

Latent Transforming Growth Factor β -Binding Proteins-2 and -3 Inhibit the Proprotein Convertase 5/6A^{*[5]}

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The basic amino acid-specific proprotein convertase 5/6 (PC5/6) is an essential secretory protease, as knock-out mice die at birth and exhibit multiple homeotic transformation defects, including impaired bone morphogenesis and lung structure. Some of the observed defects were attributed to impaired processing of the TGF β -like growth differentiating factor 11 precursor (proGdf11). In this work we present evidence that the latent TGF β -binding proteins 2 and 3 (LTBP-2 and -3) inhibit the extracellular processing of proGdf11 by PC5/6A. This is partly due to the binding of LTBPs in the endoplasmic reticulum to the zymogen proPC5/6A, thus allowing the complex to exit the endoplasmic reticulum and be sequestered as an inactive zymogen in the extracellular matrix but not at the cell surface. This results in lower levels of PC5/6A in the media, without affecting those of PACE4, Furin, or a soluble form of PC7. The secreted soluble protease-specific activity of PC5/6A or a variant lacking the C-terminal Cys-rich domain (PC5/6- Δ CRD) is significantly decreased when co-expressed with LTBPs in cells. A similar enzymatic inhibition seems to apply to PACE4 and Furin. *In situ* hybridization analyses revealed extensive co-localization of PC5/6 and LTBP-3 mRNAs in mice at embryonic day 15.5 and post partum day 1. In conclusion, this is the first time that a zymogen of the proprotein convertases was shown to exit the endoplasmic reticulum in the presence of LTBPs, representing a potential novel mechanism for the regulation of PC5/6A activity, e.g. in tissues such as bone and lung where LTBP-3 and PC5/6 co-localize.

The mammalian proprotein convertases (PCs)² form a family of nine serine proteinases related to subtilisin that primarily modify the activation state of a wide range of bioactive proteins. Seven PCs, PC1 (also known as PC3), PC2, Furin, PC4, PC5/6 (also known as PC5 or PC6), PACE4, and PC7, cleave protein precursors at basic sites during their transit through the secre-

tory pathway and/or at the cell surface. Among these basic amino acid-specific PCs, Furin, PC5/6, PACE4, and PC7 are ubiquitous or are widely distributed, although they exhibit characteristic patterns of expression in specific tissues and cells.

PC5/6 is the only member of the PC family that exists as two isoforms: soluble PC5/6A (1) and membrane-bound PC5/6B, which has an extended C-terminal Cys-rich domain (CRD) (2). Except in the small intestine and kidney, PC5/6A is the major isoform in all other tissues (3). PC5/6A is synthesized as an inactive zymogen (proPC5/6A). It undergoes a first autocatalytic processing in the endoplasmic reticulum (ER) at RTKR¹¹⁶ \downarrow (supplemental Fig. S1), resulting in a tight binding complex of the inhibitory prosegment with the protease, allowing the protein to exit the ER. It is then activated by a second autocatalytic cleavage within the prosegment at RTIKR⁸⁴ \downarrow (supplemental Fig. S1), which mostly occurs on the cell surface, where PC5/6A is anchored through its CRD that binds to heparan sulfate proteoglycans (HSPGs) (4).

PC5/6 knock-out (KO) mice die at birth, and the newborn pups recapitulate all the phenotypes observed in mice lacking growth and differentiation factor 11 (Gdf11, also known as bone morphogenic protein 11 (BMP11)) (5), including an altered antero-posterior patterning with extra thoracic and lumbar vertebrae, lack of tail, and kidney agenesis (6, 7). Gdf11 thus seems selectively cleaved by PC5/6 during development. In agreement, *in vitro* and *ex vivo* analyses showed a high selectivity of PC5/6 for Gdf11 compared with the other constitutively secreted PCs, PACE4, Furin, and PC7 (6).

Gdf11 belongs to the transforming growth factor β (TGF β) superfamily, which includes activins, nodals, BMPs, growth differentiating factors, and canonical TGF β s. These TGF β -like factors form homodimers that are cleaved at site 1 (S1) intracellularly or extracellularly into N-terminal inhibitory prodomains and mature C-terminal domains that remain non-covalently associated. The prodomain of canonical TGF β s interacts with latent TGF β -binding proteins (LTBPs) that facilitate the secretion of the ligand and target the latent complex to the extracellular matrix (ECM) (8). LTBPs belong to the LTBP/fibrillin superfamily, a group of high molecular weight ECM protein that contains several eight-cysteine repeats. LTBP-1, -3, and -4 form a disulfide bond with TGF β prodomains through a Cys in their third 8-cysteine repeat, resulting in a large complex (9). Upon secretion, this complex accumulates in the ECM (10), likely via binding of LTBPs to fibrillin-1 (11), and awaits a local activation that requires a second cleavage at site 2 (S2) within the prodomain to release the mature and active TGF β ligand.

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

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² The abbreviations used are: PC, proprotein convertase; PC5/6, proprotein convertase 5/6; Ab, antibody; ER, endoplasmic reticulum; ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; LTBP, latent TGF β binding protein; Gdf11, growth differentiating factor 11; BMP, bone morphogenic protein; pSmad2, phospho-Smad2; CRD, cysteine-rich domain; S1, site 1; S2, site 2; pC, protein C; SP, signal peptide.

Latent TGF β -Binding Proteins-2 and -3 Are Natural Inhibitors of PC5/6A

Despite the identification of PC5/6 as the enzyme responsible for the first cleavage of proGdf11, little is known about how and where the cleavage occurs. The interaction between Gdf11 and LTBP has never been investigated. Myostatin (also known as Gdf8) is the closest member to Gdf11, sharing 92% amino acid identity in their mature domain and 49% identity in their prodomain (12). It was recently demonstrated that LTBP-2 and -3 interact non-covalently with pro-myostatin early along the secretory pathway. LTBP-3 furthermore reduces pro-myostatin secretion by retaining it in the ECM and, therefore, inhibits its cleavage by membrane-bound Furin (13). As a consequence, LTBP-3 negatively regulates myostatin signaling. Whether this inhibitory effect also occurs with Gdf11 is not known. We thus analyzed the effect of LTBP-2 or LTBP-3 on Gdf11 trafficking, processing, and activity.

Herein, we demonstrated that PC5/6A cleaves proGdf11 extracellularly and that LTBP-2 and -3 inhibit this cleavage and consequently decrease the Gdf11-mediated Smad2 signaling. We also showed that inhibition of Gdf11 cleavage is partly due to proPC5/6A sequestration in the ECM.

EXPERIMENTAL PROCEDURES

Expression Constructs—Mouse 7B2 (14, 15), PC1/3, PC2 (16), PC5/6A (17), PC5/6B (18), PC5/6- Δ CRD, PC5/6A^{R116A} (17), human PACE4 (17), human Furin (19), and soluble rat PC7 (20) with or without a C-terminal V5 tag were expressed using pIRES2-EGFP vectors (Clontech). Mouse Gdf11 (21) and HA epitope-tagged human LTBP-2 and mouse LTBP-3 (13) were expressed using pcDNA3 vectors.

Cell Culture and Transfection—COS-1 and HEK293 cell lines were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Invitrogen). All cells were maintained at 37 °C under 5% CO₂. At about 80–90% confluence, COS-1 and HEK293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and Effectene (Qiagen), respectively. Stable transfectants of proGdf11 were obtained in HEK293 and COS-1 cells upon hygromycin B selection.

Cell Surface Biotinylation, Immunoprecipitation, and Western Blotting—Cultured cells were washed with serum-free medium 24 h post-transfection and incubated with serum-free medium for the following 24 h. Media were then collected, and cells were lysed in 50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in the presence of a mixture of protease inhibitors (Roche Applied Science). Lysates were centrifuged, and supernatants were collected. For media swap experiments, serum-free media were collected at 48 h post-transfection and swapped. After 24-h incubations, media and cells were collected.

For cell surface biotinylation, HEK293 cells were transiently transfected. 48 h post-transfection, the cells were washed with cold PBS adjusted to pH 8.0 and biotinylated with 0.2 mg/ml sulfo-NHS-LC-Biotin (Pierce) for 30 min at 4 °C. The cells were then incubated with 100 mM glycine for 5 min to quench the reaction. As a control, the cells are treated with 0.05% trypsin for 15 min on the ice to remove all cell surface protein. Trypsin was then inactivated with 10% fetal bovine serum. The cells were finally washed with PBS and harvested as described above. The cell lysates were then incubated with streptavidin coupling

with agarose (Fluka) for 12 h. The beads were washed six times by lysis buffer and then resolved by Western blotting.

For immunoprecipitation, cell lysates and media were incubated overnight at 4 °C with a PC5/6 prosegment rabbit polyclonal antibody (Ab) pPC5 (18) (1:100), anti-V5 (1:500; Sigma), or anti-HA (1:500; Covance). Protein A-agarose beads were then added to the antigen-antibody complexes, incubated for 3 h, and washed 6 times with the above lysis buffer and 1 time with cold PBS buffer.

Protein samples were heated in reducing Laemmli buffer, resolved on SDS-polyacrylamide gels, electrotransferred onto PVDF membranes, incubated with specific primary and secondary antibodies, and revealed by chemiluminescence (Amersham Biosciences). The following Ab were used: anti-pPC5 (1:2,000), anti-PC7 (1:10,000 (22)), anti-mPC1-NT (1:2,000, (16)), anti-mPC2 (1:2,000, (16)), anti-Furin (1:1,000, Alexis), β -actin (1:5,000; Sigma), anti-pSmad2 (1:1,000; Cell signaling), anti-protein C (1:3,000; Roche Applied Science), anti-HA (1:3,000; Covance), and horseradish peroxidase (HRP)-conjugated mouse anti-V5 (1:10,000; Sigma). Bound primary Abs were detected using anti-mouse IgG-HRP or anti-rabbit-IgG-HRP secondary Ab (both at 1:10,000; Amersham Biosciences).

Immunocytochemistry—COS-1 cells were plated on glass-bottom culture dishes (MatTek) and transfected the following day. After 24 h, cells were washed 3 times with PBS and fixed in 3.7% paraformaldehyde for 10 min at room temperature. For intracellular labeling, cells were permeabilized in methanol for 3 min at –20 °C, then washed in PBS and incubated for 5 min in 150 mM glycine. Cells, either permeabilized or not, were washed once in PBS, incubated for 30 min in 1% BSA in PBS (blocking solution), and further incubated overnight at 4 °C with monoclonal mouse anti-V5 (1:200), anti-pPC5/6 (1:100), anti-protein C (1:200), or anti-HA (1:500) in blocking solution. The next day cells were washed four times with PBS and incubated for 45 min with secondary Ab: anti-rabbit IgGs or anti-mouse IgGs coupled to either Alexa-fluor-488 (*green*), 555 (*red*), or 647 (*blue*) (Molecular Probes, Eugene, OR). Cells were then washed four times with PBS and mounted in glycerol 1,4-diazabicyclo[2.2.2]octane (DABCO; Sigma). Immunofluorescence analyses were performed with a Zeiss LSM-710 confocal microscope.

In Situ Hybridization—To generate mouse LTBP-3 cRNA, a fragment of cDNA corresponding amino acids 995–1257 was amplified by PCR using the primers 5'-CATATTGTTTGGGGCAGAGATCT and 5'-GCCAGCTTTGCAGACACAG and then subcloned into pDrive vector (Qiagen). Mouse PC5/6A cRNA probes corresponding to the coding region for amino acids 20–348 were described previously (23). Both probe were synthesized using ³⁵S-UTP and ³⁵S-CTP (>1000 Ci/mmol; Amersham Biosciences). Cryosections (8–10 μ m) were fixed for 1 h in 4% formaldehyde and hybridized overnight at 55 °C. For autoradiography, the sections were dipped in photographic emulsion (NTB-2, Eastman Kodak Co.), exposed for 5 days, and developed in D19 solution (Kodak).

In Vitro Activity Assay—Enzymatic *in vitro* assays were performed in 100 μ l of buffer (2 mM CaCl₂, 25 mM Tris-HCl, pH 7.0) at 37 °C in the presence of 100 μ M PC-substrate pyroglu-

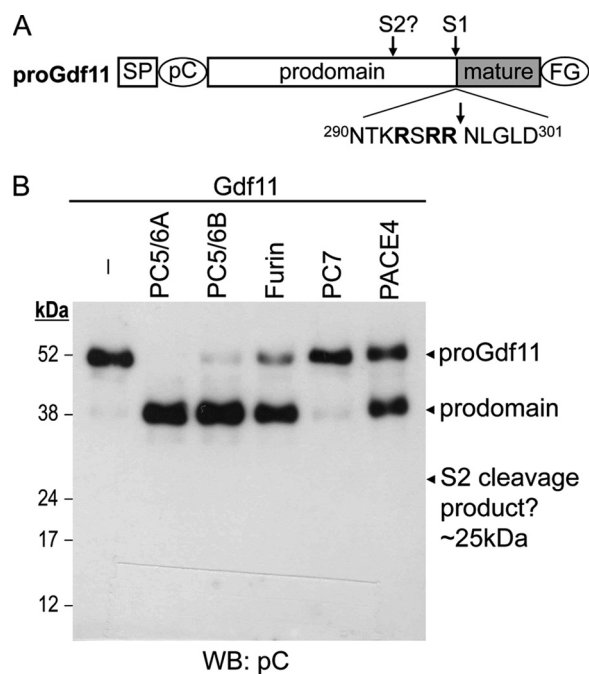


FIGURE 1. **PC5/6A does not cleave proGdf11 at site S2.** *A*, a schematic representation of the proGdf11 structure shows the signal peptide (SP), position of the N-terminal pC and C-terminal FLAG (FG) tags, the amino acid sequence surrounding the cleavage site at Arg²⁹⁶ ↓ (site S1) and the speculated site 2. *B*, shown is expression of proGdf11 in HEK293 cells alone (–) or with different PCs, including PC5/6A, PC5/6B, Furin, PC7, and PACE4. At 48 h post-transfection the media were analyzed by Western blotting using anti-pC Ab. The migration positions of proGdf11 (53 kDa) and its prodomain (37 kDa) are shown.

tamic acid-RTKR-7-amido-4-methyl-coumarin and 60 μ l of medium from HEK293 cells. The release of free 7-amino-4-methylcoumarin was detected with a Spectra MAX GEMINI EM microplate spectrofluorimeter (Molecular Devices; excitation, 360 nm; emission, 460 nm) (24). To calculate the relative fluorescence units, the background fluorescence was subtracted from fluorescent readings of each well. The enzymatic activity was determined by the slope of the linear portion of the data plot of relative fluorescence units *versus* time and expressed in relative fluorescence units/min.

RESULTS

PC5/6A Is Not Responsible for proGdf11 Processing at S2—It was previously reported that PC5/6 can cleave TGF β -like factors and BMP4 at both S1 and S2 (25). To examine whether proGdf11 could be cleaved by PC5/6A and/or PC5/6B at S2, we co-expressed proGdf11 carrying an N-terminal protein C (pC) and C-terminal FLAG tags (Fig. 1*A*) with PC5/6A or PC5/6B in HEK293 cells. Analysis of the media 48 h post-transfection by Western blotting using a pC-specific Ab revealed the efficient cleavage by both PC5/6 isoforms of the Gdf11 precursor (proGdf11, 53 kDa) into a 37-kDa species corresponding to the N-terminal prodomain (Fig. 1*B*). We did not observe any lower molecular mass product (expected ~25 kDa), indicating that PC5/6A and PC5/6B do not cleave proGdf11 at another site (S2) within the prodomain (Fig. 1*B*). PC5/6 seems to be the unique PC responsible for Gdf11 processing *in vivo* during embryonic development (6, 7). However, the ability of the three other constitutively secreted PCs, Furin, PC7, and PACE4, to

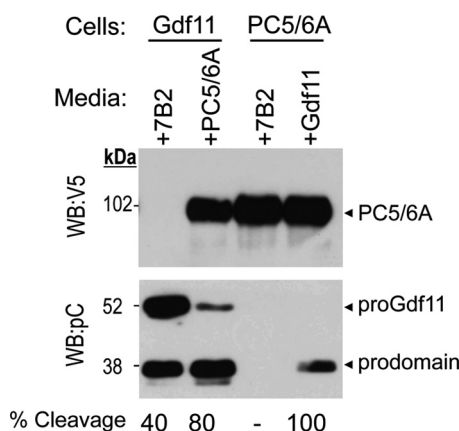


FIGURE 2. **ProGdf11 is cleaved extracellularly by PC5/6A.** The media from HEK293 cells transfected individually with V5-tagged PC5/6A, pC-tagged proGdf11, or an unrelated protein 7B2 were collected 24 h after transfection and incubated with HEK293 cells overexpressing either proGdf11 or PC5/6A for 12 h. Western blot analysis of the media used anti-pC Ab to reveal proGdf11 and its prodomain and anti-V5 Ab to reveal PC5/6A.

cleave proGdf11 at S1 and possibly at another site was also assessed. Although Furin and PACE4 both cleaved proGdf11 at S1, albeit to a lesser extent than PC5/6, PC7 did not. Here also as for PC5/6, no cleavage at another site by these PCs was observed (Fig. 1*B*), suggesting that none of the PCs are responsible for cleavage at S2. However, a second cleavage of the prodomain of Gdf11 at S2 has been reported to be performed by the metalloprotease BMP-1/Tolloid (21, 26).

ProGdf11 Is Cleaved by PC5/6A Extracellularly—Previous results showed that PC5/6A is activated at the cell surface upon a second autocatalytic cleavage of its prosegment (4). We thus hypothesized that proGdf11 could be cleaved extracellularly either at the cell surface or in the media. We first separately expressed in HEK293 cells proGdf11, PC5/6A, or an unrelated protein 7B2 as control. 7B2 is a neuroendocrine-specific protein (14, 27). Its primary function is to specifically bind the zymogen proPC2 in the ER and consequently to allow the productive folding of proPC2 and its exit from this compartment (28, 29). 7B2 is first cleaved in the Golgi by Furin (30) and the C-terminal domain acts as an inhibitor of mature PC2 until the complex reaches immature secretory granules, where the C-terminal domain of 7B2 is further cleaved by PC2, thereby liberating the active enzyme allowing it to act *in trans* on other substrates (27, 31).

After 24 h the media were collected and incubated for 12 h with HEK293 cells transiently transfected with cDNAs coding for either proGdf11 or PC5/6A and then analyzed the media by immunoblotting. On one hand, the addition of a medium containing PC5/6A to cells expressing proGdf11 led to a ~80% cleavage *versus* the basal control ~40% cleavage observed with 7B2 (Fig. 2). On the other hand, addition of a medium containing proGdf11 to cells expressing PC5/6A led to its complete cleavage into Gdf11 (Fig. 2). These data demonstrate that PC5/6A can cleave proGdf11 at the cell surface and/or in the medium.

LTBP-2 and -3 Interact with proGdf11 without Affecting Its Secretion—LTBP-2 and -3 were shown to interact intracellularly with pro-myostatin, the closest TGF β -like member to Gdf11, and to sequester it in the ECM (13). To examine whether

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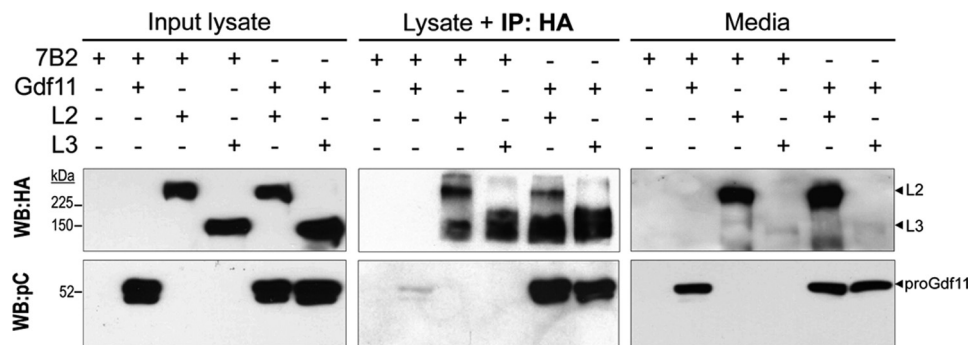


FIGURE 3. LTBP-2 and -3 interact with proGdf11 without affecting its secretion. ProGdf11, HA-tagged LTBP-2 (L2), or LTBP-3 (L3), and/or 7B2, a negative control protein, were co-expressed in HEK293 cells. Cell lysates were immunoprecipitated with anti-HA Ab, and the immunoprecipitated (IP) proteins or lysates and media were analyzed by Western blotting using anti-HA to reveal LTBP-2 or -3 and anti-pC to reveal Gdf11. Note that in cell extracts proGdf11 migrates as two bands with the upper one only seen in the media, likely due to terminal N-glycosylation trimming of secreted proGdf11 in the Golgi apparatus.

LTBPs play a similar role on proGdf11, we individually co-expressed in HEK293 cells their HA-tagged forms with proGdf11 or 7B2 as a control (Fig. 3). The migration positions of proGdf11 (~53 kDa), LTBP-2 (~250 kDa), and LTBP-3 (~150 kDa) and the levels of their co-expression were first detected by Western blotting of the input lysates (Fig. 3, *left panel*). Evidence for binding of proGdf11 with LTBP-2 or -3 was obtained by immunoprecipitation of cell lysates with the HA Ab and revelation by the pC Ab (Fig. 3, *middle panel*). The small amount of proGdf11 seen in cells only expressing Gdf11 and 7B2 is likely due to a nonspecific pulldown of proGdf11 by the HA Ab. In contrast to myostatin (13), proGdf11 in the media of cells co-expressing either 7B2, LTBP-2, or LTBP-3 was found at similar levels, indicating that it is not retained in cells or ECM by these LTBPs (Fig. 3, *right panel*).

To further investigate the cellular interaction between Gdf11 and LTBPs, they were transiently expressed either alone or together in COS-1 cells, and immunocytochemistry was performed using pC and HA Ab. Under permeabilizing conditions, we observed an almost complete co-localization of LTBP-2 or -3 with proGdf11 in a perinuclear ER-like compartment (*supplemental Fig. S2, left panel*). When the cells were not permeabilized, proGdf11 and LTBP-2 or -3 co-localized at the cell surface (*supplemental Fig. S2, right panel*). However, Gdf11 can bind the cell surface even in the absence of either LTBPs (*supplemental Fig. S2, top panels*), suggesting that the Gdf11-LTBP interaction is not critical for the intracellular and cell surface localization of Gdf11.

LTBP-2 and -3 Retain proPC5/6A in the ECM and Prevent Its Maturation—Although LTBP-2 and -3 do not affect proGdf11 levels in the media, their co-expression with PC5/6A in HEK293 or COS-1 cells reduced the levels of soluble PC5/6A in the media by 30–50%, as assessed by a C-terminal V5-Ab (Fig. 4A, *upper panels*). It is important to note that soluble PC5/6A in the media is only immunoreactive to the V5 and not to the prosegment (pPC5) Ab (see Fig. 7, *bottom panel*), suggesting an activated form that lost its prosegment (4). We also noted that no intracellular PC5/6A accumulation was observed in either cell line. Interestingly, removal of the HSPG binding C-terminal CRD domain (PC5/6- Δ CRD) (17) abrogated the reduction of the level of PC5/6A in the media by either LTBP-2 or -3 (Fig. 4A, *middle panel*). The effect seems to be specific to PC5/6A, as in HEK293 media the levels of either PACE4 (the closest mem-

ber to PC5/6A) or a soluble form of PC7 (sPC7) (32) are not affected by the co-expression of LTBP-2 or -3 (Fig. 4A, *lower panels*). Because both LTBP-2 (33) and -3 (34) were shown to assemble onto fibrillar structures in the ECM, we investigated whether the lower soluble levels of PC5/6A were due to its sequestration within the ECM. For this, PC5/6A was co-expressed with the unrelated protein 7B2 or LTBP-2 or -3 in COS-1 and HEK293 cells. After removing the cells in lysis buffer, plastic-bound proteins were extracted in boiling Laemmli buffer with a cell scraper. Western blotting revealed that upon co-expression with LTBP-2 or -3, ~1.5–3.6-fold more proPC5/6A was trapped within the ECM, based on the molecular mass (~110 kDa) and the immunoreactivity to both pPC5 and the C-terminal V5-tag Ab (Fig. 4B). Because the increased proPC5/6A retention in the ECM was not associated with an increased cell lysis in the presence of LTBP-2 or 3 (absence of β -actin in the ECM or media (not shown)), this suggests that LTBP-2 or -3 facilitates the exit of non-cleaved proPC5/6A zymogen from the ER, which normally does not exit this compartment (4, 17). More LTBP-3 than LTBP-2 was associated with the ECM in COS-1 cells but not in HEK293 cells (Fig. 4B), which may explain the lower levels of media LTBP-3 (Fig. 3). However, the amount of sequestered proPC5/6A was similar with both LTBPs (Fig. 4B). Thus, it seems that the level of LTBPs is not limiting for the ability of proPC5/6A to exit the ER and to be sequestered in the ECM. We presume that in a similar fashion both LTBPs primarily allow exit of the zymogen proPC5/6A from the ER, and the complex with LTBPs is sequestered very efficiently in the ECM. The retention of proPC5/6A in the ECM probably occurs via both the C-terminal CRD of PC5/6A (4) and LTBP-2 (35) or -3, all of which likely bind HSPGs and/or fibrillin-containing microfibrils (8). This is consistent with the fact that the media level of PC5/6- Δ CRD, which does not bind HSPGs, is not affected by LTBP-2 or -3. Finally, cell surface biotinylation revealed that LTBP-2 and -3 reduce the levels of cell surface-associated PC5/6A by ~20–30% (Fig. 4C), as it did in the media (Fig. 4A).

To confirm that LTBPs facilitate the exit of proPC5/6A from the ER and its sequestration in the ECM, we co-expressed LTBP-2 with the non-cleavable proPC5/6A-R116A mutant (*supplemental Fig. S1*), which results in a proPC5/6A form that does not exit the ER (17). Indeed, we can only detect proPC5/6A in cells and not in media (Fig. 4D). How-

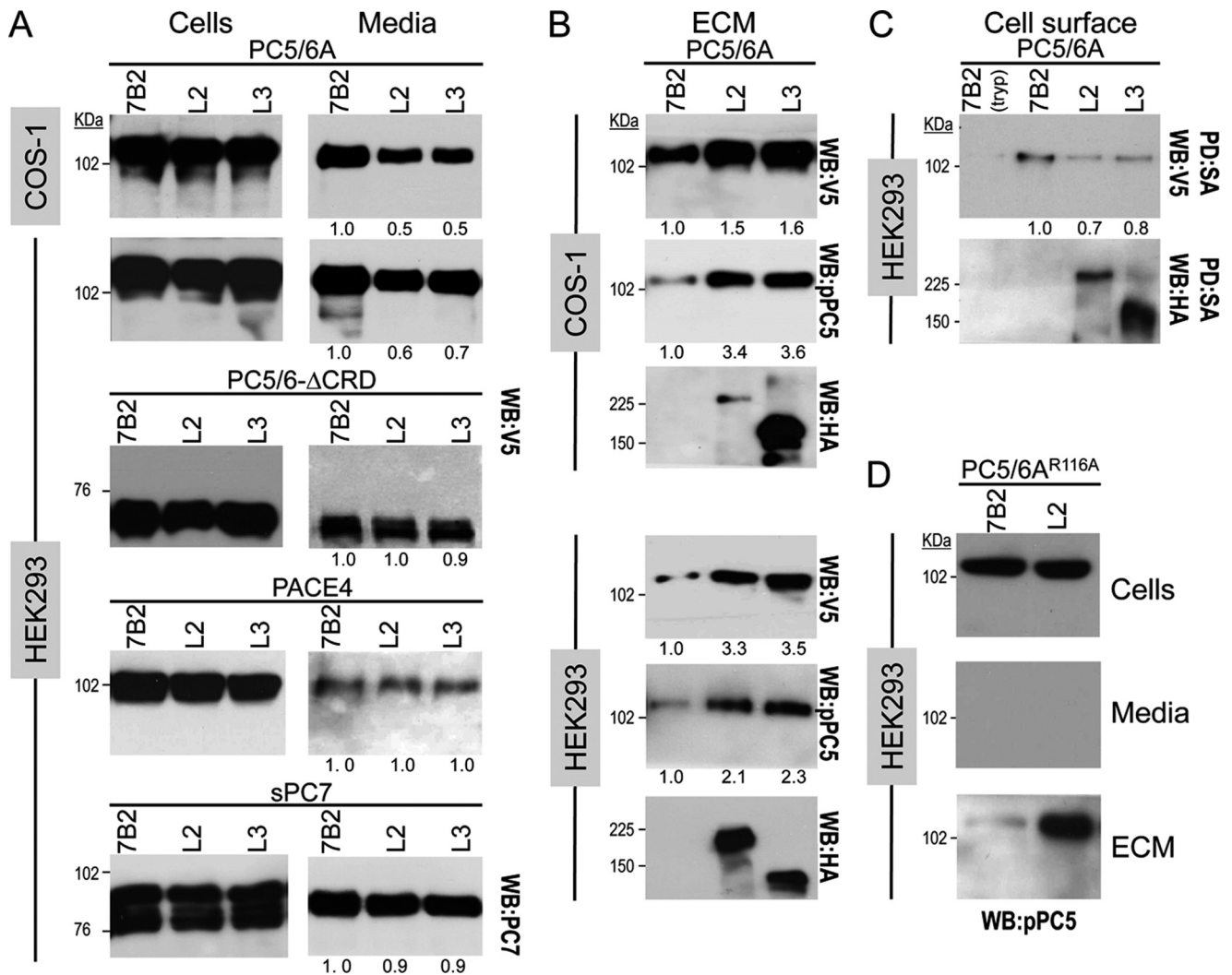


FIGURE 4. LTBP-2 and -3 allow the exit of proPC5/6A from the ER, and the complex is then secreted and retained in the ECM. A, V5-tagged PC5/6A and HA-tagged LTBP-2 (L2) or LTBP-3 (L3) or the negative control protein 7B2 were co-expressed in COS-1 or HEK2993 cells. PC5/6A was assessed by Western blotting using anti-V5 and anti-pPC5 Ab in the media and cell lysates (A) as well as ECM fractions (B). LTBP-2 or -3 were also detected in the ECM fractions using HA Ab. As controls, V5-tagged PACE4 and a soluble form of PC7 (sPC7) were quantified by Western blotting in media and cell lysates from HEK2993 using V5 and PC7 Ab, respectively. C, HEK2993 cells co-transfected with PC5/6A with 7B2 or LTBP-2 or -3 were biotinylated and pulled down (PD) with streptavidin-agarose (SA). The pulled down proteins were then analyzed by Western blotting using V5 and HA Ab. As the negative control for cell surface proteins, the cells co-expressing PC5/6A and 7B2 were treated with trypsin (Tryp), which removes all cell surface proteins. D, PC5/6A mutant R116A was either co-transfected with 7B2 or LTBP2 in HEK2993 cells. The cell lysates, media, and ECM fractions were analyzed by Western blotting using pPC5 Ab. Immunoblots were submitted to quantitative analysis using the ImageQuant software. The intensity was calculated relative to that of the control, which was fixed at 1. These data are representative of at least three independent experiments giving consistent and reproducible results.

ever, the cellular co-expression with LTBP-2 clearly enhanced the levels of proPC5/6A-R116A retained in the ECM as compared with those of the 7B2 control (Fig. 4D). The small amount of proPC5/6A in the ECM in the absence of co-expressed LTBP-2 may be due to endogenous expression of these proteins in HEK2993 cells.

Altogether, these data suggest that the decreased levels of PC5/6A in the media and cell surface are likely due to a pool of proPC5/6A that escaped autocatalytic cleavage at the primary RTKR¹¹⁶ ↓ site (supplemental Fig. S1) in the ER and that is subsequently secreted and trapped in the ECM and cannot be readily autoactivated into PC5/6A under basal conditions.

LTBP-2 and -3 Inhibit the Cleavage of proGdf11 by PC5/6A—It was previously shown that the sequestration of pro-myostatin in the ECM by LTBP-3 can inhibit its processing by Furin (13). Based on the fact that the both cellular and media levels of

proGdf11 are not affected by LTBP-2 or -3 (Fig. 3), we concluded that proGdf11 is not appreciably sequestered by LTBPs in the ECM, whereas proPC5/6A was. To examine whether proGdf11 processing was affected by LTBP-2 or -3, the latter were co-expressed with PC5/6A in HEK2993 or COS-1 cells that stably express Gdf11. Because mature Gdf11 (17 kDa) was almost undetectable with an anti-FLAG Ab (21) despite the presence of three FLAG epitopes at its C terminus, we rather analyzed proGdf11 or its remaining prodomain after cleavage (53 and 37 kDa; see Fig. 1) using protein C Ab.

In COS-1 cells, proGdf11 cleavage was modulated by the quantity of transfected PC5/6A cDNA. It was almost complete with 50 ng (96%) and reduced to 56 and 19% with 10 and 1 ng, respectively (Fig. 5, left panel). When LTBP-2 or -3 were co-expressed with 50 ng of PC5/6A, cleavage of proGdf11 was reduced from 96% to 40 and 18%, respectively. Similar data

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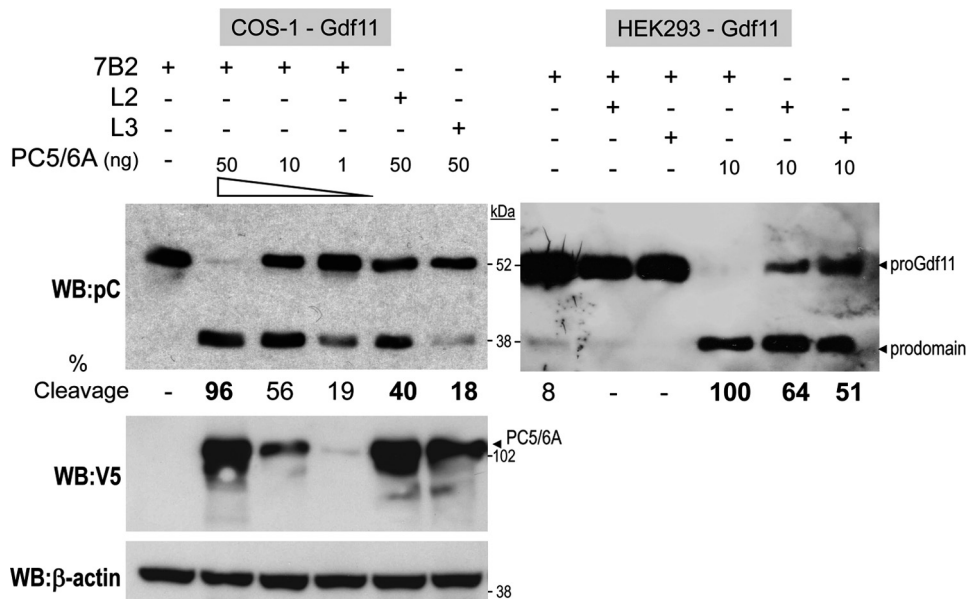


FIGURE 5. LTBP-2 and -3 partially and dose dependently inhibit the cleavage of proGdf11 by PC5/6A. COS-1 cells (*left*) and HEK293 cells (*right*) stably expressing proGdf11 were transfected with PC5/6A and/or LTBP-2 (L2) or -3 (L3). Conditioned media were analyzed by Western blotting using anti-pC Ab that recognizes the proGdf11 and its prodomain. The total quantity of cDNA used for transfection was kept constant by using a vector expressing an unrelated protein, 7B2. The quantity (ng) of transfected PC5/6A cDNA was indicated for both cell lines. The protein level of PC5/6A and β -actin were revealed in total cell lysates by Western blotting using V5 Ab and anti- β -actin Ab. The immunoblots were submitted to quantitative analysis by using ImageQuant. The percentage cleavage was calculated from the ratio of prodomain/(proGdf11 + prodomain). These data are representative of at least three independent experiments.

were obtained in HEK293 cells (Fig. 5, *right panel*). Herein, the 100% cleavage obtained with 10 ng of PC5/6A-expressing vector was reduced to 64 and 51% upon LTBP-2 or -3 co-expressions, respectively. In both cell lines, LTBP-2 and, to a larger extent, LTBP-3 inhibited the PC5/6A-mediated proGdf11 cleavage into mature Gdf11.

PC5/6A Inhibition by LTBP-2 and -3 Requires Their Intracellular Interaction—To determine whether the LTBP inhibited the overall PC5/6A activity or specifically the proGdf11 cleavage, we measured PC5/6A activity on a fluorogenic substrate pERTKR-7-amido-4-methylcoumarin (24) in the media of HEK293 cells that co-expressed PC5/6A with a control 7B2 or with LTBP-2 or -3. Although PC5/6A protein levels in the media of HEK293 cells were reduced by \sim 30% upon co-expression with LTBP-2 and -3, PC5/6A activity was reduced by \sim 80 and \sim 70%, respectively (Fig. 6A). Normalization of PC5/6A activity to its V5-immunoreactive protein quantity revealed that LTBP-2 and -3 achieved a \sim 75 and \sim 65% inhibition of activity, respectively (Fig. 6A). However, no inhibition of pERTKR-7-amido-4-methylcoumarin cleavage was obtained when media containing PC5/6A were co-incubated with those containing LTBP-2 or -3 (Fig. 6A), suggesting that the inhibitory complex formation occurs intracellularly before secretion. In agreement, proGdf11 cleavage was not affected when media containing proGdf11, PC5/6A, and LTBP-2 or -3 were co-incubated (Fig. 6B). We, therefore, conclude that PC5/6A inhibition requires a prior intracellular interaction with LTBP-2 or -3. Furthermore, the *in vitro* activity of soluble PC5/6- Δ CRD construct lacking the C-terminal CRD and, hence, cannot bind HSPGs, is also inhibited by co-expression with LTBP-2 or -3 (Fig. 6C). Interestingly, although the level of PC5/6- Δ CRD in the media is not affected by the co-expression of LTBP-2 or -3 (Fig. 4), the latter inhibit the *in vitro* activity of PC5/6- Δ CRD by

\sim 55–60% (Fig. 6C). Notably, because their media levels are unchanged, PACE4 (Fig. 4A) and shed Furin (supplemental Fig. S3A) are presumably not retained in the ECM in the presence of LTBP-2 or -3. In contrast, their activities in the medium are reduced by \sim 70% for PACE4 (Fig. 6D) and 50–60% for shed Furin (supplemental Fig. S3B). As negative controls, we have also compared the effects of LTBP-2 or -3 on the secreted pro-protein convertases PC1/3 or PC2 (supplemental Fig. S3). The media levels and activities of these two convertases were not affected by either LTBP-2 or -3.

Therefore, inhibition of PC5/6A by LTBP is a consequence of two additive effects. One is through reducing active PC5/6A media levels, and another one occurs via reduction of its enzymatic activity (Fig. 6A). However, we did not observe a reduction in media levels of PC5/6- Δ CRD (Figs. 4A and 6C). This suggested that the reduction of PC5/6A levels by LTBP in the media is CRD-dependent. It is consistent with the fact that PC5/6A- Δ CRD is less inhibited by LTBP (Fig. 6C).

We conclude that LTBP-2 and -3 can selectively reduce the media levels of full-length PC5/6A, which binds HSPGs but not those of PC5/6- Δ CRD. Furthermore, they do not affect the media levels of Furin, PACE4, or sPC7. In addition, LTBP-2 and -3 can inhibit the activity of PC5/6A (as well as PACE4 and Furin) independent of its ability to bind HSPGs, suggesting that they can also inhibit the catalytic subunit of these convertases independently from their ability to be sequestered in the ECM.

ProPC5/6A Forms an Intracellular Complex with proGdf11 and LTBP-2 or -3—To understand the mechanism of PC5/6A inhibition by LTBP, we analyzed the interactions between the enzyme, substrate, and inhibitors by co-transfection in HEK293 cells. Immunoprecipitation of PC5/6A with a V5 Ab and Western blots using various Ab demonstrated the interaction of proPC5/6A with proGdf11 or LTBP-2 and -3 (Fig. 7). Further-

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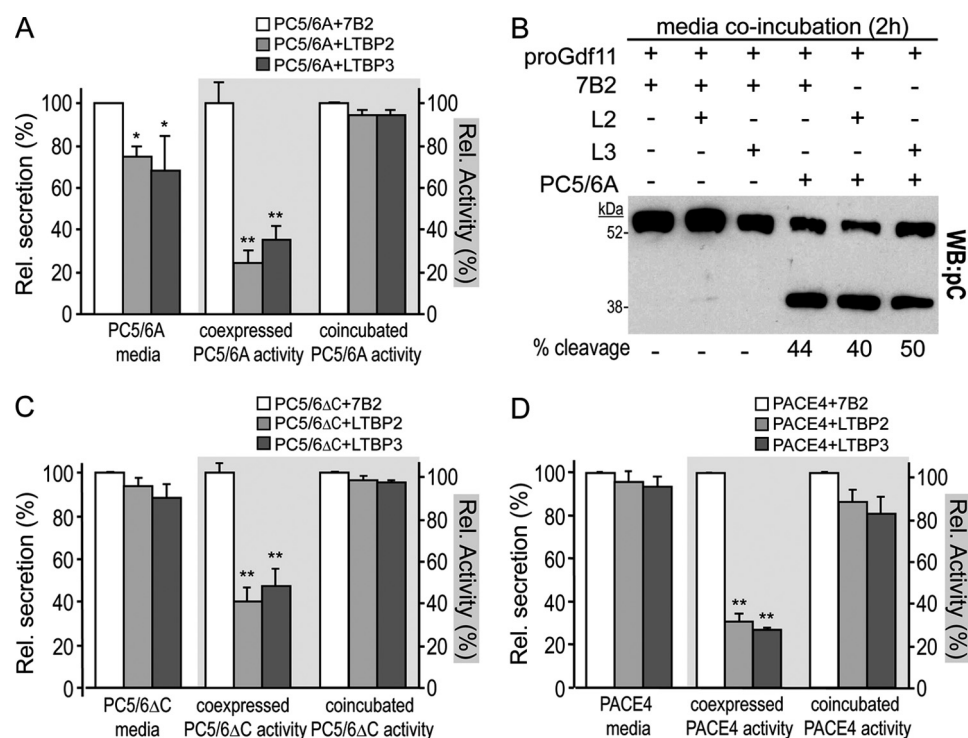


FIGURE 6. LTBP-2 and -3 reduce secreted PC5/6A and PACE4 activities when co-expressed in the same cells. The conditioned media from HEK293 cells co-expressing PC5/6A (A), PC5/6- Δ CRD (C), or PACE4 (D) with LTBP-2 or -3 were first analyzed by Western blotting to quantify the level of these PCs (left bars). *In vitro* enzymatic assay using a fluorogenic substrate, pERTKR-7-amido-4-methylcoumarin, was used to measure the activity of either the above media or those expressing individually PCs, LTBP-2, or LTBP-3 that were then mixed and incubated for 2 h. For equal amounts of PCs, the relative activity was normalized to relative fluorescence units in media containing only PCs fixed to 100% (shaded area). The error bars indicate S.E. of three independent experiments. *, $p < 0.05$; **, $p < 0.005$ (Student's *t* test). B, conditioned media from HEK293 cells expressing proGdf11 were incubated with those expressing individually PC5/6A or LTBP-2 or -3. Cleavage of proGdf11 was revealed by Western blotting using anti-pC Ab. The intensity was quantified by ImageQuant. The % cleavage was calculated from the ratio of prodomain/(proGdf11+prodomain).

more, in tripe-transfected cells, proPC5/6A can co-immunoprecipitate with proGdf11 and either LTBP-2 or -3. This suggests that these three proteins form an intracellular complex. Note that the levels of V5-immunoprecipitated proPC5/6A in cells are lower in the presence of either proGdf11, LTBPs, or both (Fig. 7, left panel). Because direct Western blots did not show this difference (Fig. 4A), we presume that complex formation partially interferes with the recognition of the V5 epitope in the C terminus of PC5/6A. In the media, however, only PC5/6A was found, lacking its prosegment, and neither Gdf11 nor LTBP-2 or -3 was precipitated with PC5/6A (Fig. 7).

Our previous results showed that in the cell the majority of PC5/6A immunoreactivity is associated with its zymogen proPC5/6A (4, 17). PC5/6A is then fully activated at the cell surface and released into the medium free of its prosegment (4). This was confirmed by the analysis of V5-immunoprecipitated proteins with the prosegment pPC5 Ab, which generated a similar pattern to that obtained with V5 Ab (Fig. 7; lower panels). These data suggest that proPC5/6A, and not PC5/6A, stably interacts with proGdf11 and LTBP-2 or -3 in the cells.

Further evidence for the existence of a ternary complex was obtained upon subcellular co-localizations by immunofluorescence. PC5/6A was co-expressed with proGdf11 or LTBP-2 or -3 in COS-1 cells. Under permeabilizing conditions, PC5/6A primarily co-localized with Gdf11 and LTBP-2 or -3 (Fig. 8) in an ER-like compartment (20). Under non-permeabilizing conditions, PC5/6A co-localized with Gdf11 or LTBP-2 or -3 at the

cell surface (Fig. 8). Note that we also showed that Gdf11 co-localized with LTBP-2 or -3 in the cell and at the cell surface (supplemental Fig. S2). Taken together, these data demonstrate that proPC5/6A, Gdf11, and LTBP-2 or -3 form intracellular and cell surface complexes. This may enhance the efficiency of proGdf11 processing by PC5/6A at the cell surface and its regulation by LTBP-2 and -3.

LTBPs Regulate the Activity of Gdf11—It has been demonstrated that Gdf11 was able to activate the downstream Smad signaling pathway by inducing Smad2 phosphorylation in *Xenopus* (36). Additionally, a second cleavage of the prodomain of Gdf11 at S2 by BMP-1/Tolloid has been shown to be important for Gdf11-dependent activation of Smad2 phosphorylation (37). We decided to examine the effect on Gdf11 activity of (i) the cleavage at S1 by PC5/6A and (ii) PC5/6A inhibition by LTBP-2 and -3.

The biological activity of Gdf11 can be measured by the extent of Smad2 phosphorylation, which was assessed by immunoblotting using an Ab to phosphorylated Smad2 (pSmad2). HEK293 cells transfected with proGdf11 exhibited a ~2.5-fold higher pSmad2 signal than those transfected with LTBP-2, LTBP-3, or PC5/6A alone (Fig. 9), which indicated the basal level of pSmad2. Although proGdf11 is likely activated by endogenous PC5/6A in HEK293 cells (3), the co-expression of PC5/6A with proGdf11 increased pSmad2 levels by 8.6- and 3.4-fold as compared with that observed with the expression of either PC5/6 or proGdf11 alone (Fig.

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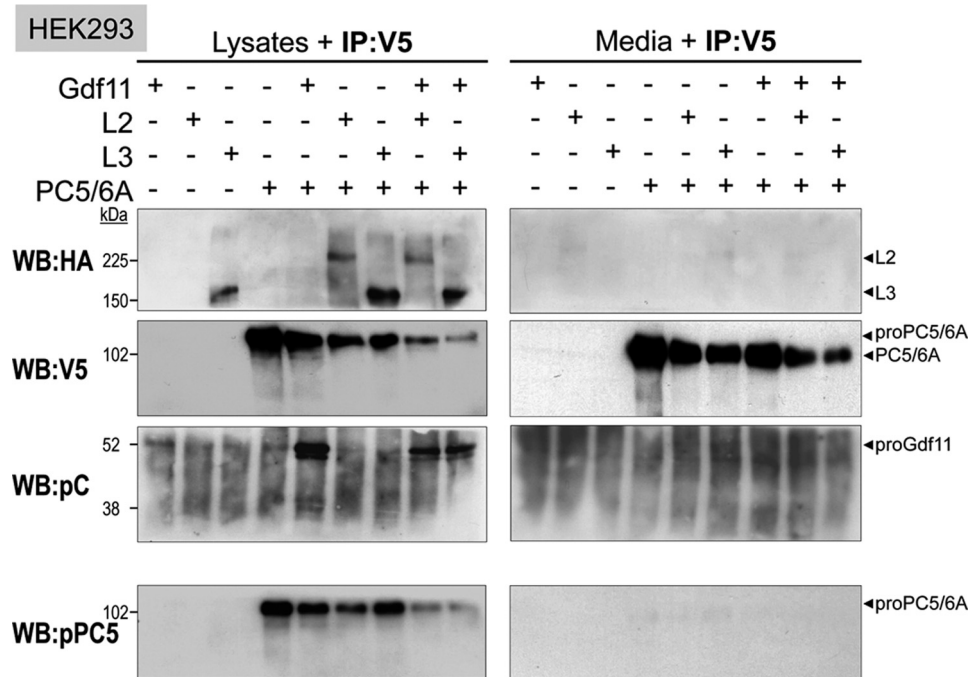


FIGURE 7. **ProPC5/6A forms an intracellular complex with proGdf11, LTBP-2, or LTBP-3.** In HEK293 cells, PC5/6A was expressed either with 7B2 or co-expressed with proGdf11 and 7B2 and/or LTBP-2 (L2) or -3 (L3) as indicated. The quantity of transfected PC5/6A cDNA was kept constant (10 ng), and the total level of cDNAs transfected was also kept constant (50 ng) by including a corresponding quantity of 7B2 cDNA, except for the triple transfections. PC5/6A from cell lysates or media was immunoprecipitated (IP) using anti-V5 Ab, and the immunoprecipitated proteins were detected by Western blotting using anti-HA, anti-pC, anti-V5, and anti-pPC5.

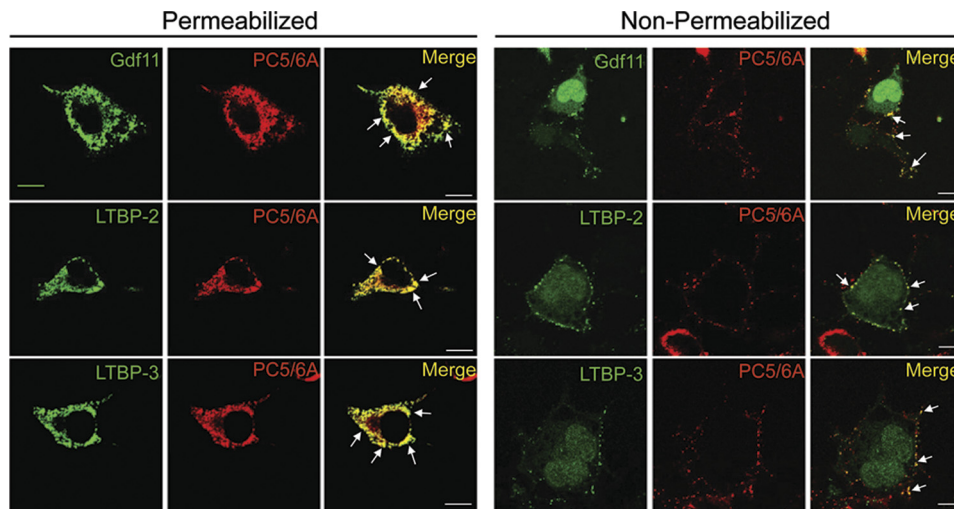


FIGURE 8. **PC5/6A co-localizes with ProGdf11 and LTBP-2 or -3 in cells and on the cell surface.** PC5/6A was co-expressed transiently with either proGdf11 or LTBP-2/-3 in COS-1 cells. The intracellular and cell surface immunofluorescence staining was performed in permeabilized and non-permeabilized COS-1 cells, respectively, using anti-HA Ab against LTBP-2 and -3 (green), anti-pC Ab against Gdf11 (green), and anti-V5 Ab against PC5/6A (red). Arrows point to sites where co-localizations were evident. Bar = 10 μ m. These data are representative of three independent experiments.

9). In the absence or presence of exogenous PC5/6A, LTBP-2 or -3 reduced pSmad2 levels by 1.3–2.9-fold (Fig. 9), demonstrating that PC5/6A inhibition by LTBP-2 and -3 led to reduced signaling of Gdf11.

Co-localization of PC5/6 and LTBP-3—To probe the physiological significance of the down-regulation of PC5/6 by LTBPs, we compared by *in situ* hybridization histochemistry their expression in mouse both at embryonic day 15.5 (E15.5) and at postpartum day 1 (P1) (Fig. 10). The data show an extensive co-localization of PC5/6 mRNA with those of LTBP-3 at E15.5 in various tissues and organs, including vertebrae, ribs,

blood vessels, and vertebral bodies of the tail. At postpartum day 1, clear co-localizations were seen in the stomach, kidney, lung, femur and humerus bones, ciliary bodies, and alveolar bones. Thus, LTBP-3 and PC5/6A co-localize at various developmental stages in bones and other tissues, befitting the deduced function of PC5/6 from its knock-out phenotype in bone morphogenesis as well as lung and tail formation (6, 7).

DISCUSSION

Previous work (6, 7) showed that PC5/6 is the proGdf11 convertase through cleavage at S1, and our present work further

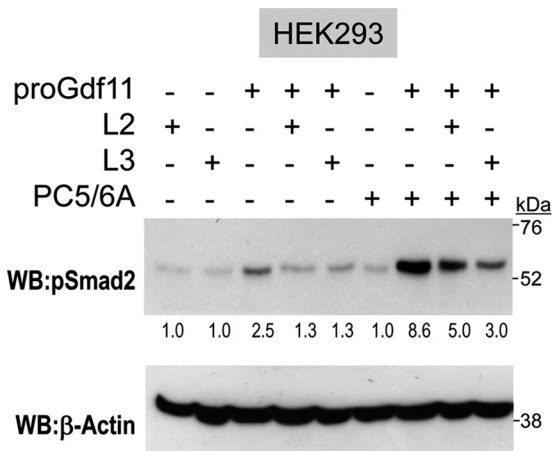


FIGURE 9. ProGdf11 processing regulates the phosphorylation of Smad2. ProGdf11, LTBP-2, LTBP-3, or PC5/6A were expressed alone or co-expressed in HEK293 cells as indicated. The cell lysates were detected by Western blotting using anti-phosphoSmad2 Ab and anti- β -actin Ab. The immunoblots were submitted to quantitative analysis by using ImageQuant.

demonstrates that neither PC5/6A nor any of the other PCs cleave at S2 (Fig. 1). The metalloprotease BMP1/Tolloid was reported to cleave proGdf11 within the prodomain at the S2 site Gly¹¹⁹ \downarrow Asp¹²⁰, leading to the dissociation of the prodomain from mature Gdf11 (21). Interestingly, it was suggested that PC5/6 activates BMP1/Tolloid (38) via cleavage at the PC-like site RSRR¹²⁵ \downarrow AA (39). We can thus conclude that proGdf11 activation requires two sequential cleavages; the first one at S1 by PC5/6 and the second one by activated BMP1/Tolloid at S2. Both occur extracellularly (Fig. 2 and Ref. 21).

The zymogens of the PCs do not exit the ER until a first autocatalytic cleavage takes place (40). An exception is proPC2 that can exit the ER as a complex with the inhibitory pro7B2 (27, 29, 41). Our data revealed a second exception whereby proPC5/6A (amino acids 35–915) can exit the ER by forming a complex with LTBPs without undergoing the canonical autocatalytic cleavage at Arg¹¹⁶ \downarrow (supplemental Fig. S1). Cleavage in the ER leads to an enzymatically inactive complex of the prosegment (amino acids 35–116) and mature PC5/6A (amino acids 117–915) (1, 42). Subsequently, a second autocatalytic cleavage at Arg⁸⁴ \downarrow (supplemental Fig. S1), occurring at the cell surface or in the ECM, releases the active form of PC5/6A into the medium (4). Upon co-expression of full-length PC5/6A and LTBP-2 or -3, proPC5/6A was found in the ECM, and ~30–50% lower levels of mature PC5/6A were detected in the medium (Fig. 4A). In contrast, the levels of mature PC5/6- Δ CRD in the medium were not affected by either LTBP-2 or -3 (Fig. 4A), suggesting that binding to HSPGs is important for ECM sequestration. The co-localization (Fig. 8) and co-immunoprecipitation of cellular proPC5/6A and LTBP-2 or -3 (Fig. 7), likely originating from the ER where they are mostly localized, suggested that these proteins form a complex very early along the secretory pathway. This complex may allow the exit of proPC5/6A from the ER and its accumulation in the ECM but not at the cell surface (Figs. 4, B and C). This was confirmed with the PC5/6A-R116A mutant, which remains as a zymogen that accumulated in the ECM in the presence of LTBP-2 (Fig. 4D). Whether this ECM-bound pool of proPC5/6A can be subsequently activated locally is not clear.

LTBP-2 and -3 inhibit the processing of proGdf11 by PC5/6A (Fig. 5) by reducing the levels of PC5/6A in the media but also by lowering the specific enzymatic activity of PC5/6A (Fig. 6A). Indeed, when equal quantities of secreted PC5/6A, obtained from cells either expressing it alone or co-expressing it with LTBP-2 or -3, were assessed, *in vitro* PC5/6A enzymatic activity was reduced by ~65% in the presence of co-expressed LTBPs (Fig. 6A). However, mixing media containing LTBP-2 or -3 and PC5/6A did not affect the protease activity (Fig. 6A). Thus, inhibition requires both PC5/6A and LTBP-2 or -3 to be co-expressed in the same cells. This suggests that LTBP-2 or -3 can inhibit PC5/6A activity, likely via a prior intracellular complex formation. The complex may remain stable and be directed to the ECM as an inactive zymogen or it could dissociate and allow PC5/6A activation, albeit less efficiently than in the absence of LTBPs. The fact that inhibition of PC5/6A activity can only be observed when LTBPs are co-expressed with the convertase suggested that the intracellular interaction between LTBPs and PC5/6A can induce some conformational change in the protease early in the secretory pathway, which would lead to reduced secreted enzymatic activity.

Conversely, the levels of PACE4 and shed Furin in the media of HEK293 cells were not affected upon their co-expression with either LTBP-2 or -3 (Figs. 4A and supplemental Fig. S3A). This suggested that different from PC5/6A, LTBPs do not sequester proPACE4 or proFurin in the ECM, which may be due to unique sequences in their prosegment that need to be identified (supplemental Fig. S1). However, the protease activity of PC5/6A (-70–80%), PACE4 (-70%), and Furin (-50 to 60%) are reduced in the presence of LTBPs (Figs. 6, supplemental Fig. S3), suggesting that the overexpression of the latter can also inhibit the catalytic activity of these enzymes in the media but not those of PC1/3 or PC2 (supplemental Fig. S3). Under limiting amounts of endogenous levels of LTBP-2 or -3, it is possible that the co-expressed PC5/6A (Fig. 10) would be mostly affected in both its secretion level and protease activity.

In the presence of LTBP-2 or -3, the media levels of proGdf11 were not affected (Fig. 3, right panel), suggesting that LTBPs do not sequester proGdf11 in the ECM. In contrast, LTBP-2 or -3 were shown to retain pro-myostatin in the ECM (13), thereby reducing the levels of mature myostatin in the medium. The abundance of the convertase PACE4 in skeletal muscle (43, 44) suggests that it could cleave pro-myostatin. In contrast, the levels of secreted mature TGF β 1 in the media of HEL cells were reported to be enhanced by the presence of LTBPs (45). The difference may be due to the fact that proTGF β 1 is best processed by membrane-bound Furin (46), whereas proGdf11 and pro-myostatin are likely to be best processed by PC5/6A (6) and possibly PACE4, both of which bind HSPGs at the cell surface and ECM via their C-terminal CRD domain (40).

Gdf11 globally regulates antero-posterior axial patterning by controlling the spatial-temporal expression of *Hox* genes. The loss of Gdf11 in mice causes anterior homeotic transformation of the axial skeleton and the absence of caudal segments (5). During embryonic development, the concentration of active Gdf11 is critical for its patterning function. Here we provided evidence for a mechanism by which PC5/6 and LTBP-2 or 3 tightly regulate Gdf11 activation, thereby modulating the gra-

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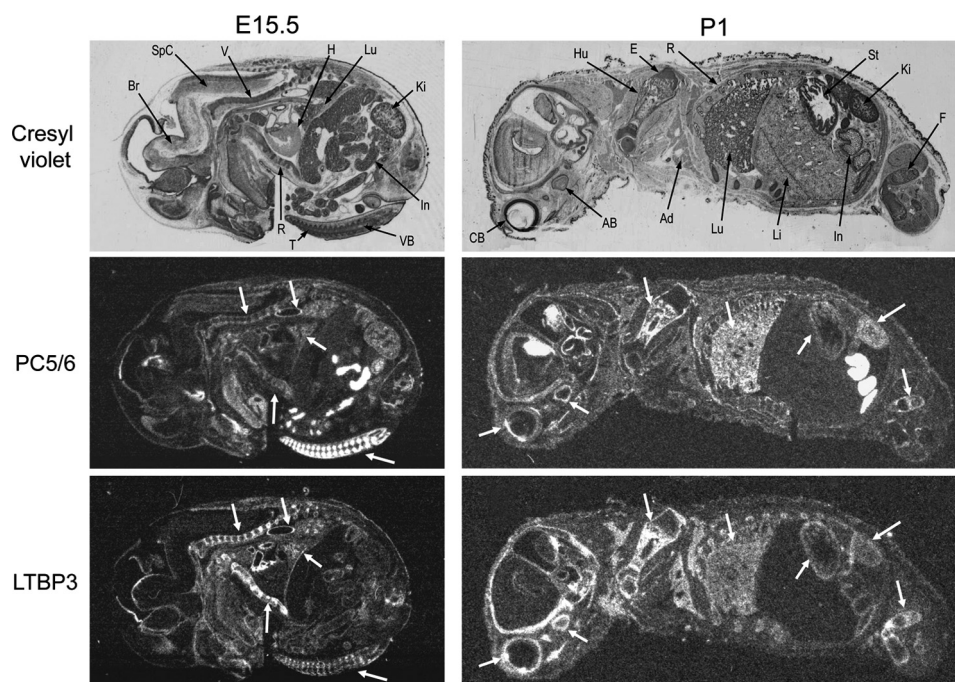


FIGURE 10. **Comparative *in situ* hybridization shows co-localization of PC5/6 and LTBP3 mRNA in E15.5 mouse embryo and newborn mouse.** E15.5 mouse embryo and newborn (P1) mouse were cut at lateral plan into 6 μm sections. The sections were stained with cresyl violet (top) or hybridized with ^{35}S -labeled mPC5/6 (middle)- and mLTBP-3 (bottom)-specific probes. PC5/6 and LTBP mRNA labeling were seen as bright under dark field illumination in the x-ray film autoradiogram. The white arrows indicate the major tissues of co-localization. Br, brain; H, heart; In, intestine; Ki, kidney; R, ribs; SpC, spinal cord; T, tail; V, vertebrae; VB, vertebral bodies; AB, alveolar bone; Ad, adipose tissue, intercapsular; E, epiphysis; F, femur; CB, ciliary body; Hu, humerus; Li, liver; Lu, lung; St, stomach.

dient of active Gdf11. First, the production of active Gdf11 depends on the level of PC5/6A (Fig. 5). Second, the quantity of extracellular active PC5/6A is negatively regulated by LTBP-2 and -3. These regulations of mature Gdf11 production were reflected in its downstream signaling, as reflected by Smad2 phosphorylation, which is reduced in the presence of LTBP3 (Fig. 9). We also noted that the reduction in pSmad2 signal caused by LTBP-3 is more significant than that by LTBP-2. This is consistent with the fact that inhibition of proGdf11 processing is more drastic with LTBP-3 than that with LTBP-2 (Fig. 5). Our work provided the first evidence that PC5/6A processing of proGdf11 into Gdf11 is important for its downstream activity, and thus, inhibition of this cleavage reduces Gdf11 activity.

Very recently, the first natural inhibitor of Furin was identified as plasminogen activator inhibitor 1 (47). Herein, we identified LTBP-2 and -3 as natural inhibitors of PC5/6A. What is the physiological significance of the observed inhibition of PC5/6A activity by LTBP3 and in what tissues will this be operating? First, we noted that in some tissues *Pcsk5* KO mice (6, 7) exhibit opposite phenotypes to those observed in *Ltbp-3* KO mice (48). For example, although lung alveoli are collapsed in *Pcsk5* KO mice, *Ltbp-3* KO mice exhibit a much larger volume of alveoli. In addition, *Pcsk5* KO mice exhibit ossification defects (6, 7), whereas *Ltbp-3* KO mice exhibit an osteopetrotic-like phenotype with increased bone deposition and excessive trabecular mass (48). Thus, it is possible that LTBP-3 down-regulates PC5/6 activity, specifically in lung and bones. In support of this hypothesis, *in situ* hybridization of PC5/6 and LTBP-3 mRNAs during development at E15.5 and at postpartum day 1 revealed a large degree of co-localization in the lung,

various bones including humerus, femur, and alveolar bones as well as kidney and tail (Fig. 10).

Interestingly, microarray data on hypophosphatemic mice that exhibit bone mineralization defects suggested that in bone osteocytes PC5/6 activates the bone forming BMP1/Tolloid (39) and inactivates the bone-desorbing fibroblast growth factor FGF23 by cleavage at the RHTR¹⁷⁹ \downarrow SA site (38). Further studies will be needed to confirm that PC5/6 is involved in bone mass up-regulation and that LTBP-3 may down-regulate this activity.

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