type or doubly null MEF medium (Fig. 5C). Siliconized tubes and carrier protein were used throughout sample preparations, and thus it seems unlikely that mature GDF11 was lost during the preparation of samples. A probability, supported by evidence provided below, is that mature, active GDF11 was

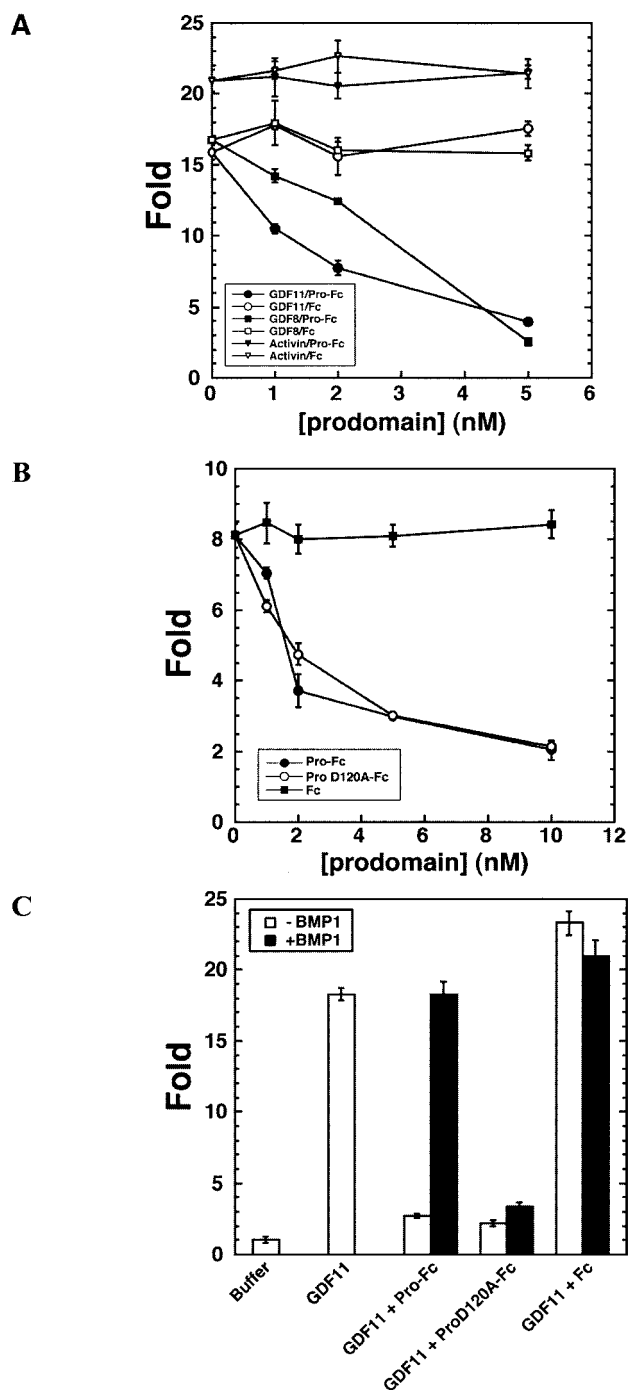


FIG. 4. Inhibition of GDF11 signaling by addition of exogenous GDF11 prodomain-Fc fusion proteins. (A) A204 cells transfected with the pGL3(CAGA)₁₂ reporter vector were incubated with 10 ng/ml of active GDF11, GDF-8/myostatin, or activin and increasing amounts of either GDF11 prodomain-Fc fusion protein (Pro-Fc) or recombinant Fc domain (Fc), the latter as a negative control. (B) In the same type of assay, 10 ng/ml active GDF11 was incubated with increasing amounts of wild-type or mutant prodomain-Fc fusion protein, or recombinant Fc domain (as a negative control), with results demonstrating that GDF11 prodomain-Fc fusion protein bearing the D120A mutation inhibits active GDF11 as effectively as does the wild-type prodomain-Fc fusion protein. (C) In the same type of assay, preincubation of wild-type GDF11 prodomain-Fc fusion protein with BMP1 destroys its ability to inhibit signaling by active GDF11, whereas pre-

present in these samples, but that it was not detected by anti-Flag antibody due to loss or inaccessibility of the Flag epitope.

To test for possible effects of endogenous BMP1-like proteinases on levels of GDF11 signaling, wild-type and *Bmp1/Tll1* doubly null MEFs were transfected with a construct for expression of recombinant GDF11 resembling that in Fig. 5A, except that it lacked a COOH-terminal epitope tag, since COOH-terminal tags have been found to interfere with signaling by TGF- β -like proteins. Transfections were followed by tests of conditioned media for levels of active GDF11, using the A204 cell/luciferase gene reporter assay described above. As can be seen in Fig. 5D, expression construct-transfected wild-type MEF medium induced levels of signaling far higher than those induced by medium from expression construct-transfected doubly null MEF medium, and media from both types of expression construct-transfected MEFs induced signaling levels markedly higher than the basal levels induced by the media of wild-type or doubly null MEFs transfected with empty vector. Thus, recombinant GDF11 was responsible for the majority of signaling detected by the reporter gene assay, and GDF11 signaling was at markedly higher levels in wild-type than in *Bmp1/Tll1* doubly null MEFs. A control experiment (Fig. 5E) demonstrated that differences in signaling activity between transfected wild-type and doubly null MEF cultures was due to differences in latent complex activation, rather than differences in recombinant GDF11 expression levels, since upon 10-min heat (80°C) activation, which would effectively activate all latent complexes, both types of cells had similar levels of signaling activity.

The data of Fig. 5D and E are consistent with the probability that, although mature GDF11 was not detected by anti-Flag antibody in the conditioned media samples of Fig. 5C, it was nonetheless present. The overall results of Fig. 5 are consistent with the probability that proteinases encoded by the *Bmp1* and *Tll1* genes are involved in GDF11 activation by cells.

Effects of formation and BMP1 activation of the GDF11 latent complex on NGF-induced differentiation to a neuronal-like phenotype. Previously, GDF11 has been shown to inhibit neurogenesis in olfactory epithelium (43). PC12 pheochromocytoma cells differentiate into cells with a phenotype similar to that of noradrenergic sympathetic neurons upon treatment with NGF and are widely used as a model system for neuronal differentiation (10). Thus, we employed PC12 cells to assay for possible effects of GDF11 in regulating neural differentiation in a system other than olfactory epithelium and to determine how such effects might be modulated by the formation and activation of GDF11 latent complexes. Towards this end, PC12 cells were incubated with NGF in the presence of increasing amounts of active GDF11. This assay showed GDF11 to have a potent and dose-dependent effect in countering NGF-induced neurite outgrowth in PC12 cells (Fig. 6A), with reductions of 66%, 78%, 80%, and 87% in PC12 cell differentiation in the presence of 2.5, 5, 10, and 20 ng/ml GDF11, respectively. The extents of inhibition of neurodifferentiation by 5 and 20

incubation of prodomain-Fc fusion protein bearing the D120A substitution with BMP1 does not have a marked effect on its ability to inhibit active GDF11. Numbers on the ordinate axes in panels A to C represent fold increases in signaling compared to buffer alone.

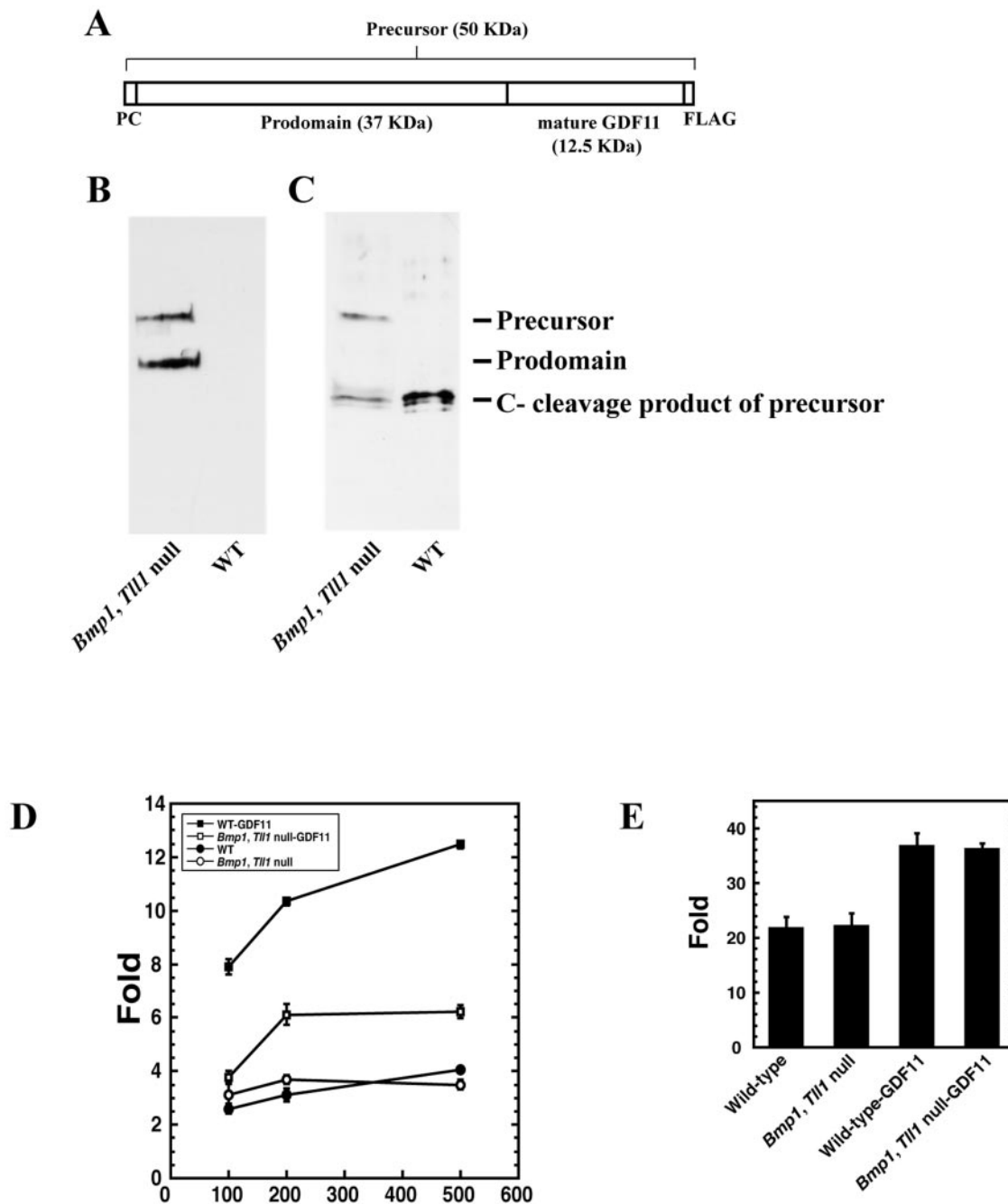


FIG. 5. MEFs employ BMP1/Tolloid-like proteinases to cleave propeptide sequences, thus activating GDF11. (A) Schematic of recombinant precursor GDF11 expressed by transfected MEFs in panels B and C. Small boxes on the NH₂ and COOH termini represent protein C and Flag epitope tags, respectively. A vertical line represents the site at which propeptide and mature GDF11 sequences are separated by a recognition site for cleavage by furin-like proprotein convertases. (B and C) Western blot assays using anti-protein C antibodies (B) or anti-Flag antibodies (C) were employed to monitor pro-GDF11 processing in transfected cultures of wild-type MEFs (WT) or MEFs derived from embryos doubly homozygous null for the *Bmp1* and *Tll1* genes (*Bmp1 Tll1* null). (D and E) Conditioned media from wild-type (WT) or *Bmp1 Tll1* doubly null MEFs (*Bmp1 Tll1* null) transfected with empty vector, or from the same types of cells transfected with a pro-GDF-11 expression vector (WT-GDF11 and *Bmp1 Tll1* null-GDF11), were tested for GDF11 signaling activity in the A204 cell pGL3(CAGA)₁₂ reporter gene assay. (D) Aliquots of 100, 200, and 500 μ l were compared for each sample of conditioned medium from wild-type or doubly null MEF cells that had been transfected with the expression construct or with empty vector. Final volumes for the reporter gene assay were in each case 500 μ l, with DMEM added as necessary. (E) Aliquots (200 μ l) of samples of conditioned media were heat activated for 10 min at 80°C and then added to 300 μ l DMEM prior to testing in the reporter gene assay. Numbers on the ordinate axis in panels D and E represent fold increases in signaling compared to buffer alone.

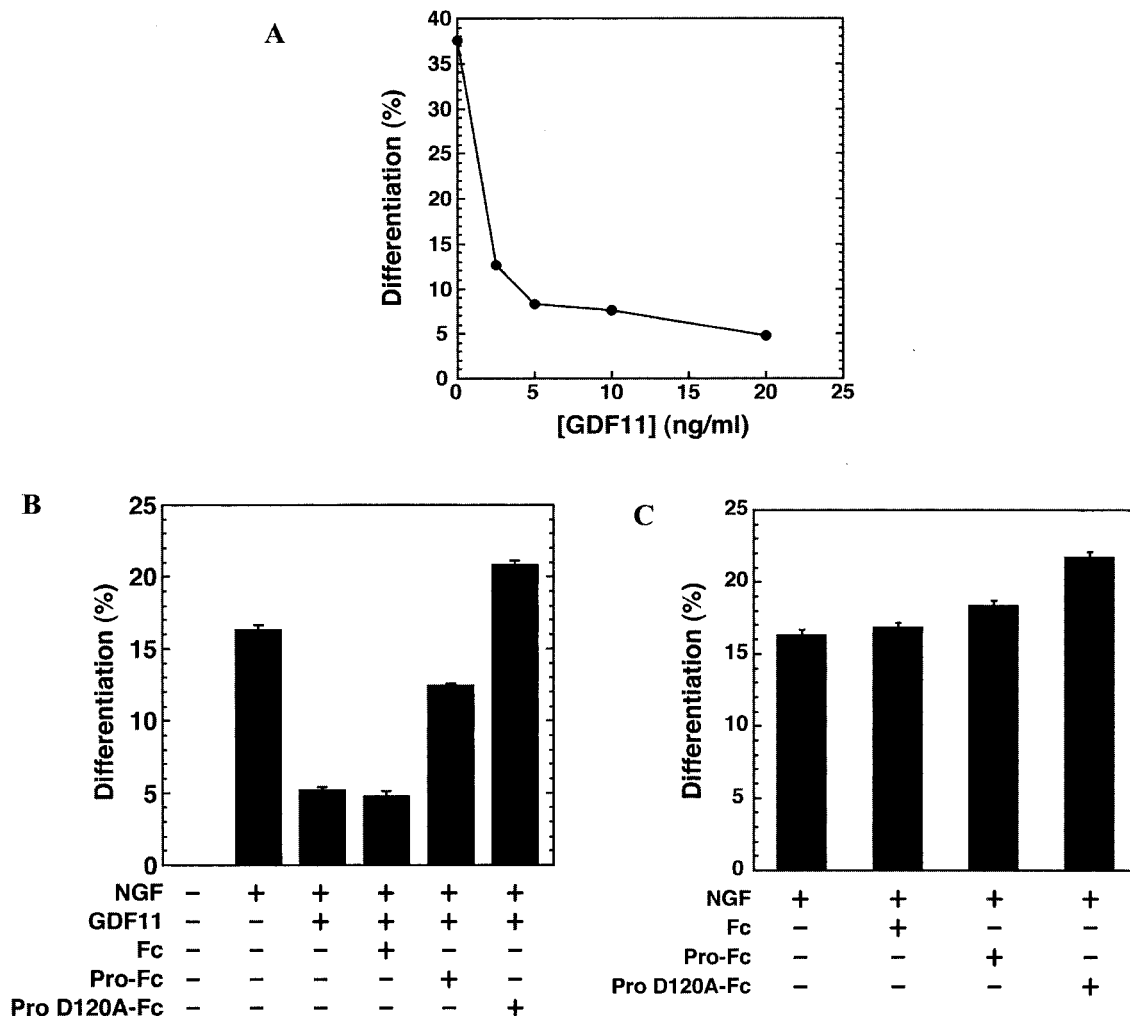


FIG. 6. Active GDF11 is a potent, dose-dependent inhibitor, and GDF11 prodomain, especially if impervious to cleavage by BMP1-like proteinases, is a potentiator of NGF-induced PC12 cell differentiation. (A and B) PC12 cells were incubated in the presence of 50 ng/ml NGF plus 0, 2.5, 5, 10, or 20 ng/ml active GDF11 (A) or plus 5 ng/ml active GDF11 (B) in the presence of 250 ng/ml wild-type (Pro-Fc) or D120A-substituted (Pro D120A-Fc) GDF11 prodomain Fc fusion protein, or 80 ng/ml Fc domain (as a negative control). (C) Conditions for the experiment shown in panel C were the same as for panel B, except that exogenously added active GDF11 was not used. Percentages of PC12 cells undergoing differentiation to a neural-like phenotype under the various conditions were determined as described in Materials and Methods.

ng/ml GDF11 were similar enough that 5 ng/ml GDF11 was used in subsequent experiments.

We next tested for possible effects of the GDF11 prodomain on NGF-induced PC12 cell differentiation. As can be seen in Fig. 6B, the ability of active GDF11 to inhibit neurite outgrowth was greatly reduced in the presence of wild-type GDF11 prodomain-Fc fusion protein (Fig. 6B). Interestingly, prodomain-Fc fusion protein with the D120A substitution was considerably more effective than wild type in rescuing neurite outgrowth from the inhibitory effects of active GDF11. This implies that PC12 cells secrete one or more proteinases capable of cleaving the ¹¹⁹Gly-Asp¹²⁰ bond and thereby inactivating wild-type, but not mutant, GDF11 prodomain sequences. In fact, the observation that PC12 cells treated with both active GDF11 and the mutant prodomain-Fc fusion protein had greater NGF-induced neurite outgrowth than did PC12 cells treated with NGF alone (Fig. 6B) implies that the exogenously

added prodomain may have bound and inhibited endogenous, as well as exogenously added, active GDF11. To assay for this possibility, wild-type and D120A-substituted prodomain-Fc fusion proteins were added to PC12 cells in the presence of NGF but in the absence of exogenously added active GDF11. As can be seen in Fig. 6C, both wild-type and mutant prodomain fusion proteins increased the percentage of PC12 cells differentiating to a neural-like phenotype. This finding is consistent with the possibility that some level of GDF11 signaling is normally involved in limiting the NGF-induced differentiation of PC12 cells. The observation that the mutant prodomain was more effective than wild type in enhancing differentiation is again consistent with the probability that endogenous proteinase(s) secreted by PC12 cells can activate GDF11 via cleavage at the prodomain ¹¹⁹Gly-Asp¹²⁰ site. To determine whether such endogenous proteinases might be members of the BMP1/TLD family of proteinases, PC12 cells were incubated in the

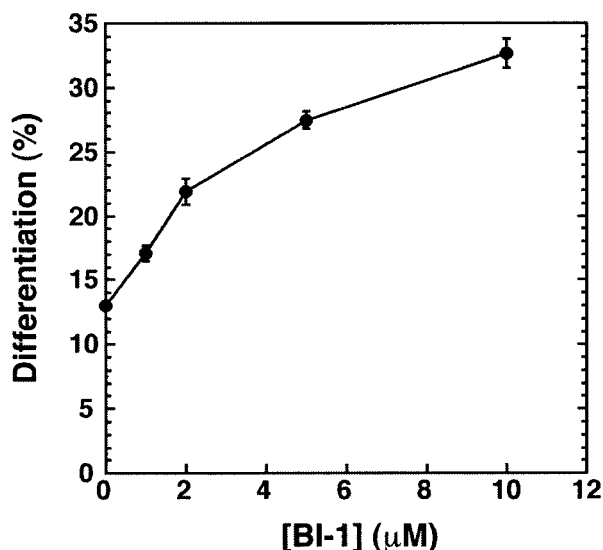


FIG. 7. A highly specific inhibitor of BMP1-like proteinases potentiates the NGF-induced differentiation of PC12 cells. PC12 cells were incubated in the presence of 50 ng/ml NGF and 0, 1, 2, 5, or 10 μ M BI-1 inhibitor.

presence of NGF and increasing concentrations of the previously described hydroxamic acid-based inhibitor BI-1, which is highly specific for BMP1/TLD-like proteinases (41). As can be seen in Fig. 7, increasing concentrations of the inhibitor led to a large increase in the percentage of PC12 cells undergoing differentiation to a neural-type phenotype. This result is highly supportive of the possibility that PC12 cells secrete endogenous BMP1/TLD-like proteinases and that such proteinases play a role in inhibiting NGF-induced differentiation. The difference between percentages of PC12 cells differentiating in the absence of inhibitor or presence of 10 μ M inhibitor in Fig. 7 (13% versus 32%) is a relatively large one, compared with the difference between percentages of cells differentiating in the presence of wild-type or D120A prodomain fusion proteins (18% versus 22%). This difference is explainable by the probability that endogenous BMP1/TLD-like proteinases more efficiently cleave endogenous GDF11, with which they are cosecreted at high initial concentration, than exogenously added prodomain fusion proteins, which are distributed throughout the culture medium. The latter probability is consistent with previous studies showing that BMP1/TLD-like proteinases cleave substrates at or near the cell surface (11).

We next sought to determine whether GDF11 is actually expressed by PC12 cells and/or whether PC12 cells express the closely related protein GDF8/myostatin, which can mimic the effects of GDF11 in some systems (18, 43). We also sought to determine whether BMP1 and/or mTLL-1, the two proteinases found in this study to efficiently cleave the GDF11 prodomain ¹¹⁹Gly-Asp¹²⁰ site (see above), are actually expressed by PC12 cells. As can be seen in Fig. 8A, RNAs for GDF11 and BMP1 were readily detectable by RT-PCR in PC12 cells and the level of each RNA was essentially similar in PC12 cells either treated or untreated with NGF. Neither mTLL1 nor GDF8/myostatin RNAs were detectable under the RT-PCR conditions used in this study. Thus, RNAs for GDF11 and for one of

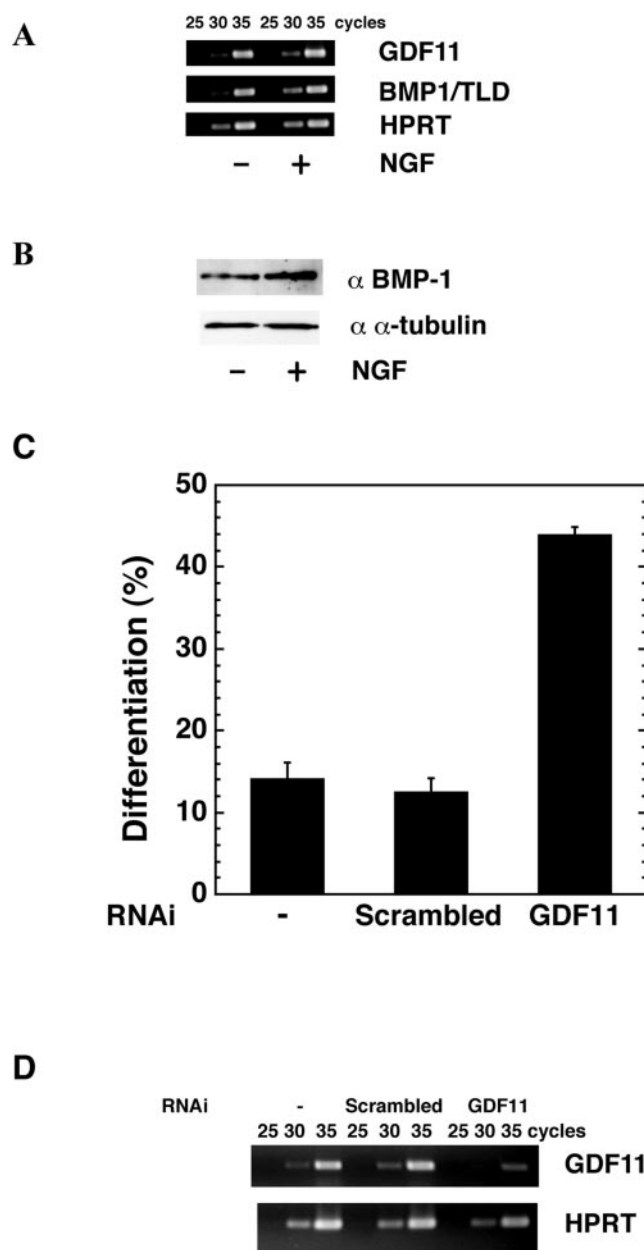


FIG. 8. Expression of endogenous GDF11 and BMP1, but not GDF8 or mTLL1, by PC12 cells. (A) RT-PCR was performed on the RNA from PC12 cells incubated in the presence (+) or absence (-) of 50 ng/ml NGF. (B) The lysates of PC12 cells incubated in the presence (+) or absence (-) of 50 ng/ml NGF were analyzed by Western blotting for the presence of endogenous BMP1. The same blot was reprobed with anti- α -tubulin antibody as a control for loading. (C) PC12 cells were mock transfected (-) or transfected with synthetic RNAi duplexes for GDF11 or for scrambled sequences. Cells were then treated with NGF and assayed for percent differentiation. (D) RT-PCR was performed on RNA from the PC12 cells of the experiment shown in panel C. PCR was for 25, 30, and 35 cycles for each sample.

the two BMP1/TLD-like proteinases that can activate it are expressed by PC12 cells, but neither up- or down-regulation of steady-state levels of these RNAs appears to be involved in NGF-induced PC12 differentiation to a neural-like phenotype.

Moreover, none of the effects ascribed to GDF11 signaling or inhibition in this study are due to endogenous GDF8/myostatin, which is not expressed by PC12 cells.

To more directly assay the possibility of a role for endogenous GDF11 in NGF-induced PC12 cell differentiation, levels of endogenous GDF11 RNA were reduced in PC12 cells via RNAi and effects on NGF-induced differentiation were analyzed. As can be seen in Fig. 8C, reduction of endogenous GDF11 RNA via RNAi resulted in a large increase in the percentage of differentiated PC12 cells, subsequent to treatment with NGF. In contrast, RNAi with a scrambled sequence showed no effect on levels of NGF-induced differentiation. Thus, endogenous GDF11 is normally involved in modulating NGF-induced PC12 cell differentiation. RNAi-mediated reduction in levels of endogenous GDF11 RNA had no apparent effects on the morphology of PC12 cells untreated with NGF, nor did D120A-substituted prodomain-Fc fusion protein or the BI-1 inhibitor (data not shown).

We next employed Western blot analysis using an antibody that recognizes sequences in the highly homologous protease domains of BMP1, mTLD and mTLL1 (antibody 517) (41) to determine whether any of these proteins are detectable in PC12 cells. The antibody readily detected a single band in PC12 cells (Fig. 8B), and this band had a mobility corresponding to that expected for the pro- (intracellular) form of BMP1. Thus, PC12 cells synthesize BMP1, a protein that we have shown to efficiently activate GDF11 (see above). The band representing BMP1 is more intense (~2.5-fold) in sample from PC12 cells treated with NGF than in sample from untreated cells (Fig. 8B), although it remains to be determined whether a change of this magnitude is significant in regard to regulation of NGF-induced differentiation of PC12 cells. We were not able to ascertain levels of secreted BMP1. This is because conditions for NGF-induced differentiation of PC12 cells required serum, which resulted in large amounts of background in Western blot assays of media samples, due to nonspecific binding of antibodies to serum proteins that had electrophoretic mobilities similar to that of BMP1 (not shown).

GDF11 and NGF both induce PC12 cell cycle arrest with increased levels of p27^{Kip1}, but with opposite phenotypic effects. GDF11 has been reported to inhibit neurogenesis in olfactory epithelium via inducing reversible cell cycle arrest in neural progenitor cells (43). Similarly, NGF-induced differentiation of PC12 cells to a neural-like phenotype is associated with cell cycle arrest (10). Thus, we sought to determine whether the opposite phenotypic effects induced by NGF and GDF11 in PC12 cells in the current study were both indeed accompanied by cell cycle arrest. As can be seen in Fig. 9A, both NGF and GDF11 reduced the percentage of PC12 cells that incorporated BrdU, consistent with induction of cell cycle arrest. NGF alone reduced BrdU-positive PC12 cells from 57% to 32%, and GDF11 reduced BrdU-positive cells from 57% to 40%. Interestingly, the two factors had an additive effect in inducing cell cycle arrest, since in the presence of NGF and GDF11 together only 21% of cells were BrdU positive.

GDF11-induced cell cycle arrest in olfactory epithelium is accompanied by increased expression of the cyclin-dependent kinase inhibitor p27^{Kip1} (43); thus, we next assayed for levels of p27^{Kip1} in cells treated with NGF, GDF11, or both. As can be

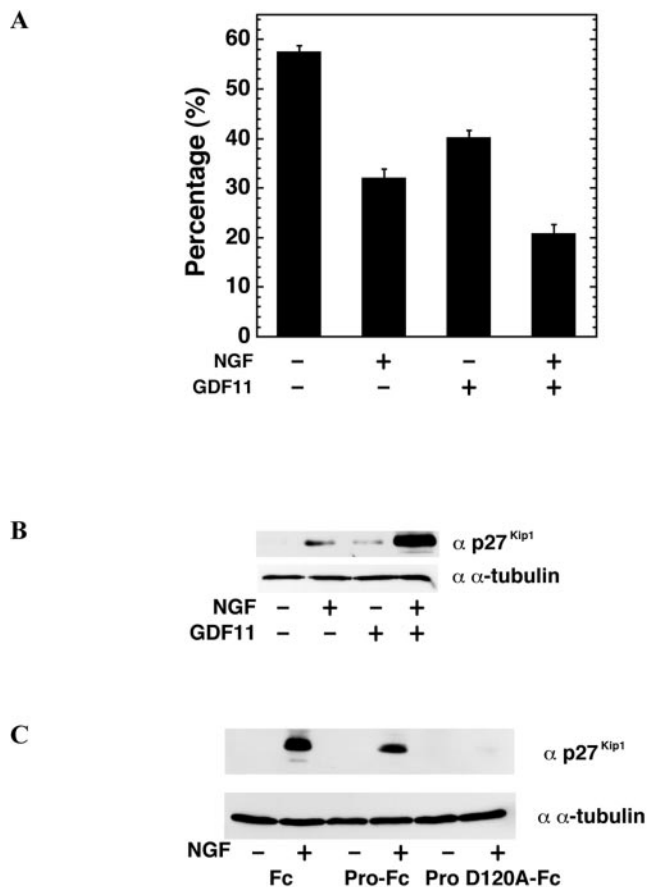


FIG. 9. Although they have opposite effects on differentiation, both NGF and active GDF11 induce PC12 cell cycle arrest and p27^{Kip1} expression. (A and B) PC12 cells incubated in the presence (+) or absence (-) of 50 ng/ml NGF and/or 5 ng/ml active GDF11 were assayed for the percentage of cells incorporating BrdU (A), or were lysed and assayed by immunoblotting for levels of p27^{Kip1} expression (B). Levels of α -tubulin were also determined by immunoblotting, as a control for loading. (C) PC12 cells incubated in the presence (+) or absence (-) of 50 ng/ml NGF and/or 250 ng/ml wild-type (Pro-Fc) or D120A-substituted (Pro D120A-Fc) GDF11 prodomain-Fc fusion protein, or 80 ng/ml Fc domain (as a negative control), were assayed for levels of p27^{Kip1} expression.

seen in Fig. 9B, both NGF and GDF11 induced increased levels of p27^{Kip1}, and these levels were appreciably higher in cells incubated in the presence of both factors. The similar effects of NGF and GDF11 in inducing cell cycle arrest, but the opposite phenotypic consequences, are consistent with the probability that the two factors are inducing cell cycle arrest in two different populations of cells. The most straightforward explanation of the various data is that GDF11 induces cell cycle arrest in progenitor cells, prior to commitment to terminal differentiation, such that a neural-tissue-like phenotype is not achieved, whereas NGF induces cell cycle arrest in cells further along in the progression towards terminal differentiation, such that a neural-tissue-like phenotype is achieved. The seemingly synergistic effect in p27^{Kip1} induction in cells treated with NGF and GDF11 together (Fig. 9B) may result from a greater effect of GDF11 on morphologically undifferentiated

cells that have been started down the differentiation pathway by NGF.

In a related experiment, addition of wild-type prodomain-Fc fusion protein to NGF-treated PC12 cells resulted in slightly decreased levels of p27^{Kip1}, whereas addition of D120A-substituted fusion protein produced a markedly greater decrease, such that only a faint band of p27^{Kip1} was visible (Fig. 9C). These results are again consistent with the probability that endogenous GDF11 and BMP1 are involved in NGF-induced differentiation of PC12 cells to a neural-tissue-like phenotype. The seemingly greater effect of D120A-substituted fusion protein on PC12 cell p27^{Kip1} levels (Fig. 9C) than on PC12 cell differentiation (Fig. 9B) may relate to the fact that NGF-induced PC12 cell differentiation and cell cycle arrest are also mediated via additional pathways that do not involve p27^{Kip1} (15, 30).

DISCUSSION

Vertebrate BMP1/TLD-like proteinases have previously been shown to play multiple roles in morphogenetic events, via positive regulation of extracellular matrix formation and via activation of BMPs 2 and 4, which are important in patterning and organogenesis (11, 27, 28, 33). More recently, BMP1/TLD-like proteinases were shown to activate myostatin/GDF8, via freeing mature myostatin sequences from a latent, noncovalent association with its cleaved prodomain (42). Here we demonstrate that GDF11 forms a noncovalent latent complex with its cleaved prodomain and describe a new role for mammalian BMP1/TLD-like proteinases in activating GDF11 via cleavage within latent complex prodomain sequences.

Myostatin, the TGF- β superfamily member most closely related to GDF11 by sequence homology, appears to limit skeletal muscle growth during development and in the adult (13, 16, 23, 25, 45) via a negative feedback mechanism that involves induction of Cdk inhibitor p21-mediated cell cycle arrest in muscle progenitor cells (22). Myostatin has thus been referred to as a "chalone" (45), hypothetical factors secreted by tissues to negatively regulate their own growth, via negative feedback (3). GDF11 appears to play a number of roles in development and in the adult. Unlike myostatin, GDF11 may play a role in patterning, since knockout mice homozygous null for the *gdf11* gene show anteriorly directed homeotic transformations of the axial skeleton, consistent with a role for GDF11 in anterior-posterior patterning (24), and GDF11 signaling may be involved in assigning positional identities to spinal motor neurons (18). GDF11 also appears to play a role in organogenesis of the kidneys (24) and endocrine pancreas (12), and it has been suggested that GDF11 may play a role in the induction of mesoderm (7, 24). In the context of the present study, it is of particular interest that GDF11 appears to play the role of chalone in controlling the growth of the olfactory epithelium, since evidence has been presented that GDF11 secreted by mature neurons inhibits further neurogenesis within that tissue, via induction of cell cycle arrest in neural progenitors (43). Here we demonstrate that GDF11 inhibits the NGF-induced differentiation of PC12 cells to a neural-tissue-like phenotype, thus supporting the possibility of a wider and more general role for GDF11 in neurodifferentiation. The demonstrated expression of GDF11 in diverse neural tissues, which include devel-

oping spinal cord and dorsal root ganglia and various portions of embryonic and postnatal brain (7, 26), is also consistent with a widespread role in neural tissue development and homeostasis.

GDF11 inhibition of neurogenesis in olfactory epithelium appears to occur via induction of cell cycle arrest, accompanied by increased expression of the Cdk inhibitor p27^{Kip1} (43). Here we demonstrate that GDF11 inhibits NGF-induced PC12 cell differentiation in a similar fashion, via induction of cell cycle arrest, accompanied by increased p27^{Kip1} levels. GDF11 blockage of neurodifferentiation in olfactory epithelium appears to involve the cell cycle arrest of neuronal precursors, prior to full progression down the neurodifferentiation pathway (43). In fact, GDF11 appears to inhibit the differentiation of pancreatic β -cells in a similar way, via the induction of cell cycle arrest in progenitor cells (12). Thus, although terminal differentiation of cells into neurons, or β cells, is itself accompanied by cell cycle arrest, cell cycle arrest of precursors prior to sufficient progression down differentiation pathways is a mechanism for the prevention of differentiation. Since NGF-induced differentiation of PC12 cells to a neural-like phenotype is also associated with cell cycle arrest (10), shown here to be accompanied by induction of p27^{Kip1}, we conclude that GDF11 inhibition of PC12 differentiation occurs via induction of p27^{Kip1}-accompanied cycle arrest at a point in the differentiation pathway early enough to preclude full progression to a neural-like phenotype.

Demonstration here that mature GDF11 forms a noncovalent, latent complex with its cleaved prodomain, expands the range of TGF- β -like proteins known to form such complexes. Previously, this small subgroup of the more than 30 known TGF- β -like molecules has consisted of TGF- β s 1, 2, and 3 (2) and myostatin (13, 16, 39, 42, 45). It remains to be determined which other TGF- β -like proteins may similarly be regulated via noncovalent complex formation with their cleaved prodomains. The present report also expands the known range of TGF- β -like proteins activated via BMP1/TLD-like proteinases. Previously, this small subgroup has consisted of myostatin (42), BMPs 2 and 4, and the *Drosophila melanogaster* BMP 2/4 orthologue decapentaplegic, the latter three proteins being activated via cleavage of the extracellular antagonist chordin/SOG (21, 27, 29, 33). Addition of myostatin and GDF11 to the list of substrates increases the range of morphogenetic processes known to be regulated by BMP1/TLD-like proteinases to include chalone activity, in addition to previously known regulatory roles in patterning and extracellular matrix formation.

Of great potential interest, are the results reported here that suggest possible use of exogenously added GDF11 propeptide sequences to stimulate proliferation/differentiation of neuronal precursors. Such uses conceivably include enhancement of the ex vivo derivation of neurons from progenitor cells. In vivo, GDF11 propeptide sequences have potential uses in the induction of neural growth, with implications for therapeutic interventions in neural injuries and neurodegenerative diseases. In support of the latter possibilities, myostatin prodomain-Fc fusion protein, analogous to GDF11 prodomain-Fc fusion proteins described herein, has been demonstrated to induce marked increases in muscle mass when administered to normal adult mice (42). Interestingly, mutant myostatin prodomain-Fc fusion, made impervious to cleavage by BMP1/TLD-like pro-

teinasas by a single amino acid substitution, but not wild-type myostatin prodomain sequences, had this *in vivo* effect (42). Thus, the GDF11 propeptide-Fc fusion protein described herein, which a single amino acid substitution renders impervious to cleavage by BMP1/TLD-like proteinases, is a potential reagent for the enhancement of neural growth *in vivo*.

The finding that BMP1-like proteinases cleave/activate GDF11 suggests that one or more of these proteinases are potential targets for therapeutic interventions in aid of neural growth. It remains to be determined which of these proteinases are engaged in activating GDF11, and myostatin, *in vivo*. However, there may be functional overlap in this regard, since although the proteinases mTLL1 and mTLL2 have relatively high levels of expression in neural tissue and muscle, respectively (33, 37), the proteinases BMP1 and mTLD show relatively broad distributions of expression that overlap the expression domains of both mTLL2 and mTLL1 (33, 37, 38).

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