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BMP-4 is proteolytically activated by furin and/or PC6 during vertebrate embryonic development

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Bone morphogenetic protein-4 (BMP-4) is a multifunctional developmental regulator. BMP-4 is synthesized as an inactive precursor that is proteolytically activated by cleavage following the amino acid motif -Arg-Ser-Lys-Arg-. Very little is known about processing and secretion of BMPs. The proprotein convertases (PCs) are a family of seven structurally related serine endoproteases, at least one of which, furin, cleaves after the amino acid motif -Arg-X-Arg/Lys-Arg-. To examine potential roles of PCs during embryonic development we have misexpressed a potent protein inhibitor of furin, α_1-antitrypsin Portland (α_1-PDX) in early *Xenopus* embryos. Ectopic expression of α_1-PDX phenocopies the effect of blocking endogenous BMP activity, leading to dorsalization of mesoderm and direct neural induction. α_1-PDX-mediated neural induction can be reversed by co-expression of downstream components of the BMP-4 signaling pathway. Thus, α_1-PDX can block BMP activity upstream of receptor binding, suggesting that it inhibits an endogenous BMP-4 convertase(s). Consistent with this hypothesis, α_1-PDX prevents cleavage of BMP-4 in an oocyte translation assay. Using an *in vitro* digestion assay, we demonstrate that four members of the PC family have the ability to cleave BMP-4, but of these, only furin and PC6B are sensitive to α_1-PDX. These studies provide the first *in vivo* evidence that furin and/or PC6 proteolytically activate BMP-4 during vertebrate embryogenesis.

Keywords: α_1-antitrypsin Portland/BMP-4/furin/proprotein convertase/*Xenopus*

Introduction

The process of embryonic induction, in which one population of cells influences the developmental fate of another, plays an essential role in establishing the basic body plan of all multicellular organisms. These inductive events, as well as subsequent patterning events, rely heavily upon cell–cell interactions mediated by secreted proteins, including members of the transforming growth factor-β (TGF-β) family.

Bone morphogenetic proteins (BMPs) are members of the TGF-β family that have been implicated in the development of nearly all organs and tissues (reviewed by Hogan, 1996). One of the earliest and best-documented roles for BMPs is in establishment of the dorsoventral axis (reviewed by Graff, 1997). In *Xenopus*, expression of BMP-4 is restricted to cells on the ventral side of gastrula-stage embryos, where it plays a central role in specifying ventral mesodermal (e.g. blood) and ectodermal (i.e. skin) fates. When cells on the dorsal side of the embryo are made to misexpress BMP-4, they differentiate as blood rather than notochord, or form skin rather than brain. Conversely, when endogenous BMP signaling is blocked in ventral cells, by introduction of dominant interfering forms of either the BMP ligand or receptor, blood formation is eliminated and these cells form muscle instead. Furthermore, blockade of the BMP signaling pathway in explanted ectoderm causes these cells to differentiate as neural tissue. Thus, BMP-4 is required for ventral mesoderm formation and for the induction of epidermal fate at the expense of neural tissue.

As with all TGF-β family members, BMP-4 is synthesized as an inactive precursor and is proteolytically activated by cleavage following the multibasic amino acid motif -Arg-Ser-Lys-Arg- to yield a C-terminal mature protein dimer (Aono *et al.*, 1995). This processing event has been proposed to regulate the secretion and/or diffusion of BMPs, thereby controlling the range over which these molecules can signal during embryonic development (Jones *et al.*, 1996). In general, very little is known about intracellular assembly, processing and secretion of BMPs.

Members of a family of higher eukaryotic endoproteases, named proprotein convertases (PCs) (reviewed by Steiner *et al.*, 1992), are good candidates for endogenous BMP convertases. In mammals, seven members of this family have been characterized and designated furin, PC2, PC1/3 (hereafter called PC3), PACE-4, PC4, PC5/6A and B (hereafter called PC6A and B), and LPC/PC7/PC8 (hereafter called PC7) (Seidah and Chrétien, 1997). Individual PCs exhibit overlapping, but distinct, substrate specificities (Breslin *et al.*, 1993; Creemers *et al.*, 1993). Furin, the first member of this family to be characterized, is a membrane-associated, calcium-dependent serine endoprotease that proteolytically activates proprotein molecules at the C-terminal side of the consensus sequence Arg-X-Arg/Lys-Arg (Molloy *et al.*, 1992). Many precursor proteins, including those for TGF-β (Dubois *et al.*, 1995) and other growth factors, receptors, serum proteins, viral envelope proteins and bacterial toxins, share this cleavage site and can be efficiently cleaved by furin in gene transfer and *in vitro* digestion studies (reviewed by Bresnahan *et al.*, 1993; Nakayama, 1997).

Expression patterns of distinct PCs have been examined in a variety of species. In mammals, furin and PC7 are ubiquitously expressed throughout development (Constam *et al.*, 1996). In contrast, PC2 and PC3 transcripts are confined to neuroendocrine tissues, and specifically cleave

neuropeptides and other hormones (reviewed in Steiner *et al.*, 1992), while PC4 is restricted to testicular germ cells (Nakayama *et al.*, 1992). Interestingly, although PACE-4 and PC6A/B are expressed at low levels in all embryonic tissues, both genes display dynamic expression patterns throughout development and are upregulated in some tissues that exhibit high-level expression of BMPs (Constam *et al.*, 1996; Zheng *et al.*, 1997). This observation has led to the hypothesis that PACE-4 and PC6 may act in combination to locally modulate BMP activity (Constam *et al.*, 1996). However, direct evidence that BMPs are substrates for PC-like endoproteases is lacking.

To begin to test the hypothesis that BMP-4 is proteolytically activated by a member of the PC family, we have used a protein-based inhibitor, termed α_1-PDX, to block endogenous PC activity *in vivo*. This inhibitor is a genetically engineered mutant form of the naturally occurring serine protease inhibitor, α_1-antitrypsin (Anderson *et al.*, 1993). α_1-PDX contains in its reactive site the amino acids -Arg-Ile-Pro-Arg-, the minimal consensus motif for efficient processing by furin. This protein has been shown to be a potent inhibitor of furin and PC6B *in vitro* (Anderson *et al.*, 1993; Jean *et al.*, 1998).

In the present study, we demonstrate that ectopic expression of α_1-PDX phenocopies the effect of blocking endogenous BMP-4 activity in *Xenopus* embryos, and can rescue ventralization caused by overexpression of exogenous BMP-4. Furthermore, α_1-PDX-mediated patterning defects can be blocked by co-expression of downstream components of the BMP-4 signaling pathway. These results demonstrate that α_1-PDX can inhibit BMP activity upstream of receptor binding, and suggest that α_1-PDX blocks the activity of the endogenous protease(s) responsible for proteolytic activation of BMP-4. Consistent with this hypothesis, α_1-PDX completely blocks cleavage of BMP-4 in an *in vivo* oocyte translation assay. Furthermore, we find that while furin, PACE-4, PC6B, and PC7 all have the potential to cleave BMP-4, only furin and PC6B are sensitive to inhibition by α_1-PDX. Taken together, these studies provide the first *in vivo* evidence that proteolytic maturation of BMP-4 is achieved by furin and/or PC6, and demonstrate the feasibility of using a selective protease inhibitor as a tool to investigate the developmental functions of PCs in a whole-animal model.

Results

Misexpression of α_1-PDX respecifies the fate of ventral mesodermal cells

To begin to test the possibility that BMP-4 is a substrate for an endogenous furin-like endoprotease(s), we used a ventral marginal zone (VMZ) assay to ask whether misexpression of the PC inhibitor, α_1-PDX, can inhibit BMP-4 function in these cells. Overexpression of known antagonists of BMP-4, such as dominant negative receptors or ligands, can convert the fate of VMZ cells from blood and mesenchyme to more dorsal derivatives, such as muscle (reviewed by Graff, 1997). Thus, if α_1-PDX is sufficient to block the activity of an endogenous convertase that is required for proteolytic activation of BMP-4, overexpression of α_1-PDX should dorsalize the fate of VMZ cells.

Approximately 500 pg of synthetic RNA encoding either α_1-PDX or a dominant mutant truncated BMP receptor (tBR, as a positive control; Graff *et al.*, 1994) was injected near the ventral midline of four- or eight-cell embryos as illustrated at the top of Figure 1. VMZ tissue was dissected out of early gastrula stage embryos, cultured in isolation until sibling embryos reached the tailbud stage (stage 26) and analyzed for the presence of dorsal mesoderm by immunostaining with muscle- (Figure 1A–D) or notochord- (Figure 1E–H) specific antibodies.

The majority of VMZ explants from uninjected embryos remained rounded and formed neither muscle nor notochord (Figure 1B and F; Table I). An identical phenotype was observed in explants made to express α_1-PIT (Table I; data not shown), a naturally occurring mutant form of α_1-antitrypsin. α_1-PIT contains the residues -Ala-Ile-Pro-Arg- in its reactive site, and can inhibit thrombin but not PCs (Anderson *et al.*, 1993; Jean *et al.*, 1998). In contrast, all of the α_1-PDX- or tBR-expressing VMZs formed immunoreactive muscle (Figure 1C–D, arrowheads; Table I). Small patches of immunoreactive notochord were observed in some α_1-PDX-expressing explants (Figure 1G, arrowheads; Table I) and, less frequently, in tBR-expressing explants (Figure 1H; Table I).

These results demonstrate that misexpression of α_1-PDX in VMZ tissues phenocopies the effect of inactivating BMP-4 signaling, consistent with the possibility that α_1-PDX can inhibit the activity of the endogenous BMP-4 convertase(s).

Misexpression of α_1-PDX neuralizes the fate of ectodermal cells

In addition to inducing ventral mesodermal cells to adopt a dorsal fate, BMP antagonists can directly induce ectodermal cells to form neural tissue (reviewed by Graff, 1997). To test further the possibility that α_1-PDX can antagonize BMP-4 function, we misexpressed α_1-PDX in prospective ectodermal cells by injection of synthetic RNA (500 pg) as illustrated above Figure 2. Ectodermal explants (animal caps) were isolated at the blastula stage and cultured until sibling embryos reached the tadpole stage, at which point they were analyzed for gross morphology and for expression of neural-specific genes.

As shown in Figure 2, animal caps from uninjected or α_1-PIT-injected embryos formed spheres of ciliated epidermis (Figure 2A), while animal caps from α_1-PDX-injected embryos elongated and formed an anterior ecto-

Table I. α_1-PDX-expressing ventral cells form muscle and notochord

Explant	RNA injected	Muscle	Notochord
VMZ	none	7 (28)	0 (12)
VMZ	α_1-PDX	100 (18)	50 (36)
VMZ	tBR	100 (20)	10 (32)
VMZ	α_1-PIT	ND	0 (19)
DMZ	none	ND	98 (31)

The DMZ or VMZ region was isolated from stage 10 embryos that had been injected with synthetic RNAs as indicated above the table. These explants were cultured until sibling embryos reached stage 26 and were immunostained with muscle- or notochord-specific antibodies. Data are expressed as percentage of explants showing positive staining for muscle or notochord, as indicated at the top of the table, followed by the number of explants examined, in parentheses. ND: not determined.

dermal organ, the cement gland (Figure 2B, arrowheads). RT–PCR analysis revealed that α_1-PDX-injected animal caps, but not uninjected or α_1-PIT injected animal caps, expressed cement gland (XAG)-, anterior neural (OtxA)- and pan-neural (NCAM)-specific genes but did not express a posterior neural-specific gene (Xlhbox6; Figure 2C). Animal caps explanted from α_1-PDX-injected embryos did not form muscle nor did they express the mesodermal gene *Xbra*, demonstrating that neural induction is direct, i.e. it occurs in the absence of mesoderm induction (data not shown). Specific blockade of the BMP signaling pathway within isolated ectodermal cells leads to an identical direct induction of anterior, but not posterior, neural tissue (reviewed by Wilson and Hemmati-Brivanlou, 1997).

α_1-PDX inhibits the activity of exogenously expressed BMP-4

To begin to determine whether the patterning defects caused by ectopic expression of α_1-PDX are due to blockade of endogenous BMP activity, we assayed for the ability of α_1-PDX to directly antagonize the activity of exogenous BMP-4. As previously shown (reviewed by Graff, 1997), microinjection of BMP-4 RNA (3 ng) into dorsal cells led to a complete loss of all anterior (Figure 3C) and dorsal mesodermal (e.g. notochord; Figure 3D) structures in most embryos (79% of embryos completely lacked immunoreactive notochord, $n = 47$). Co-injection of RNA encoding α_1-PDX along with BMP-4 significantly rescued the formation of anterior structures (Figure 3E) as well as the formation of immunoreactive notochord (Figure 3F; 63% of embryos showed extensive immunoreactive notochord, $n = 51$). In contrast, embryos co-injected with RNAs encoding α_1-PIT and BMP-4 appeared identical to those injected with BMP-4 alone (82% of co-injected embryos lacked notochord staining, $n = 36$; data not shown). Expression of α_1-PDX alone did not inhibit notochord staining in any embryos (data not shown). This result supports the possibility that α_1-PDX inhibits an endogenous convertase that is directly required for proteolytic processing of BMP-4.

α_1-PDX-mediated neural induction is blocked by co-expression of intracellular transducers of BMP-4 signals

If neural induction mediated by α_1-PDX is due to inhibition of BMP processing, and therefore to antagonism of signaling upstream of receptor activation, then co-expression of a downstream component of the BMP signaling cascade should block this phenotype. The intracellular protein Smad1 has been shown to transduce BMP signals from the membrane to the nucleus (reviewed by Massagué *et al*., 1997). As with other Smads, Smad1 contains three domains (Figure 4A): an inhibitory (MH1) domain, a linker domain of unknown function, and an effector (MH2) domain. Previous work in other laboratories has shown that the MH2 domain in isolation is constitutively active and can transduce BMP signals in the absence of ligand (reviewed by Massagué *et al*., 1997). More recent work has shown that this activity can be augmented by co-expression of Smad4, presumably due to the fact that endogenous Smad4 is present in rate-limiting amounts *in vivo* (Candia *et al*., 1997).

To determine whether activation of the Smad1 signaling cascade can block α_1-PDX-mediated neural induction, RNA encoding α_1-PDX alone (500 pg) or together with RNA encoding Smad4 and the MH2 domain of Smad1 (500 pg each) was injected near the animal pole of two cell embryos. Animal caps were isolated at the blastula

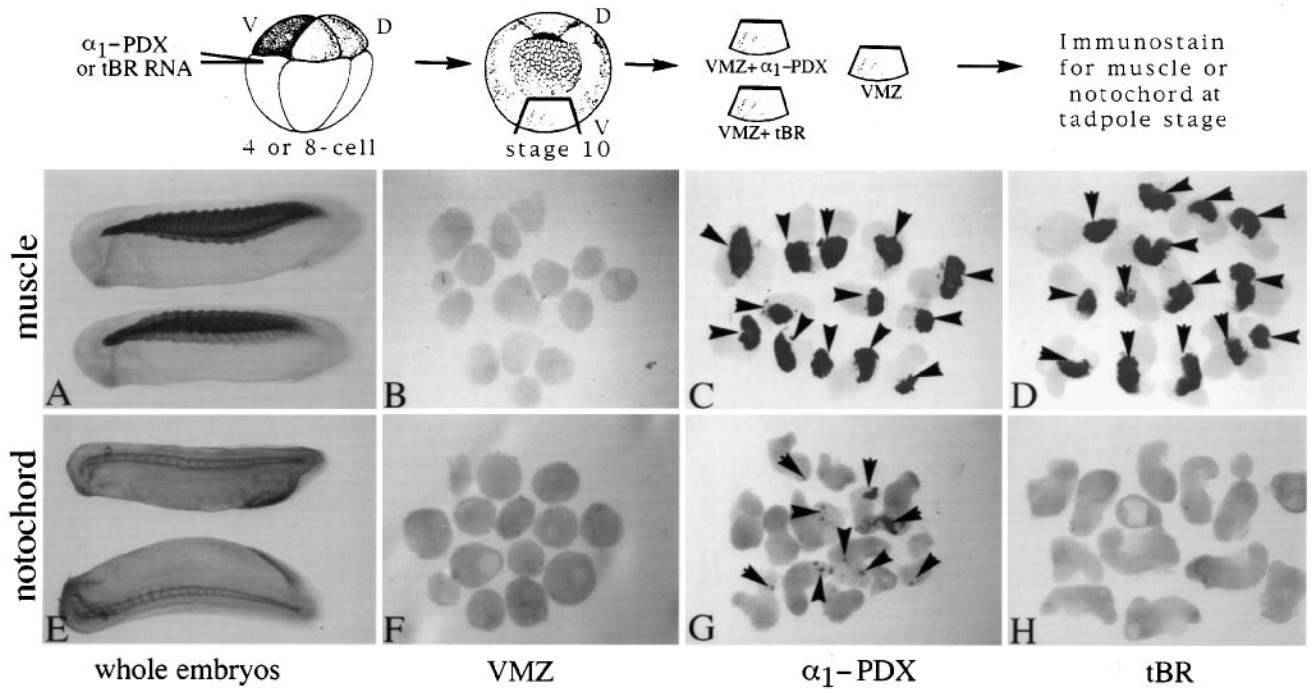

Fig. 1. Dorsal mesoderm formation in α_1-PDX and tBR-expressing VMZs. RNAs encoding α_1-PDX or tBR were injected into VMZs of four- to eight-cell embryos, and VMZs were explanted at stage 10 as illustrated at the top of the figure. VMZs (**B**–**D**, **F**–**H**) were cultured until sibling embryos (**A** and **E**) reached tailbud stages at which time they were immunostained with muscle- (A–D) or notochord- (E–H) specific antibodies. Specific staining is indicated by arrowheads.

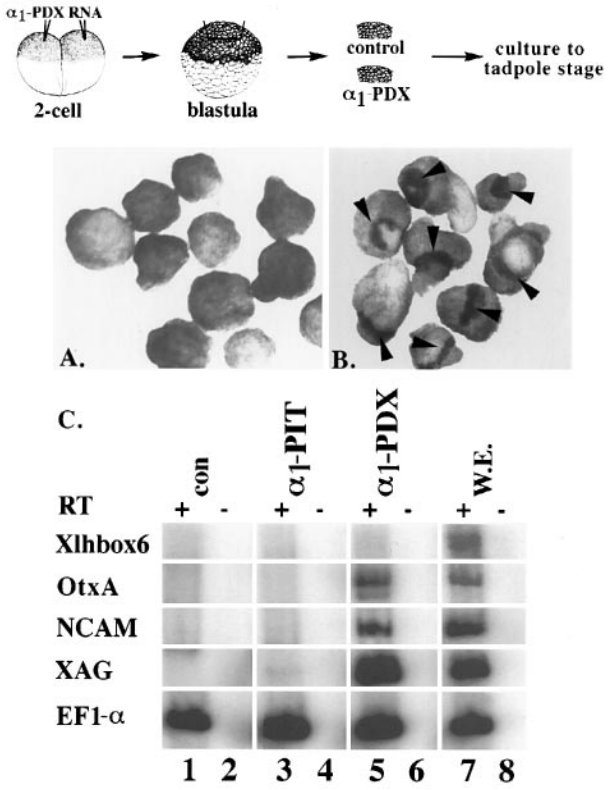

Fig. 2. Neural induction in α_1-PDX-expressing ectodermal explants. RNA encoding α_1-PDX or α_1-PIT was injected near the animal pole of two-cell embryos, animal caps were explanted at the blastula stage and cultured until sibling embryos reached the tadpole stage as shown at the top of the figure. Animal caps from control embryos (**A**) retain an epidermal morphology while animal caps from α_1-PDX-expressing embryos (**B**) form cement gland (arrowheads). (**C**) RNA samples from tadpole stage animal caps or whole embryos (W.E.) were analyzed for expression of neural- (Xlhbox6, OtxA, NCAM) or cement gland- (XAG) specific genes by RT–PCR in the presence (+) and absence (–) of reverse transcriptase (RT). The faint XAG signal in α_1-PIT-expressing animal caps is not reproducible. EF1-α is a control for equivalent amounts of RNA in each sample. Note OtxA was consistently detected as double bands.

stage, cultured to the tadpole stage, and analyzed for expression of neural- or cement gland-specific genes. As shown in Figure 4B, co-expression of the MH2 domain of Smad1, Smad4 and α_1-PDX nearly completely repressed α_1-PDX mediated induction of neural-specific genes in isolated animal caps and led to a 41-fold reduction in expression of the cement gland-specific gene, *XAG*. Thus, α_1-PDX can inhibit BMP signaling upstream of receptor activation, consistent with the possibility that it directly blocks the function of an endogenous protease(s) required for cleavage of the BMP-4 precursor protein.

α_1-PDX blocks processing of Flag-tagged BMP-4 in Xenopus oocytes

An *in vivo Xenopus* oocyte translation assay was used to directly test the possibility that α_1-PDX blocks proteolytic activation of BMP-4. RNA encoding epitope (Flag)-tagged BMP-4 (50 ng) was injected into *Xenopus* oocytes either alone, or together with RNA encoding α_1-PDX or α_1-PIT (5 ng). Oocytes were labeled with [^{35}S]methionine and BMP-4Flag protein was immunoprecipitated from oocyte lysates using a Flag-specific antibody. As shown in Figure

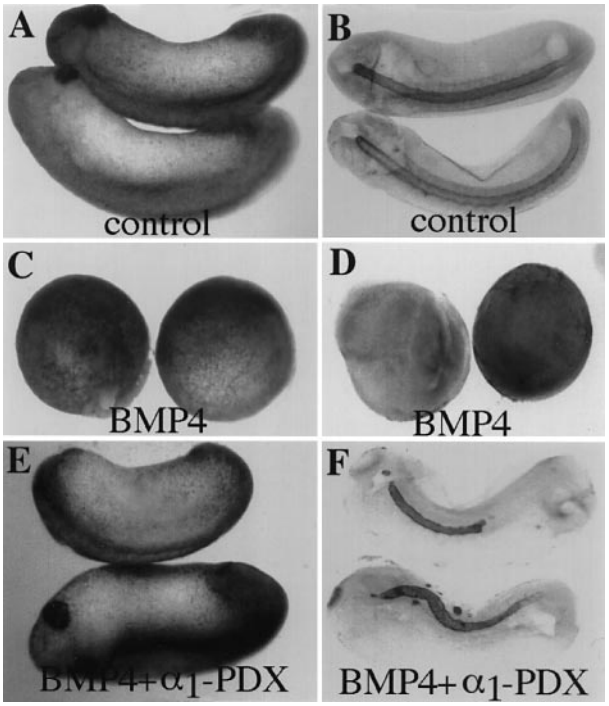

Fig. 3. α_1-PDX inhibits the activity of exogenously expressed BMP-4. RNA encoding BMP-4 was injected alone (**C** and **D**), or in combination with α_1-PDX (**E** and **F**), into dorsal blastomeres of four-cell embryos. Injected embryos were cultured until control siblings (**A** and **B**) reached the tailbud stage, at which time they were scored for gross morphology (left panels) or for the presence of immunoreactive notochord (right panels).

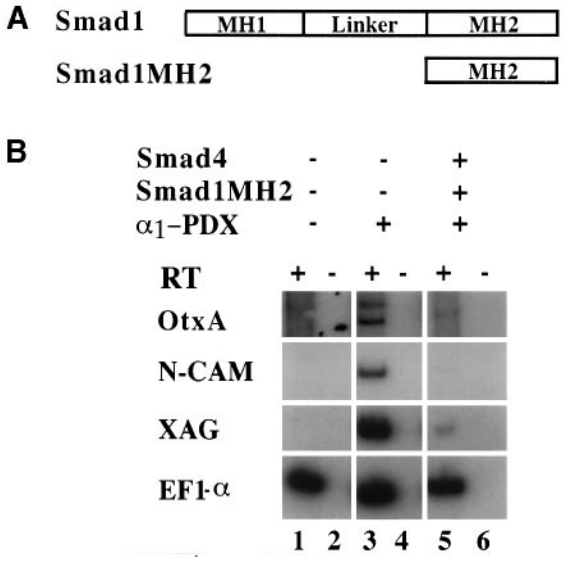

Fig. 4. Activation of the intracellular BMP-4 signal transduction cascade represses α_1-PDX-mediated neural induction. (**A**) Schematic diagram showing the domain structure of wild-type Smad1 and the deletion mutant, Smad1MH2. (**B**) α_1-PDX RNA alone, or in combination with Smad1MH2 and Smad4 RNA, was injected near the animal pole of two-cell embryos. Ectoderm was explanted at blastula stages as shown at the top of Figure 2, cultured until siblings reached the tadpole stage and analyzed for expression of neural- (OtxA, NCAM) or cement gland- (XAG) specific genes by RT–PCR in the presence (+) or absence (–) of reverse transcriptase (RT). Specific, reproducible bands corresponding to OtxA are observed only in ectodermal cells made to express α_1-PDX alone.

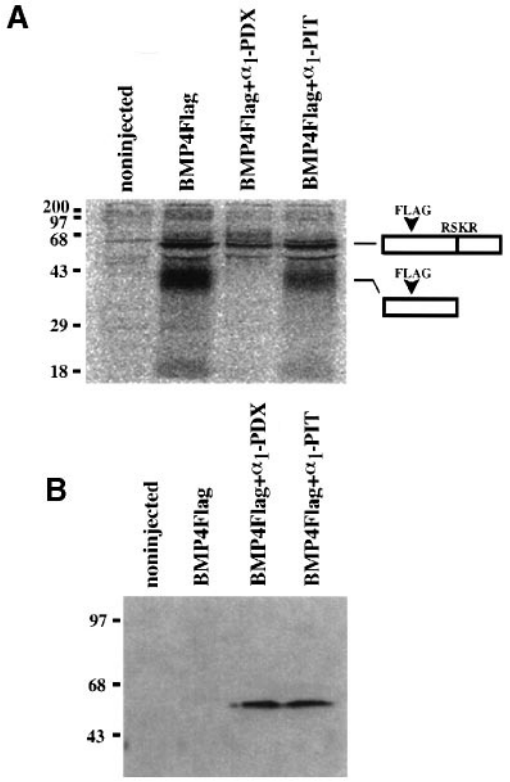

Fig. 5. α_1-PDX blocks proteolytic processing of BMP-4 precursor *in vivo*. (**A**) RNA encoding BMP-4Flag alone, or in combination with α_1-PDX or α_1-PIT, was injected into stage VI oocytes. Oocytes were labeled with [^{35}S]methionine and the lysates were subjected to immunoprecipitation with a Flag-specific antibody. The position of uncleaved BMP-4 precursor protein and the position of the cleaved proregion are illustrated schematically on the right side of the gel. (**B**) Western blot of protein extracts from uninjected oocytes, or from oocytes injected with the RNAs indicated above each lane, probed with an antibody which recognizes α_1-antitrypsin.

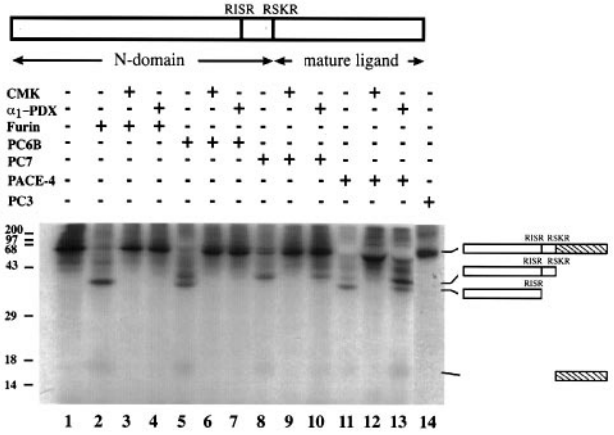

Fig. 6. α_1-PDX inhibits the ability of furin and PC6B, but not PACE-4 or PC7, to cleave the BMP-4 precursor *in vitro*. Radiolabeled BMP-4 precursor protein was incubated for 6 h with purified PCs, or with PCs that had been preincubated with purified α_1-PDX protein (1 μM) or CMK (10 μM) as indicated above each lane. Aliquots of each reaction were separated electrophoretically on a 12% polyacrylamide gel and proteolytic fragments were detected by fluorography. Bands predicted to correspond to the uncleaved precursor; intact N-terminal domain following cleavage at the -R-S-K-R- site (illustrated schematically above the figure), N-terminal domain following cleavage at the -R-I-S-R- site, and mature polypeptide are indicated to the right of the gel.

5A, α_1-PDX, but not α_1-PIT, completely inhibited cleavage of BMP-4 precursor protein. In this experiment, ~50% less BMP-4 precursor is synthesized in oocytes made to express α_1-PDX relative to control oocytes. However, the BMP-4 cleavage product remains undetectable in immunopreciptitates from α_1-PDX-injected oocytes even when the gel is overexposed, thereby demonstrating that the lack of detectable processing in these oocytes is not due to relatively lower levels of BMP-4 precursor.

To control for the possibility that the observed failure of α_1-PIT to inhibit BMP-4 processing was due to inefficient translation of α_1-PIT RNA relative to α_1-PDX RNA, Western blots of protein extracts isolated from injected oocytes were probed with an α_1-antitrypsin-specific antibody. As shown in Figure 5B, α_1-PIT and α_1-PDX proteins were expressed at comparable levels. Together, these results demonstrate that α_1-PDX, but not the related inhibitor α_1-PIT, can block proteolytic cleavage of BMP-4 *in vivo*, consistent with the hypothesis that BMP-4 is cleaved by an endogenous PC(s).

Cleavage of BMP-4 precursor by furin and PC6B, but not by PACE-4 or PC7, is blocked by α_1-PDX

An *in vitro* digestion assay was used to identify PCs capable of cleaving BMP-4. Radiolabeled BMP-4Flag precursor protein was incubated with purified PCs in solution and proteolytic cleavage of the precursor was assayed after 6 h of incubation. As shown in Figure 6, BMP-4 precursor protein incubated in the absence of PCs (lane 1), or in the presence of the neuroendocrine-specific PC3 (lane 14), was not cleaved. In contrast, PC6B (Figure 6, lane 5) and PC7 (Figure 6, lane 8) cleaved the BMP-4 precursor protein to yield fragments of ~15 kDa (more readily visible on longer exposures) and 35 kDa, consistent with the predicted M_r of the mature bioactive BMP-4 protein, and of the intact N-domain, respectively. The same 35 kDa band was observed following shorter (1 h) incubations of the precursor with either furin or PACE-4 (data not shown). Intriguingly, furin (Figure 6, lane 2), PC6B (lane 5) and PACE-4 (lane 11), but not PC7 (lane 8), cleaved BMP-4 at a second site, probably within the proregion, generating a 32 kDa fragment. The size of this product is consistent with cleavage at a minimal furin consensus sequence (-Arg-Ile-Ser-Arg-) located ~30 amino acids upstream of the primary cleavage site (-Arg-Ser-Lys-Arg-).

Since α_1-PDX can block proteolytic activation of BMP-4 *in vivo*, one criterion for candidate BMP-4 convertases is that they must be sensitive to inhibition by α_1-PDX. To determine whether furin, PC6B, PC7 or PACE-4 meet this criterion, a parallel set of *in vitro* digestions was performed in which purified PCs were preincubated with α_1-PDX for 30 min prior to assaying their ability to cleave radiolabeled BMP-4 precursor. Alternatively, purified PCs were preincubated with Decanoyl-Arg-Val-Lys-Arg-CH_2Cl (CMK), an active-site directed inhibitor of all PC family members (Jean *et al*., 1998), as a positive control for inhibition. As shown in Figure 6, CMK inhibited the ability of all PCs to cleave BMP-4 (lanes 3, 6, 9 and 12), while α_1-PDX selectively prevented furin (lane 4) and PC6B (lane 7), but not PC7 (lane 10) or PACE-4 (lane 13), from efficiently cleaving BMP-4. These results,

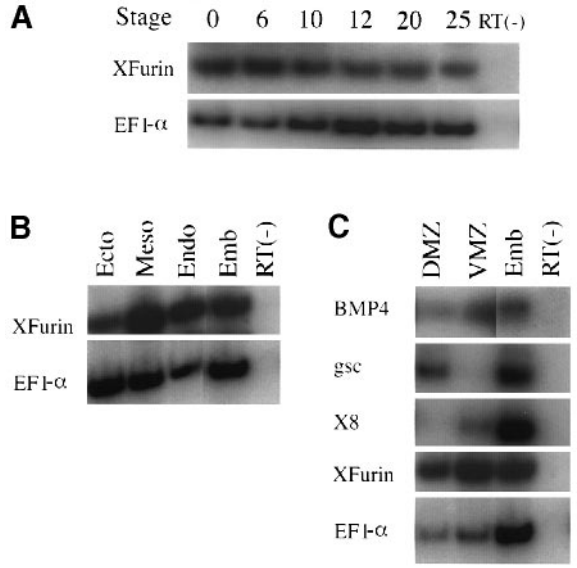

Fig. 7. Furin is ubiquitously expressed in gastrula stage embryos. (**A**) RNA isolated from oocytes (stage 0), blastulae (stage 6), early or late gastrulae (stages 10 and 12, respectively), neurulae (stage 20) or tailbud stage embryos (stage 25) was analyzed for expression of *Xenopus* furin (Xfurin) by RT–PCR. (**B**) RNA isolated from ectodermal (Ecto), mesodermal (Meso) or endodermal (Endo) portions of gastrulae (stage 10), or from whole stage 10 embryos (Emb) was analyzed for expression of *Xenopus* furin (Xfurin) by RT–PCR. (**C**) The dorsal marginal zone (DMZ) or VMZ region was dissected from gastrulae at stage 10 and RNA isolated from each group of explants, or from whole embryos (Emb), was analyzed for expression of BMP-4, goosecoid (gsc), Xwnt-8 (X8) or furin by RT–PCR. In all panels, EF-1α serves as a loading control and representative samples were analyzed in the absence of reverse transcriptase [RT(–)] to demonstrate absence of genomic contamination.

together with the observation that α_1-PDX inhibits processing of BMP-4 *in vivo*, argue that endogenous BMP-4 is proteolytically activated by furin and/or PC6.

Furin is ubiquitously expressed in Xenopus gastrulae

To begin to determine whether the spatial and temporal pattern of furin expression is appropriate for an endogenous BMP-4 convertase, we used RT–PCR to detect furin transcripts in RNA isolated from developmentally staged embryos and in tissues dissected from various regions of early gastrulae. Furin transcripts are present in oocytes, as previously shown (Korner *et al.*, 1991), and throughout embryonic development (Figure 7A). Transcripts encoding furin are detected in all three germ layers of gastrulae (Figure 7B). RNA isolated from the DMZ and VMZ was analyzed for expression of the dorsal- and ventral-specific genes, *goosecoid* (gsc; Cho *et al.*, 1991) and *Xwnt-8* (X8; Christian *et al.*, 1991), respectively, to confirm the accuracy of dissections (Figure 7C). The same RNA was analyzed for the presence of BMP-4 and furin transcripts. BMP-4 transcripts are enriched within ventral cells of early gastrulae (Figure 7C), and become restricted to ventral cells by mid-gastrula stages (Fainsod *et al*., 1994). Transcripts encoding furin are detected at fairly equivalent levels in dorsal and ventral cells of gastrulae when normalized to expression of EF-1α (Figure 7C). These results are consistent with previously published reports that furin is ubiquitously expressed throughout embryonic development in other vertebrates (Constam *et al.*, 1996) and confirm that furin is present at an appropriate time and place to proteolytically activate BMP-4.

Discussion

PCs as potential regulators of embryonic patterning

Members of the PC family of serine endoproteases are likely to be essential participants in the process of embryonic patterning, since many secreted or membrane-bound developmental regulators derive from inactive precursors that require proteolytic maturation. Consistent with this possibility, most PCs are expressed ubiquitously throughout embryogenesis, although tissue-specific enrichment of some transcripts has been observed (Constam *et al.*, 1996; Zheng *et al.*, 1997). An absolute requirement for proteolytic processing during development has recently been demonstrated in *Caenorhabditis elegans* where disruption of the *bli-4* gene, which encodes a PC-like endoprotease, leads to embryonic arrest (Thacker *et al.*, 1995).

The wide tissue distribution of most PCs during vertebrate development, coupled with the essential embryonic roles of potential substrates, make it likely that targeted deletions of ubiquitously expressed PC genes in mice will produce complex phenotypes and early lethality. For this reason, conditional or tissue-specific loss of PC function is an attractive strategy for investigating the developmental roles and substrate specificity of these endoproteases. We have taken advantage of a well characterized furin inhibitor, α_1-PDX, to block furin-like PC function within a subset of cells in early *Xenopus* embryos. Recent analysis of the ability of α_1-PDX to inhibit PC-mediated hydrolysis of a synthetic peptide substrate has shown that this protein is a potent and highly selective inhibitor of furin and PC6B, but not of other PCs (Jean *et al.*, 1998). Specifically, α_1-PDX inhibits both furin and PC6B at nanomolar concentrations (K_i = 0.6 nM and 2.3 nM, respectively) but is orders of magnitude (<2000- to 8000-fold) less effective at blocking PACE-4 and PC7. Our studies provide the first *in vivo* evidence that proteolytic maturation of BMP-4 is achieved by a member of the PC family, and demonstrate the feasibility of using α_1-PDX as a tool to investigate developmental functions of PCs in a whole-animal model.

Taken together, our results demonstrate that the protease responsible for maturation of BMP-4 *in vivo* is most likely to be furin and/or PC6. Although we did not specifically assay PC2 or PC4 for their ability to cleave BMP-4, these enzymes are not appropriate candidate convertases since they are confined to neuroendocrine tissues (Zheng *et al.*, 1994, 1997; reviewed in Steiner *et al.*, 1992) or to adult testicular germ cells (Nakayama *et al.*, 1992; Mbikay *et al.*, 1997), respectively, while BMP-4 is widely expressed. PACE-4 has been suggested to be an endogenous BMP-convertase, based on the observation that it is co-expressed with BMPs in a number of embryonic tissues (Constam *et al.*, 1996). Our results do not support a role for this convertase in processing BMP-4, since it is not sensitive to inhibition by α_1-PDX, but do not rule out the possibility that it proteolytically activates other members of the BMP family.

One potential concern with our experiments is the possibility that we are overexpressing α_1-PDX at sufficiently high levels that the specificity of this inhibitor for PC6 and furin is lost. Several controls suggest that this is not the case. First, α_1-PIT is capable of inhibiting furin if it is expressed at very high (micromolar) levels (Anderson *et al.*, 1993). In our studies, ectopically expressed α_1-PIT is inactive against the endogenous BMP-4 convertase whereas the same concentration of α_1-PDX efficiently inhibits this enzyme, suggesting that we are not achieving micromolar concentrations of either inhibitor. Secondly, we have characterized activin as a substrate for PACE-4 and have shown that levels of α_1-PDX that inhibit processing of BMP-4 *in vivo* do not inhibit processing of activin (Y.Cui and J.L.Christian, manuscript in preparation). Taken together, our studies strongly support the hypothesis that BMP-4 is proteolytically activated by furin and/or PC6. While the spatial and temporal pattern of expression of furin is appropriate for this proposed role, expression of PC6 has not been analyzed in *Xenopus* embryos. Given, however, that patterns of expression of other PCs have been shown to be conserved across species, we anticipate that PC6 is ubiquitously expressed throughout early development in *Xenopus* as it is in other vertebrate species (Constam *et al.*, 1996; Zheng *et al.*, 1997).

In addition to BMP-4, a number of other candidate PC substrates including Vg1, activin βA, lunatic fringe and *Xenopus* nodal-related (Xnr) proteins are activated by proteolytic cleavage after the furin consensus motif, -Arg-X-Arg/Lys-Arg-, and have been proposed to play essential roles in patterning the early embryo. Blockade of endogenous activin and/or Vg1 signaling, for example, leads to a complete loss of mesoderm (Hemmati-Brivanlou and Melton, 1992; Kessler and Melton, 1995). Vg1 has additionally been hypothesized to function as a dorsal determinant that is proteolytically activated in dorsal, but not ventral, cells (reviewed by Vize and Thomsen, 1994). Lunatic fringe has also been implicated in the process of mesoderm induction, and like Vg1 its activity has been proposed to be restricted to certain regions of the embryo by regulated proteolytic processing (Wu *et al.*, 1996). Finally, the nodal-related proteins Xnr1 through Xnr4 are restricted to dorsal cells of gastrulae and have been suggested to function as neural- or mesoderm-inducing molecules, or as dorsalizing factors (Jones *et al.*, 1995; Smith *et al.*, 1995; Joseph and Melton, 1997). Interestingly, global misexpression of α_1-PDX in one-cell *Xenopus* embryos does not perturb mesoderm induction, and misexpression of α_1-PDX in dorsal blastomeres does not lead to a loss of dorsal or neural fate (Y.Cui and J.L. Christian, unpublished). Due to the fact that the process of mesoderm induction is initiated at very early stages (Jones and Woodland, 1987), it is conceivable that activin, Vg1 and/or lunatic fringe protein(s) is/are processed during oogenesis, and that pre-cleaved forms of these molecules are present and ready for secretion at the one-cell stage, prior to the time that injected α_1-PDX is active. In contrast, Xnrs function during gastrulation and thus should be sensitive to processing inhibitors. Our results suggest that Xnrs are either proteolytically activated by an endoprotease which is insensitive to α_1-PDX, or that they are not required for induction or patterning of the mesoderm or central nervous system. While the substrate specificity of most PCs is not well established, our findings that PC3 does not cleave BMP-4, and that PC7 fails to recognize a second cleavage site within BMP-4 that is efficiently cleaved by PACE-4, PC6 and furin (Figure 6), suggest that individual PCs have unique roles in proteolytic activation of discrete signaling molecules. Furthermore, the identification of a potential second cleavage site within the pro-domain of BMP-4 raises the possibility that proteolysis liberates a novel, bioactive peptide that is distinct from BMP-4 itself, or that this second cleavage in some way regulates the bioactivity of BMP-4.

Proprotein processing: a novel mode of regulating BMP-4 activity?

Consistent with its multifunctional nature, BMP-4 activity is regulated at both transcriptional and post-transcriptional levels (reviewed by Hogan *et al.*, 1994). Post-transcriptionally, two secreted proteins (noggin and chordin) have been identified which bind BMP-4 with high affinity and thereby block BMP-mediated activation of cognate cell-surface receptors (reviewed by Hogan, 1996; Graff, 1997). BMP-4 function may also be limited by competition with related signaling pathways for shared components of the intracellular signal transduction cascade, as has been shown to be the case with Smad4 (Candia *et al.*, 1997). Finally, Smad-related proteins have been shown to function within BMP-responsive cells to downregulate the amplitude and/or duration of BMP signaling (Nakayama *et al.*, 1998; reviewed by Heldin *et al.*, 1997).

One final level at which BMP activity may be regulated is proprotein processing. While furin and PC6 are ubiquitously expressed during embryogenesis, it is not known whether they are constitutively active in all cells at all times. Indirect evidence that ectopically expressed BMPs are not cleaved until the gastrula stage (Candia *et al.*, 1997), despite the presence of transcripts encoding furin many hours prior to this time, supports the possibility that the activity of these convertases is regulated post-transcriptionally. Our results provide a framework for future studies into the substrate specificity and regulation of activity of members of the PC family during vertebrate development.

Materials and methods

Embryo culture and manipulation

Xenopus eggs were obtained, and the embryos were injected with synthetic RNAs and cultured as described (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (1967). The coding regions of α_1-PDX and α_1-PIT cDNAs (Anderson *et al.*, 1993) were subcloned into the expression vector pSP64T (Krieg and Melton, 1984). A cDNA encoding the MH2 domain of Smad1 was generated by subcloning the *Ava*I fragment of pSP64TEN-Xmad1 (gift of Dr D.Melton) into the expression vector pCS2+ (Turner and Weintraub, 1994) to generate pCS2+MH2. Capped synthetic RNA was produced by *in vitro* transcription of linearized pSP64T–α_1-PDX, pSP64T–α_1-PIT, pSP64T–tBR (Graff *et al.*, 1994), pCS2+MH2, DPC4(FL)/pSP64TEN (DPC4, gift of Dr J.Massagué), and pSP64T–BMP-4Flag (gift of Dr K.Cho). Embryonic explants were isolated and cultured as described in Cui *et al.* (1996).

Whole mount immunostaining

Whole mount immunostaining using the muscle specific antibody 12/101 (Kintner and Brockes, 1984) or the notochord-specific antibody

Tor70 (Bolce *et al.*, 1992; gift of R.Harland) was performed according to Moon and Christian (1989).

RT–PCR analysis

RT–PCR analysis of RNA samples was performed as described previously (Cui *et al.*, 1996). The sequences of BMP-4 (Fainsod *et al.*, 1994), XlHbox6 (Wright *et al.*, 1990), NCAM, EF1-α, OtxA, goosecoid and XAG (Kengaku and Okamoto, 1995) primers have been published previously. The sequence of the furin primers used in this paper are listed as follows: upstream 5′-GTTATGTTGAGAAAATCG-3′; downstream 5′-TAACATTAGCAGCAAAGT-3′. Number of cycles of PCR was determined empirically to be in the linear range for each primer pair. Amplified bands were visualized with a Molecular Dynamics PhosphorImager and quantified using the Macintosh IP lab. gel program.

Oocyte injections and analysis of proteins

Ovaries were isolated from mature female frogs and stage VI oocytes were manually defolliculated and injected with *in vitro* synthesized RNAs. Groups of 10 oocytes were pooled and cultured in oocyte culture medium (50% L15 medium supplemented with 15 mM HEPES pH 7.8, 1 mM glutamine, 1 mM BSA, 1 μg/ml of bone pancreatic insulin, and 100 μg/ml of Gentamicin) in microtiter plates in the presence of 0.1 mCi/ml translabel (NEN) for 48 h. ^{35}S-labeled oocytes (10 per group) were homogenized in RIPA buffer (Harlow and Lane, 1988) and BMP-4FLAG protein was immunoprecipitated using the Flag-specific antibody D8 (Santa Cruz Biotech) and protein A–Sepharose as described (Harlow and Lane, 1988). Precipitated proteins were boiled in 1× SDS buffer and separated by electrophoresis on a 12% polyacrylamide gel. The gel was dried, and radiolabeled proteins visualized with a Molecular Dynamics 8500 PhosphorImager.

For Western blot analysis, proteins were extracted from a group of 10 oocytes as described by Moon and Christian (1989). Proteins were separated by electrophoresis on a 12% polyacrylamide gel and transferred to a nitrocellulose membrane. The Western blot was probed with an antibody directed against α_1-antitrypsin (Calbiochem) which was visualized by a chemiluminescence kit (Pierce) according to manufacturer's instructions.

In vitro digestion assay

BMP-4Flag protein was immunoprecipitated from [^{35}S]methionine labeled oocytes which had been injected with RNAs encoding BMP-4 Flag (50 ng) and α_1-PDX (5 ng). Flag epitope-tagged furin, PC3, PC6B, PACE-4 and PC7 proteins were produced by infecting cultured cells with the corresponding vaccinia virus (VV) recombinant [VV:human fur713t/f (Molloy *et al.*, 1994; hereafter named hfurin/f), VV:mPC6B/f, VV:hPACE-4/f and VV:hPC7/f (secreted soluble Flag-tagged human PC7; Jean *et al.*, 1998). Secreted/shed enzymes were collected from culture media as described previously (Molloy *et al.*, 1992; Jean *et al.*, 1998), concentrated [Biomax filter, 30 kDa cut-off (Millipore)] and stored at –70°C until use. hfurin, mPC3, mPC6B and hPC7 were expressed in BSC-40 cells, while hPACE-4 was expressed in LoVo cells.

The activity of each purified PC was tested using the fluorogenic substrate pGlu-Arg-Thr-Lys-Arg-methylcoumaryl-7-amide (pERTKR-MCA; Peptide International). Enzyme assay data were obtained using a FluoroMax-2 spectrofluorometer equipped with a 96-well plate-reader (Instrument SA, Inc.) using excitation/emission wavelengths of 370/460 nm to measure released AMC (7-amino-4-methylcoumarin). Furin, PC6B, PC7, and PACE-4 assays were performed in 100 mM HEPES pH 7.5, containing 0.5% Triton X-100 and 1 mM $CaCl_2$. PC3 assays were performed as described (Jean *et al.*, 1995). Each enzyme preparation was enzymatically pure based on the absence of PC activity in medium from replicate cells infected with wild-type VV (data not shown).

The concentration of each PC was determined by tight-binding titration using the active-site-directed irreversible inhibitor Dec-RVKR-CH_2Cl. PCs were incubated with increasing amounts of Dec-RVKR-CH_2Cl for 30 min at room temperature. pERTKR-MCA (100 μM) was added to determine residual PC activity. Values for E_0 were obtained by fitting the data (*v* and I) to the equation for equilibrium binding:

$$v = SA\ (E_0 - 0.5\{(\ E_0 + I + K_i) - [(E_0 + I + K_i)^2 - 4E_0I]^{1/2}\})$$

(v = reaction velocity with the substrate concentration, s; SA = specific activity; E_0 = enzyme concentration; and I = inhibitor concentration) by non-linear regression (ENZFITTER, Elsevier-Biosoft, Cambridge, UK) (Knight, 1995; Jean *et al.*, 1998). *In vitro* digestion of BMP-4Flag was conducted at room temperature using hfurin (5.0 nM), mPC6B (2.0 nM), hPC7 (2.0 nM), hPACE-4 (5.0 nM) and mPC3 (19 nM).

To test the sensitivity of individual PCs to α_1-PDX, furin, PC6B, PACE-4 and PC7 were preincubated with α_1-PDX (1 μM final concentration) for 30 min at room temperature prior to addition of BMP-4 precursor. Reactions were allowed to proceed at 25°C for 6 h, at which time cleavage of BMP-4 by each PC was essentially complete. Aliquots of each reaction were analyzed by SDS–PAGE and fluorography.

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