CDMP1/GDF5 Has Specific Processing Requirements That Restrict Its Action to Joint Surfaces*

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CDMP1/GDF5 has not demonstrated biological activity in *Xenopus* embryos when overexpressed by mRNA injection. We provide biological and biochemical evidence that to become active, the protein requires cleavage by two distinct proteolytic enzymes. We demonstrate a specific overlap in the expression patterns of CDMP1/GDF5 with the proteases required to release the mature peptide at the location of the future articular surface but not in the future joint space. Taken together, these observations provide a plausible mechanism for local action of CDMP1/GDF5 consistent with requirements imposed by current models of pattern formation in the developing limb.

As clinical applications of cellular therapies and engineered tissues become more widespread, improved understanding of the molecular processes underlying their mode of action will be needed. Greater biological insight will be important not only for devising improved therapeutic approaches but also for refining manufacturing processes and testing of therapeutic products to achieve consistently high quality. This has particular relevance to joint resurfacing and repair, an area of increasing interest for potential therapeutic interventions. (see, for example, www.fda.gov/ohrms/dockets/ac/05/briefing/2005-4093B1_01.pdf/, www.fda.gov/ohrms/dockets/ac/05/slides/2005-4093S2_02_files/frame.htm/, and www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4093T1.htm/, www.fda.gov/ohrms/dockets/ac/05/slides/2005-4093S1.htm, and www.fda.gov/ohrms/dockets/ac/05/transcripts/2005-4093T2_01.htm). When considering postnatal injury repair it is well recognized that there is often a recapitulation of the molecular pathways that control embryonic processes. Specification of cell and tissue fate, establishment of boundaries between developmental fields, and formation of higher order anatomic patterns are prime examples.

Several molecular entities that function primarily during embryogenesis under normal circumstances are currently being evaluated for their therapeutic potential in joint repair. Prominent among these are the bone morphogenetic proteins (BMPs3; for reviews, see Refs. 1 and 2). The BMPs comprise a large class of cystine knot-containing proteins that form homo- or heterodimers that are processed by subtilisin-like proprotein convertases (SPCs; for review, see Ref. 3) to yield mature, active secreted signaling molecules (4–6). Proteolytic cleavage of BMPs occurs at a characteristic RXR site that divides the mature peptide from the amino-terminal “pro” region (sometimes called the “canonical” proteolytic processing site). Although it is now appreciated that BMPs help control a wide variety of processes, initial attention has focused on their actions in bone and cartilage formation.

One BMP that is being examined for utility in various strategies to repair damaged joint surfaces is cartilage-derived morphogenetic protein 1 (CDMP1, also called growth and differentiation factor 5 or GDF5 in non-human species; Refs. 7–9). This growth factor has been implicated in limb patterning, is expressed predominantly in developing limbs at joint interzones, and is known to play an important role in joint formation (7–9). Its absence leads to brachypodism in mice (7) and a number of skeletal malformation syndromes in humans, including at least three forms of autosomal recessive acromesomelic chondrodysplasia (8–10) and autosomal dominant brachydactyly types C and A2 (11, 12). Increased activity leads to symphalangism, characterized by joint fusions (12). Its close association with joint formation suggests it may be useful in repairing damaged joint surfaces in vivo or in engineering artificial joint structures in vitro.

Unlike many other BMPs, GDF5/CDMP1 is processed poorly when transfected into COS cells unless the mature region is fused to the dorsalin pro-region (9, 13). Should this deficiency be true for other cell types, it may present a severe practical difficulty for the manufacture and/or potential therapeutic use of the native protein. CDMP1 is also one of only two BMPs reported to be without effect when overexpressed in *Xenopus* embryos (14, 15). In contrast, overexpression of several other BMPs induces ventral fates (for review, see Ref. 16). These observations suggest the possibility that GDF5/CDMP1 may differ from most other BMPs in its requirements for proteolytic activation.

Several studies have explored the ability of various SPCs to

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activate members of the transforming growth factor-β (TGF-β) superfamily by proteolytic cleavage at the characteristic RXRR site. SPC1, also known as Furin, enhances the processing of TGF-β1 (17). SPC1 and SPC4 (also known as PACE4) are necessary and sufficient to promote Nodal maturation (5). Furin, SPC4, SPC6, or SPC7 can process BMP4 (4), and a recent Xenopus study has implicated SPC4 in the processing of Xnr1, -2, and -3 and Vg1 (6). Moreover, several BMPs are coexpressed with different members of the SPC family. BMP2, -4, and -7 are coexpressed with SPC4 in the primitive heart, in the apical ectodermal ridge of developing limb buds, and in the interdigital mesenchyme of embryonic limbs (20). During neural tube patterning, SPC6 colocalizes with BMP4 and BMP7 in the dorsal surface ectoderm, whereas SPC4 is coexpressed with BMP6 in the floor plate (20).

Analysis of the RXRR cleavage sites of different BMPs revealed that the site in CDMP1/GDF5 is identical to that of Vg1, the only other BMP that is not efficiently processed to the mature form in vivo (14). Because of the capabilities of Xenopus assays to investigate the biology of growth factors such as the BMPs and their interactions with other molecules required for their action, we used this system to explore possible explanations for these observations. In particular, we have evaluated the possibility that stringent constraints on proteolytic processing of CDMP1/GDF5 serve to localize its field of effect.

**MATERIALS AND METHODS**

Isolation of XGDF5 cDNA—A primer (5’-TATCATTGT-GAAGGGCTTTGGACTTCCC-3’) designed to a highly conserved region within the mature domain of human, mouse, chicken, and zebrafish GDF5 was used with the SMART™ RACE cDNA amplification kit (Clontech) and cDNA obtained from stage 22–24 Xenopus embryos. A 302-bp PCR product was obtained, which encoded part of the mature region of Xenopus GDF5, including the stop codon. From this, the full-length Xenopus GDF5 (XGDF5) was isolated using 5’-SMART™ RACE cDNA amplification with specific primers and Xenopus cDNA obtained from stage 59 limbs (accession number AY685227).

Plasmids and Probes—The XGDF5 open reading frame was subcloned into pBluescript (pXGDF5) to generate probes for hybridization in situ and into CS2 (CS2XGDF5) for production of capped mRNA for injection experiments using the Megascript and mMessage mMachine kits (Ambion), respectively. A point mutation (A → C) was made in CS2XGDF5 using the Quikchange™ site-directed mutagenesis kit (Stratagene) to change the RRKR cleavage site to RRRR, producing CS2XGDF5-R. Using PCR extension, a T7-tag flanked by EarI restriction sites was subcloned into PCR4 TOPO (Invitrogen). The T7-tag was subsequently subcloned into CS2XGDF5 and CS2XGDF5-R at a unique EarI site 3’ to the RXRR cleavage site, producing CS2XGDF5-T7 and CS2XGDF5-R-T7. Xenopus Furin was obtained as a 3122-bp full-length Image clone (3397590) encoding a 326-bp 5’-UTR, a 2349-bp ORF, and a 447-bp 3’-UTR and was subcloned into CS2 as an EcoRI-NotI (blunt-ended) fragment. A near full-length expression sequence tag for Xenopus SPC4 (Image clone 6865482) was obtained. The missing 211 bp of the 5’ sequence was generated using the 5’-SMART™ RACE kit (Clontech) and Xenopus limb cDNA as template. The resulting PCR product was subcloned into PCR-4 TOPO (Invitrogen) and the full-length construct made by subcloning a 3734-bp Ndel-NotI fragment from Image clone 6865482. Xenopus SPC4 was subcloned into CS2 as a 3401-bp Pmel fragment encoding a 52-bp 5’-UTR, a 2373-bp ORF, and a 616-bp 3’-UTR. Xenopus SPC6A, obtained as Image clone 4173657, was subcloned into CS2 as a 3114-bp EcoRI-XhoI fragment encoding a 332-bp 5’-UTR, a 2373-bp ORF, and a 49-bp 3’-UTR. The Xenopus SPC7 ORF (2265 bp) was contained in 2556-bp insert in pBluescript (Image clone 3378364). The pro-region of Xenopus GDF5, isolated as a 1371-bp PCR fragment using the primers 5’-TCGGTTCTTCTTTTCAA-GAACGA-3’ and 5’-ACAGTCTTGTATCTGTGGCCAGA-3’, was subcloned into PCR4-TOPO for generating RNA probes. Mouse Furin, SPC4, and SPC6 obtained as expressed sequence tags (Image clones 6492009, 4167960, and 6836178, respectively) were subcloned into pBluescript.

Oocyte Injections and Embryo Manipulations—Enzymatically defolliculated oocytes were injected with up to 50 ng of 5’-capped mRNAs and cultured in 50 µl of oocyte Ringer’s solution (21) for 48 h in 96-well plates at a density of 5 oocytes per well before harvesting. Frogs and their embryos were maintained and manipulated using standard methods (22–24). All embryos were staged according to Nieuwkoop and Faber (25) and Keller (26). mRNA injection experiments were performed by standard procedures as described previously (27). Dorsal and ventral blastomeres were identified by size and pigment variations (25). Animal cap explants were cultured in 0.5 × Marc’s modified Ringer’s solution (24). mRNAs were injected into a single blastomere at the two-cell stage or one dorsal blastomere at the four-cell stage. For conjugated animal cap assays, animal caps were removed from stage 9 embryos, conjugated immediately, and cultured in 0.5 × Marc’s modified Ringer’s until non-injected siblings reached stage 17 or 24. Perturbations of axial patterning were scored using the dorso-anterior index (DAI) (28). Darkfield images of embryos were photographed with low angle oblique illumination and a Zeiss Stemi-6 dissecting microscope.

**Immunoblotting**—Oocyte media were collected after 18–48 h post-injection, snap-frozen on dry ice, and stored at −80 °C until analysis. Oocytes were lysed by sonication on ice in 40 mM Tris base, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride in a volume of 10 µl/oocyte. Extracts were centrifuged at 20,000 × g for 5 min. Supernatants were extracted with an equal volume of 1,1,2-trichlorotrifluoroethane to reduce the vitellogenin content (29). SDS-PAGE was done with Novex 10% NuPAGE gels using the MES buffer system. Immunoblot analysis was performed using the mini-PROTEAN II system (Bio-Rad) and Immobilon™-P polyvinylidene difluoride membranes (Millipore). Tagged proteins were detected using 17-tag horseradish peroxidase-conjugated monoclonal antibody (Novagen) and SuperSignal® West Femto maximum sensitivity substrate (Pierce).

**RT-PCR**—Separate pools of embryos or explants from at least two different fertilizations were prepared and analyzed for each condition reported. Total RNA was prepared with TRIzol™ and treated with DNA-free™ DNase removal reagent.
FIGURE 1. Characterization and expression analysis of Xenopus GDF5. A, dendrogram showing the phylogenetic relationship between members of the GDF5, -6, -7, and -16 subfamily. Full-length amino acid sequences were analyzed using GeneWorks® Version 2.2 ( IntelliGenetics, Inc.) software. B, amino acid sequence comparison of full-length human, mouse, and Xenopus GDF5. Sequence identities are boxed; sequence similarities are highlighted in gray. C, RT-PCR analysis for Xenopus GDF5 using RNA obtained from indicated stages of Xenopus development (h = hindlimb; f = forelimb). D, whole mount hybridization in situ of Xenopus GDF5 of a stage 59 Xenopus forelimb. Arrows indicate the location of positive signal at presumptive joint interzones.
Ambion). Reverse transcription was done using ThermoScript™ (Invitrogen) as described by the manufacturer, with 1 μg of total RNA per reaction; 2% of the cDNA obtained was used in each PCR. Amplification was performed, using an air thermal cycler (Idaho Technologies), in 10-μl reactions containing 40 mM Tricine-KOH, pH 8.7, 15 mM KOAc, 3.5 mM Mg(OAc)₂, 0.375% bovine albumin, 2.5% Ficoll 400, 5 mM cre- sol red, 200 μM dNTPs, 0.5 μM each primer, and 0.2 unit of Advantage® 2 polymerase (Clontech). Each cycle comprised 94 °C, 0 s; 55 °C, 0 s; 72 °C, 40 s; a 1-min denaturation at 94 °C preceded cycling, and a 2-min extension at 72 °C was included after the final cycle. PCR products were separated on 2% agarose gels in TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.3), stained with SYBR Green 1™ (Molecular Probes, Eugene, OR) and scanned using a GE Healthcare Fluorimager. PCR analysis was performed at least twice for each cDNA to confirm that the amplifications were reproducible. The Xenopus primers used for Histone H4, Brachyury, Cardiac Actin, Goosecoid, N-CAM, Xhox3, and Xvent1 were as described in the Xenbase data base.

Hybridization in Situ—cRNA probes were produced using MEGAscript T3, T7, or SP6 in vitro transcription kits (Ambion), incorporating either digoxigenin or fluorescein. For whole mount hybridization in situ on Xenopus embryos, procedures outlined by Harland were followed (30), with modifications as described (27). Xenopus and mouse paraffin sections (10 μm) were prepared for fluorescent hybridization in situ using a standard protocol (31) with the following modifications: dewaxing was carried out in Clear-Rite 3 (Richard-Allan Scientific, Kalamazoo, MI). A H₂O₂ step was included (0.5% for 20 min at room temperature) to remove any endogenous peroxidase activity. Prior to hybridization, sections were incubated for 30 min at 90 °C in 10 mM citrate buffer, pH 6.0, to enhance antigenicity (32). Hybridization was performed at 60 °C overnight in the presence of a 1 μg/ml concentration of each probe. For single-label colorimetric detection, signals were developed using alkaline phosphatase-conjugated antibodies to digoxigenin and BM-Purple (Roche Applied Science). For double-label fluorescent detection, probes were labeled with either fluorescein or digoxigenin. An alkaline phosphatase-conjugated antifluorescein antibody and a horseradish peroxidase-conjugated antidigoxigenin antibody (Roche Applied Science) were used in combination with Fast™ Red (Sigma) and tyramide fluorogenic substrates (Molecular Probes), respectively. Confocal images were obtained using a Bio-Rad Radiance confocal microscope with a krypton-argon and blue diode.

RESULTS

Isolation and Expression of Xenopus GDF5—In previous experiments, dorsal overexpression of mammalian (murine and human) CDMP1/GDF5 in Xenopus embryos produced little or no effect on axial patterning (15). To determine whether this lack of effect might result from a species-to-species variation, we isolated the Xenopus GDF5 ortholog. This cDNA sequence was more similar to mammalian CDMP1/GDF5 than to other BMPs (Fig. 1A). In the region corresponding to the mature peptide, the amino acid sequence of the Xenopus clone was 92% identical to the human sequence and 93.6% identical to the chicken sequence. In the remainder of the molecule (the amino-terminal pro-region), the sequence identities were 48% (Xenopus-human) and 58% (Xenopus-chicken; Fig. 1B).

RT-PCR analysis of Xenopus GDF5 at various stages during development failed to detect GDF5 mRNA in tadpoles or younger embryos (stages 40 and below) but revealed high levels of expression in developing limbs at stage 59 (Fig. 1C). Hybridization in situ, using Xenopus limb whole mounts, showed Xenopus GDF5 to be localized at the joint interzones (Fig. 1D),
consistent with the spatial expression pattern observed in mammals (7).

Biological Activity of Xenopus GDF5 Depends on Proteolytic Processing—Xenopus GDF5 was overexpressed in Xenopus embryos by injecting up to 100 pg of mRNA into a single blastomere at the two- or four-cell stage with dorsal targeting. Only very mild ventralization (DAI value 4) was observed in 5% of injected embryos, whereas the remainder appeared normal (Fig. 3B). Since this behavior was inconsistent with the biological effects elicited by most other BMPs, we explored possible explanations. All orthologs of CDMP1/GDF5 have the putative proteolytic cleavage recognition sequence RRKR. Of all known BMPs, only CDMP1/GDF5 and Vg1 share this sequence. Since these two proteins are the only BMPs known to have no effect in Xenopus patterning assays, we tested whether modifications to the RRKR sequence resulted in detectable biological activity (Fig. 2A).

The RXKR sequence for the highly active TGF-β superfamily member Activin (RRRR) differs by only one amino acid (Fig. 2A). We therefore engineered a Lys → Arg point mutation into GDF5 to change the cleavage site to RRKR. We could thus test whether biological inactivity of wild type GDF5 might be explained by the nature of the RXKR site. Dorsal microinjection of Xenopus GDF5 (Lys → Arg) into two- and four-cell embryos produced severe ventralization (Average DAI = 1.4, Fig. 3C). These results support the idea that the RRKR sequence can be acted upon by endogenous SPCs but the RRKR sequence cannot.

To determine which SPC(s) could be involved in GDF5 cleavage, orthologs for six of the seven SPCs known to occur in mammals were identified in Xenopus. Developmental stages when GDF5 (stage 59 limbs) is expressed were tested for SPC expression. In limbs, RT-PCR demonstrated that Furin (SPC1), SPC4 (PACE4), and SPC6 were present, whereas SPC2, SPC3 (data not shown), and SPC7 were either absent or in very low abundance (Fig. 2B). Using expressed sequence tag clones and Smart™ RACE PCR, full-length Xenopus Furin, SPC4 (accession number AY685228), and SPC6A (accession number AY685229) were obtained for use in hybridization in situ and microinjection studies. The amino acid sequence identities between human and Xenopus SPC4 (PACE4) and SPC6A were 68 and 76%, respectively. RT-PCR analysis was also conducted on SPC expression during earlier stages of Xenopus development. Consistent with a recent report (33) Furin, SPC4/PACE4, and SPC6 were all present as maternal transcripts. In addition, we also identified SPC7 as being present maternally (Fig. 2B). Furin and SPC6 were found to be present in all stages tested, whereas SPC4 expression decreased beyond stage 16 and was expressed only weakly at stages 23 and 28. SPC7 expression decreased after 23 and was not detectable at stage 28.

Coinjection of GDF5 message (60 pg) with Furin or SPC6 mRNA alone (150 pg) produced mild ventralization (average
**CDMP1/GDF5 Processing**

DAI = 4.8 and 4.7, respectively; supplemental Fig. 1) that was significantly less than that observed for GDF5 (Lys → Arg) alone (average DAI = 1.4). Ventralization was not enhanced when the amounts of injected SPC mRNA were doubled (supplemental Fig. 1), suggesting the doses of mRNA for the single SPCs were saturating. We next examined whether coinjection of wild type GDF5 with two different SPCs would result in enhanced GDF5 activity. Injection of GDF5 message together with the combination of Furin and SPC6 mRNAs produced severe ventralization (average DAI = 2.4), similar to that observed for the Lys → Arg mutant (Fig. 3D). In contrast, combinations of either Furin and SPC4 or SPC4 and SPC6 messages with GDF5 did not increase the degree of ventralization above that observed when these SPC mRNAs were injected individually (supplemental Fig. 2).

**Release of Mature GDF5 Requires the Combination of Furin and SPC6**—To confirm biochemically that Furin and SPC6 were effective in converting GDF5 to its mature form, GDF5 was coexpressed with protease combinations in *Xenopus* oocytes. For these experiments, a T7 epitope tag was introduced into GDF5 as described, so that expression of both unprocessed and mature peptides could be detected by immunoblot analysis. The predicted molecular masses for *Xenopus* pro-GDF5-T7 (58.5 kDa) and mature GDF5-T7 (16.5 kDa) were calculated. The pro-form of GDF5 (Fig. 4A) could be detected in oocyte supernatants for all treatments. However, XGDF5 mature peptide could be detected only when mRNAs encoding both Furin and SPC6 were coinjected with the growth factor message (Fig. 4A). The additional band observed migrating above the mature form of GDF5 is presumably a partially processed peptide.

**Coexpression of GDF5 and SPCs in Vivo**—We compared the expression patterns of Furin and SPC6 with that of GDF5 using double-label fluorescent hybridization in situ. For these experiments, we chose paraffin sections of mouse embryo limbs and probes, since data were technically superior to those obtained using *Xenopus* limbs. GDF5 and each of the SPCs were analyzed pairwise in serial sections so that expression could be compared directly. In 14.5 and 15.5 days post-coitum mouse embryo limbs, GDF5 was expressed throughout the developing joint interzones, consistent with earlier reports (7). Although expression of the SPCs in the digits was widespread (Fig. 5), expression of GDF5, Furin, and SPC6 overlapped in a discrete, narrowly restricted region at the distal boundary of developing phalangeal surfaces but not within the region of the future joint space (Fig. 5). Overlap was also observed within the proximal perichondrium. Consistent with earlier reports (20), SPC4 expression was not observed in the joint interzone region but was restricted to hypertrophic cartilage (data not shown).

**Evidence for Cell-autonomous Processing of GDF5 by SPCs**—In many experimental systems, following translation, BMPs are processed intracellularly, suggesting that interactions between GDF5 and various SPCs might be cell-autonomous. To address this issue we analyzed conjugated animal caps derived from embryos injected either with GDF5 or with Furin and SPC6 mRNAs. These were compared with non-injected control caps and caps derived from embryos in which the growth factor and SPC mRNAs were coinjected. GDF5 activity in the conjugated animal caps was determined by RT-PCR analysis for the pan-mesodermal marker Brachury and the ventral markers Hox3, and Xvent-1. Histone H4 is included as a loading control. *Xenopus* embryos were injected equatorially at the four-cell stage with mRNAs for either GFP (500 pg), Furin (150 pg) + SPC6 (150 pg) + GFP (200 pg), GDF5 (100 pg) + GDF6 (100 pg) + Furin (150 pg) + SPC6 (150 pg) or GDF5 Mut Lys → Arg (100 pg) + GFP (300 pg).

**DISCUSSION**

Nearly all BMPs tested to date strongly perturb embryonic patterning when overexpressed in *Xenopus* embryos. In particular, GDF6, which is 78% identical to GDF5 in amino acid sequence in the mature region of the protein, ventralizes zebrafish embryos (34) and promotes epidermis formation while inhibiting formation of neural tissue in *Xenopus* animal cap explants (35). We therefore sought to examine CDMP1/
GDF5 signaling using *Xenopus* as an assay system. Consistent with an earlier report (15), however, we found that injection of the wild type mRNA produced little effect in conventional *Xenopus* patterning assays. The finding that mammalian COS cells transfected with wild type CDMP1/GDF5 do not process the protein efficiently (9) suggested a stringent constraint on CDMP1/GDF5 proteolytic processing. All known CDMP1/GDF5 orthologs have an invariant RRKRR sequence within the putative proteolytic processing site (Fig. 2A). We therefore evaluated the possibility that this feature might render it refractory to processing by endogenous SPCs.

Support for the notion that lack of processing might explain the inactivity of wild-type CDMP1/GDF5 came from experiments with a *Xenopus* GDF5 Lys → Arg point mutant designed to mimic the cleavage site found in the highly active TGF-β superfamily member Activin (RRRR). When overexpressed dorsally, the GDF5 Lys → Arg mutant ventralized *Xenopus* embryos dramatically. Experiments were subsequently performed to determine which SPCs were able to process wild type GDF5. Overexpression of wild type GDF5 with single SPCs (Furin, SPC4, or SPC6) produced only mild ventralization when compared with the Lys → Arg mutant. In contrast, the combination of Furin and SPC6 was found to be synergistic, producing ventralization comparable with that obtained with the Lys → Arg mutant at half the dose used to test single SPCs alone. The combination of Furin with SPC4 was not synergistic, a finding consistent with apparent normal joint development in mice made hypomorphic for SPC4 by targeted deletion (36).

Our results suggest that the cleavage site of GDF5 might confer an especially stringent constraint on cleavage of this growth factor to its mature form in vivo. This conclusion is supported further by biochemical confirmation that two SPCs are required to facilitate complete proteolytic processing of GDF5 (Fig. 4A).

The expression pattern of GDF5 at joint interzones resembles the stripe-like patterns of *wingless* or *decapentaplegic* in *Drosophila*, which serve to organize compartment boundaries (37). A similar role has been suggested for CDMP1/GDF5 in establishing boundaries between developing skeletal elements (38). In this regard, the joint interzone can be viewed as a signaling center, regulating chondrocyte proliferation and differentiation and orchestrating joint formation (39). Dual-label hybridization *in situ* showed coexpression of GDF5, Furin, and SPC6 predominantly in a narrow zone near the distal boundary of GDF5 expression and within the adjacent proximal perichondrium. Expression of Furin and SPC6 is not apparent in the future joint space.

![Figure 5](image)

FIGURE 5. Dual-label hybridization *in situ* showing the spatial distribution of mRNAs for GDF5 and SPC6 in 15.5 days post-coitus mouse embryo limbs. GDF5 expression (green) is restricted to the region of joint interzones. Furin and SPC6 expression (red) is distributed more widely in the developing digit. GDF5/SPC overlapping expression (yellow) is observed predominantly at the distal boundary of GDF5 expression and within the adjacent proximal perichondrium. Expression of Furin and SPC6 is not apparent in the future joint space.
markers, was only present in caps co-injected with GDF5, Furin, and SPC6 (Fig. 4B). When caps injected with GDF5 mRNA only were conjugated with caps injected with mRNAs encoding Furin and SPC6, there was no marker induction. Production of mature CDMP1/GDF5 is thus likely to occur only in cells co-expressing the growth factor and the required proteases. Consequently, in vivo, mature, active CDMP1/GDF5 is likely to be secreted only by chondrocytes at the boundary of the developing joint surface in a very narrow zone where such coexpression was observed (Fig. 5). GDF5 expressed within the remainder of the joint interzone, in the absence of Furin and SPC6, will be the non-processed, inactive form. Such a restricted pattern of GDF5 activity provides for its specific function in the morphogenesis of individual joints. It is well established that during joint formation the proximal and distal boundaries adopt “interlocking and reciprocally shaped joint sides” (40). Our finding that GDF5 is likely to be active only in discrete regions of the developing joint surface, and not within the joint space, is consistent with this concept. Furthermore, GDF5 has been shown to signal preferentially through the BMPR1B receptor (41) that is also absent from the developing joint space. BMPR1B expression does not overlap that of CDMP1/GDF5 but is localized immediately adjacent to it in the developing cartilage (40). It is also expressed in the adjacent proximal perichondrium (41, 42).

Absence of CDMP1/GDF5 results in abnormal joint formation and ligament defects resulting in joint dislocations (7–9). GDF5 is implicated in ligament formation by studies showing that ectopic expression of GDF5 (subcutaneous or intramuscular) leads to the formation of ligament-like tissue (43). The presence of active GDF5 in the perichondrium adjacent to the joint interzone (Fig. 5) is compatible with GDF5 involvement in ligament formation. It will be of interest to determine to which cell population within the perichondrium GDF5 is signaling, as the embryonic origin of ligamentous tissue has not yet been established (44).

Normal joint formation appears to require inhibition of BMP action within the developing joint space. Overexpression of BMP activity in developing cartilage or absence of BMP inhibition results in increased chondrocyte differentiation and ablated joint formation (45–47). Accordingly, two different BMP antagonists, noggin and chordin, are both expressed within the joint interzone (47, 48). Our data suggest that an additional inhibitor of BMP activity is also present in the joint interzone: unprocessed CDMP1/GDF5. Expression of CDMP1/GDF5 in the absence of the SPCs required for processing and secretion will result in intracellular accumulation of unprocessed peptide. Previous experiments have demonstrated that unprocessed human CDMP1 is able to heterodimerize with other BMPs, including BMP2 and BMP4, inhibiting their processing and secretion (9). Thus, intra-articular expression of CDMP1/GDF5, but not Furin and SPC6, provides an additional mechanism to block the action of BMPs that would otherwise promote ossification within the joint space.

We are now also able to address the outstanding question of why, unlike many other BMPs, analyses of COS cells transfected with wild type CDMP1/GDF5 demonstrated the growth factor to be present primarily in the unprocessed form (9, 13). In these studies, CDMP1/GDF5 was efficiently processed only when a chimeric protein was used in which the pro-domain and cleavage site was derived from dorsalin (9, 13). These observations can be explained by the presence of only low levels of Furin and the absence of SPC6 in these cells (19). Consequently, in vitro, processing efficiency in transfection experiments will depend on whether the host cells coexpress the necessary levels of Furin and SPC6. When considering therapeutic applications requiring industrial scale production of the native CDMP1/GDF5 protein or the use of this growth factor in cell and gene therapies, it will be prudent to assess the levels of Furin and SPC6 within the cellular expression system.

The synergistic effect of two different SPCs on GDF5 remains to be explained. Our data are consistent with the concept of sequential cleavage at two different sites, as described for BMP4 (18), but do not account for the observation that the Lys → Arg point mutant is processed into a biologically active molecule by endogenous SPCs. One possibility is that a characteristic higher order structure associated with the RRKR sequence imposes specific requirements for proteolytic processing of the GDF5 dimer that can be met only by combinations of SPCs.

In summary, critical events in patterning of appendicular joints appear to be controlled in part by proteolytic processing of CDMP1/GDF5. Tight restriction of the expression domains of the growth factor and proteases at future articular surfaces could create a sharply limited zone of effect. Our findings also reaffirm the principle that important biological signals often do not act in isolation. Increasingly complex strategies to deliver instructive signaling molecules for therapeutic purposes, for example via gene therapy vectors, will need to address the role of any critical trans-acting factors, which may or may not be expressed at the proposed site of administration.

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