Cleavages within the prodomain regulate the signaling range and potency of mature BMP-4: a mechanistic approach

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Catherine R. Degnin

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This is certify that the Ph.D. thesis of

Catherine R. Degnin

has been approved.

[redacted]
Professor in charge of thesis

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

[redacted]
Member

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AUTHOR: Catherine R. Degnin

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This dissertation is lovingly dedicated to
Dr. Bruce King Shively
who tragically died near the summit of the North Sister
July 5, 2003.

Bruce was my friend, my mentor, and my love.
I will miss you always; I will always remember you.
Thank you for your encouragement and support through these difficult years.

I hear your laughter as you ride the winds
I feel your footsteps as together we run these woods.
You comfort me while I rest by these waters.

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Abstract

Bone morphogenetic protein (BMP)-4 is expressed in a highly dynamic fashion throughout embryogenesis. More importantly, BMP-4 signaling is regulated both temporally and spatially, with BMP-4 able to activate target gene expression in a concentration dependent fashion, at a distance from expressing cells. This dissertation characterizes one mechanism for regulating BMP-4 activity: regulated cleavage of the prodomain. We have previously shown that proBMP-4 is sequentially cleaved, first at a consensus furin motif (S1) adjacent to the mature domain, and then at an upstream, non-consensus site (S2). Cleavage at the S1 site releases mature BMP-4. However, failure to cleave at the S2 site results in reduced activity and signaling range, directly correlating with reduced levels of mature BMP-4 found in *Xenopus* embryos.

In this dissertation we provide evidence for a model of regulated cleavage at the S2 site. The S2 site is cleaved in a pH-dependent fashion, with S2 only minimally cleaved at neutral pH but efficiently cleaved under mildly acidic conditions. The pH-sensitivity of the S2 site requires a histidine residue in the P6 position, and mutation of this residue activates cleavage of the minimal site. Finally, we show that both the S1 and S2 sites of proBMP-4 are cleaved in the intracellular environment of the oocyte and that furin is necessary for S2 cleavage *in vivo*.

Cleavage of the S2 site modulates mature BMP-4 activity and signaling range. When the S1 site is cleaved, the pro and mature domains of proBMP-4 remain noncovalently associated and the complex is targeted for lysosomal/proteosomal degradation. When the S2 site is cleaved, the pro/ligand complex dissociates. This

stabilizes mature BMP-4 and allows for enhanced activity and signaling range. However, when cleavage of the S2 site is optimized there are no further increases in mature BMP-4 levels whereas there are further increases in activity and signaling range. These data suggest that optimized cleavage may directly modulate mature BMP-4 bioactivity.

We are only beginning to characterize the role for S1 cleavage. When the S1 site is not cleaved we expected proBMP-4 to be unable to traffic beyond the TGN, since we predicted that the S1 site was essential for the 'trafficking competency' of BMP-4. To the contrary, we find that the S2 site of proBMP-4 is cleaved when the S1 site is not, and that this results in production of two forms of mature BMP-4. In preliminary studies these proteins retain some bioactivity in embryo assays. Furthermore, we find the region separating the two cleavage sites is essential for proper folding of proBMP-4 dimers.

The studies in this dissertation begin to explore roles for the prodomain of proBMP-4. This region not only directs folding of the proprotein, but also modulates the activity and stability of the mature product. Our data also suggest a possible role for the prodomain in trafficking of cleaved and partially cleaved protein. Future studies will explore the essential role of the peptide separating the two cleavage sites.

Chapter 1

Introduction

BMPs are multifunctional developmental regulators

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily, and were originally purified from de-mineralized bone extracts. When implanted into animals, these proteins were found to induce ectopic cartilage and endochondral bone formation. Subsequently, BMPs have been shown to be involved in overall organogenesis and tissue development. The BMPs are subdivided into several subgroups based on sequence homology. These subgroups include the *Drosophila* Decapentapalegic (Dpp) (BMP-2-/-4) and 60A subgroups (BMP-5/-6/-7/-8) (Sebald and Mueller, 2003), the growth and differentiation factors (GDFs)-3/-5/-8/-9, and Muellerian inhibiting substance (MIS). These proteins are expressed in distinct, yet overlapping patterns during development. Because of their multifunctional nature, BMP expression and activity are tightly regulated (Dale and Jones, 1999). This dissertation focuses on how the activity of one family member, BMP-4, is regulated at the level of proteolytic activation.

In vivo role for BMPs

BMP-4 plays an essential role in early development. BMP-4 knock-out mice are early embryonic lethal, developing to the early gastrula stage and dying with little or no

mesoderm (Winnier et al., 1995). BMP-2 knock-out mice are also non-viable and have defects in amnion/chorion and cardiac development (Zhang and Bradley, 1996). BMP-7 null mice die shortly after birth showing defects in kidney and eye development, as well as skeletal abnormalities of the ribs, skull and hind limbs (Dudley et al., 1995; Luo et al., 1995).

Cell specification by BMP-4 is dose-dependent, and heterozygous null (BMP-$4^{+/-}$) mice show reduced number of primordial germ cells (PGCs), defects in lens development (Furuta and Hogan, 1998) and skeletal deformities of the limbs (Dunn et al., 1997). In addition to the defects exhibited by BMP-$4^{+/-}$ mice, mice heterozygous null for both BMP-4 and BMP-7 have minor defects in the ribcage and distal limbs, while BMP-$2^{+/-}$ /BMP-$7^{+/-}$ and BMP-$5^{+/-}$/BMP-$7^{+/-}$ mice show no additional abnormalities (Katagiri et al., 1998).

Excessive BMP-4 activity can also lead to developmental defects. Overexpression of either BMP-4 or BMP-2 in *Xenopus* embryos can cause prospective dorsal mesoderm to become ventralized (Clement et al., 1995; Dale et al., 1992; Fainsod et al., 1994; Jones et al., 1992; Wilson and Hemmati-Brivanlou, 1995). In mammals, BMP-4 overexpression in the lung causes abnormal cell proliferation and leads to aberrant patterning (Bellusci et al., 1996; Weaver et al., 1999). Mutation of BMP inhibitors also results in enhanced BMP activity. Thus, mice mutant for the BMP-2/-4 antagonists gremlin, noggin and/or chordin show early lethality and/or defects in the spinal cord, forebrain, somites, skeleton and kidney (Brunet et al., 1998; Gong et al., 1999; Khokha et al., 2003; McMahon et al., 1998). In humans, mutations in the noggin gene are responsible for multiple synostoses syndrome, a genetic disease characterized by fusion

of the joints (Gong et al., 1999). Enhanced BMP activity is also implicated in fibrodysplasia ossificans progressiva, a genetic disorder that results in ectopic bone formation throughout the body (Kaplan et al., 1998; Kaplan et al., 1990; Rao et al., 1992). Up-regulation of Smad1, a downstream effector of BMP-4 signaling, or defects in other members of the BMP signaling cascade are currently under investigation as candidates for this disease (Cohen, 2002).

In *Xenopus* embryos BMP-4 is expressed ventrally during gastrulation, where it acts to specify ventral mesodermal (e.g. blood) and ectodermal (i.e. skin) fate. Blocking endogenous BMP-4 signaling by using dominant interfering forms of the BMP-4 receptor induces a secondary dorsal axis consisting of muscle and neural tissue (Frisch and Wright, 1998; Graff et al., 1994; Maeno et al., 1994; Suzuki et al., 1994). These data suggest that BMP-4 is required for ventral mesoderm formation and suppression of neural fate (Dale and Jones, 1999). Swirl is the zebrafish ortholog BMP-2, and loss of BMP-2/swirl leads to loss of BMP-4. Consistent with other BMP-4 phenotypes, ventral cells mutant for swirl take on more dorsal fates (Kishimoto et al., 1997). Thus, BMP-4 plays a crucial role in early development and in the establishment of the dorsoventral axis (Dale and Jones, 1999).

Structure of TGF-β family members

The TGF-β superfamily is divided into two subfamilies based on both sequence similarity and the signaling pathways they activate. These include the TGFβ/Activin/nodal subfamily and the BMP/GDF/MIS subfamily (Plessow et al., 1991). All TGF-β family members share a characteristic cysteine knot structure with six

cysteines involved in intrachain disulfide bridges. Generally, a seventh cysteine residue is involved in dimerization. However, Gdf-3, Gdf-9 and BMP-15 provide an exception by forming dimers while lacking this intermolecular bond (Liao et al., 2003; McPherron et al., 1997). The crystal structures of the mature domains of BMP-2, BMP-7, activin, TGF-β2 and TGF-β3 have been solved (Daopin et al., 1992; Griffith et al., 1996; Mittl et al., 1996; Scheufler et al., 1999; Thompson et al., 2003). BMP-2 shares only 30 – 35% sequence identity with BMP-7 and yet they have very similar folding topologies (Scheufler et al., 1999). Thus, since BMP-4 shares 92% sequence identity with BMP-2, the crystal structure of BMP-4 is predicted to closely resemble that of BMP-2.

Unlike TGF-βs, BMP-2/-4/-7 have a flexible amino (N)-terminus that is not disulfide bonded to the protein core and whose crystal structure has not been resolved. The N-termini of BMP-2/-4/-7 have been shown to mediate HSPG binding (Ruppert et al., 1996) and the mobility of the tail may allow for variability in glucosaminoglycan binding partners (Scheufler et al., 1999).

BMP receptors and activation

BMPs are secreted cell-cell signaling molecules capable of activating the BMP Type I (RI) - Type II (RII) serine-threonine kinase receptor complex. There are three mammalian type I BMP receptors: BMP receptor type IA [BMPR-IA or activin receptor-like kinase, (ALK)-3], BMPR-IB (or ALK-6), and Activin receptor type I (ActR-I or ALK-2), and there are three mammalian type II BMP receptors: BMPR-II, ActR-II and ActR-IIB (Yamashita et al., 1996; Zwijsen et al., 2003). The BMPR-II differs from other TGF-β type II receptors by its extended carboxyl-terminal cytoplasmic tail, of unknown

function. These receptors oligomerize in numerous combinations of two-type I/two-type II receptors, although BMPR-II does not typically self-associate (Gilboa et al., 2000).

BMP-2/-4 are restricted in the receptors that they can bind, which include BMPR-IA, BMPR-IB, ActR-II, ActR-IIB, and BMPR-II (Gilboa et al., 2000). BMP-6/-7 can bind to all six receptor types (Zwijsen et al., 2003). Although most of the BMP receptors can promiscuously bind both BMPs and activins, BMPR-II is thought to act only in the BMP pathway (Frisch and Wright, 1998).

BMP receptors exist at the cell surface as homo- and hetero-oligomeric complexes prior to ligand binding, although ligand binding significantly enhances oligomerization. BMP-4 binds with high affinity to RI (Natsume et al., 1997; Suzuki et al., 1994; ten Dijke et al., 1994) and with low affinity to RII (Knaus and Sebald, 2001; Natsume et al., 1997; Nohno et al., 1995). This is in contrast to TGF-βs, which exhibit high affinity for their type II receptors but do not interact with isolated type I receptors (Massague, 1998). *In vivo*, the BMP-4-RI complex recruits the type II receptor, which induces a conformational shift that enables the constitutively active RII-kinase to transphosphorylate RI within a conserved glycine-serine-rich cytoplasmic region. The crystal structure of BMP-2 dimers bound to BMPR-IA (Kirsch et al., 2000b) show that BR-IA binds to the "wrist" epitope of BMP-2. BMPR-IA makes extensive contact to both BMP-2 monomers through hydrophobic interactions involving Phe85 of BMPR-IA. Phe85 participates in a'knob-into-hole' packing, pointing into a hydrophobic pocket formed at the interface between the two BMP-2 monomers. Subsequent mutational analysis of BMP-2 and BMPR-IA shows no major binding determinants at the interface, which suggests that many weak hydrophobic forces may contribute to binding (Kirsch et

al., 2000a). As the entire heteroligomeric receptor complex has not yet been crystallized, recent work has modeled the independent crystal structures of BMP-2 with BMPR-IA, and BMP-7 with BMPR-II, into a single structure. According to this model, two BMPR-II extracellular domains (ECDs) bind the two convex sides of the BMP-7 dimer through the 'knuckle' epitope, and then two BMPR-I ECDs are recruited to the complex by binding the two concave sides of the symmetrical BMP-7 dimer 'wrist' epitope (Kirsch et al., 2000b; Sebald and Mueller, 2003). In both studies, BMPR-IA and BMPR-II bind BMP-7 with their ECDs oriented towards the membrane, adding further support to this model. The modeling of these two structures does not suggest that the four receptor ECDs interact directly following ligand binding, but rather allows for the possibility that interactions may occur within the transmembrane (TM) domain to drive receptor activation (Sebald and Mueller, 2003).

Following receptor activation, BMP signals are transmitted to the nucleus by a family of cytoplasmic proteins known as Smads. The activated receptor complex drives recruitment of specific intracellular receptor-regulated Smads (R-Smads), promoting the RI-kinase to phosphorylate the R-Smad C-terminal tail (Heldin et al., 1997). TGF-β-induced signaling recruits R-Smad2/3, while BMP induced signaling recruits R-Smad1/5/8. The anchoring protein, Smad anchor for receptor activation (SARA), facilitates phosphorylation of the TGF-β pathway R-Smads. SARA, which binds directly to the RI/RII complex, contains a Fab1p, YOTB, Vac10 and EEA1 (FYVE) domain, that can also bind membranes through specific interactions with phosphatidyl inositol 3' phosphate (PI(3)P) (Itoh et al., 2002; Tsukazaki et al., 1998). For the TGF-β signaling pathway SARA acts as a scaffold for the hepatic growth factor regulated tyrosine kinase

substrate (Hrs). Hrs is another FYVE domain protein that also binds PI(3)P-rich domains in the inner leaflet of the plasma membrane (PM) or early endosomal membranes. Together, SARA and Hrs coordinately bind Smad2 and drive its recruitment to membrane structures. Following ligand binding at the cell surface, the TGF-β receptor complex is internalized and targeted to early endosomes where SARA and Hrs position Smad2 to bind activated RI. Phosphorylation of Smad2 promotes its dissociation from the SARA/Hrs complex, and this allows for Smad dimerization and recruitment of the obligate co-factor, Smad4. Although a role for SARA has not yet been demonstrated for BMP-regulated signaling, Hrs is implicated for both BMP and TGF-β signaling pathways (Itoh et al., 2002).

Mechanisms for regulating BMP-4 activity

BMPs have dynamic expression patterns, and their activity is regulated at many levels during development, including at the levels of transcription (Chen et al., 1997; Hino et al., 1999; Kim et al., 1998), translation (Fritz and Sheets, 2001), and posttranslational modification (Cui et al., 2001; Nakayama et al., 2000; von Bubnoff and Cho, 2001). In the extracellular space, BMP-4 is regulated by a number of binding proteins such as chordin, noggin and DANs that block activation of cell-surface receptors (Canalis et al., 2003), as well as the protease Tolloid, which cleaves chordin to liberate active BMP-4. BMP-4 also binds to cell surface heparin sulfate proteoglycans (HSPGs). These interactions can either promote or restrict BMP activity (Christian, 2000; Ohkawara et al., 2002; Selleck, 2001).

At the intracellular level, the inhibitory Smads (I-Smads) Smad6 and Smad7 can negatively regulate BMP signaling in receiving cells. Smad6 can directly bind activated Smad1 to generate an inactive Smad1/6 complex (Hata et al., 1998). Smad6 and Smad7 both stably bind activated BMPR-I, and compete with Smad1/5/8 for receptor binding (Hanyu et al., 2001; Imamura et al., 1997). In addition, they promote recruitment of the E3 ligases, Smad ubiquitin regulatory factor (Smurf)1 (BMP specific) and Smurf2 to the activated receptor complex (Kavsak et al., 2000). Smurf recruitment leads to the ubiquitin-dependent degradation of the RI/RII I-Smad complex, and thus attenuates signaling (Murakami et al., 2003; von Bubnoff and Cho, 2001). Smurf1 can directly ubiquitinate and promote degradation of Smad1 and 5, independent of their activation state, to regulate intracellular Smad levels (Zhu et al., 1999), however, binding and ubiquitination are greatly enhanced in the presence of I-Smads (Murakami et al., 2003). Smurf 2 can also target activated Smad1 for degradation (Zhang et al., 2001). Both Smurf 1 and Smurf 2 can bind nuclear Smad7 to promote nuclear export and further downregulate signaling (Ebisawa et al., 2001; Kavsak et al., 2000).

BMP-4 is also regulated at the level of proteolytic maturation. BMPs are synthesized as inactive precursor proteins that must be proteolytically cleaved in order to become active. Expressed initially as a prepropeptide, the signal peptide is proteolytically removed within the ER to allow proBMP to fold and traffic through the secretory system. Within a post-ER compartment, proBMP is proteolytically cleaved to generate a minimally conserved N-terminal prodomain and a more highly conserved C-terminal mature domain. Peptide sequencing of the BMP-4 mature domain revealed that cleavage occurs following a basic motif, RXKR↓ (Aono et al., 1995), which is a

consensus motif for multiple members of the proprotein convertase (PC) family of endoproteases

In mammals, seven members of the PC, or subtilisin-like proprotein convertase (SPC) family have been characterized. These include furin (SPC 1), PC2 (SPC2), PC1/3 (SPC 3), paired amino acid converting enzyme 4 (PACE4) or (SPC 4), PC4 (SPC 5), PC5/6 (SPC 6), and PC7/8 (SPC 7) or lymphoma proconvertase (LPC). These enzymes are implicated in the processing of multiple protein precursors including growth factors, receptors, multiple proteases of the coagulation and complement cascades, glycoproteins of viral envelopes, and bacterial exotoxins (Taylor et al., 2003).

Potential PC candidates for proBMP-4 cleavage were tested in an *in vitro* cleavage assay with recombinant forms of furin, PC6A, PC7, PACE4 and PC3 (Cui et al., 1998). Furin is ubiquitously expressed, while PACE-4 and PC6A/B are expressed dynamically throughout development with their expression patterns overlapping those of BMP-4 (Boheler et al., 2002; Constam et al., 1996; Rancourt and Rancourt, 1997). These PCs prefer to cleave following the motif -RXK/RR- but will also cleave following a minimal -RXXR- motif (Taylor et al., 2003). By contrast, PC2 and PC3 are restricted to neuroendocrine tissues (Steiner et al., 1992) and cleave following dibasic motifs RR or KR (Rouille et al., 1995; Thomas et al., 1991), while PC4 is limited to germ cells (Li et al., 2000; Mbikay et al., 1997). Thus, PC1/3, PC2 and PC4 are poor candidates for proBMP-4 cleavage. Furin, PC6A, PC7 and PACE4 were capable of cleaving proBMP-4 to a some extent *in vitro*, however only furin and PC6A cleavage were sensitive to α_1-PDX, a PC inhibitor selective for furin at low concentrations (Jean et al., 1998) and able to inhibit proBMP-4 processing *in vivo* (Cui et al., 1998). Taken together, this

information makes furin (Boheler et al., 2002; Roebroek et al., 1998) and PC6 (Constam et al., 1996; Cui et al., 1998; Rancourt and Rancourt, 1997) the leading candidates for proBMP-4 cleavage. Although PC6A and PC6B have the same catalytic domain, only PC6B is considered a candidate for proBMP-4 cleavage since PC6B is co-expressed with proBMP-4 in the constitutive pathway whereas PC6A is localized to the regulated secretory pathway (Taylor et al., 2003). PACE4 remains a potential candidate for pro-BMP-4 cleavage based on tissue expression patterns (Akamatsu et al., 1999; Constam et al., 1996) and cell culture studies (Constam and Robertson, 1999).

When proBMP-4 was cleaved *in vitro*, two distinct bands corresponding to the cleaved prodomain were observed, consistent with the possibility of a second cleavage site residing within the N-terminal prodomain (Cui et al., 1998). PC7 cleaved exclusively at the S1 site, consistent with its strict requirement for the P2 basic residue. Subsequent studies showed that, following cleavage at the known, preferred consensus site (RXKR; S1), proBMP-4 is cleaved at an upstream site (S2) with the minimal consensus sequence, RXXR (Cui et al., 2001) (Figure 1). Furthermore, although this second site resides within the prodomain, mutations that prevent cleavage at the S2 site led to reduced activity and signaling range and dramatically reduced the steady-state expression levels of mature BMP-4 protein in *Xenopus* embryos. By contrast, introduction of a preferred consensus cleavage motif at the S2 site led to simultaneous cleavage at both sites. This led to an increase in the activity and signaling range of mature BMP-4 without a concurrent increase in steady-state protein levels. These data suggest that cleavage at this internal site regulates the activity of the signaling molecule (Cui et al., 2001).

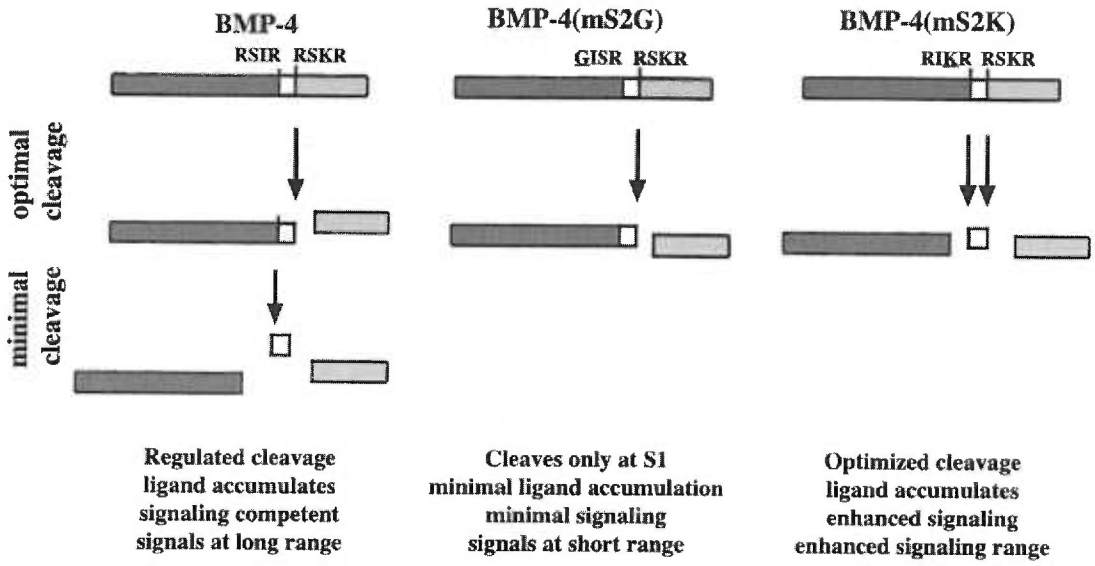

Figure 1: Cleavage products and phenotypes for BMP-4 cleavage mutants. BMP-4, BMP-4(mS2G) and BMP-4(mS2K) characterized by Cui et al, 2001.

Proprotein convertases: candidates for activating BMP-4

Members and characteristics of the proprotein convertases

Mammalian PCs act in either the regulated or constitutive branches of the secretory pathway. The PCs of the regulated pathway include PC2 and PC1/PC3, both of which are stored in dense core secretory granules (Steiner et al., 1992) for the generation of neuropeptides and peptide hormones in the brain and neuroendocrine tissues (Steiner, 1998). Other members of the PC family that function in the regulated secretory pathway

include PC4, whose expression is limited to germ cells (Li et al., 2000; Mbikay et al., 1997), and an isoform of PC6 called PC6A. Furin, PACE4, PC6B and PC7 function in the constitutive secretory pathway.

PCs share a common domain structure (Figure 2), consisting of a characteristic N-terminal propeptide (Pro), a well-conserved catalytic module, a conserved downstream domain called the 'P domain' or 'homo B-domain,' and a C-terminal domain that is unique to each PC member (Taylor et al., 2003). The Pro region acts as an intramolecular chaperone and is required for proper folding and transport of the inactive zymogen. The catalytic domain has the highest sequence conservation between family members (35% amino acid identity) and utilizes a characteristic catalytic triad consisting of active-site residues Asp, His and Ser, as well as an Asn residue that acts to stabilize the oxyanion hole in the transition state (Nakayama, 1997). Unlikethe bacterial subtilisins, PCs have a high density of negative charges within their conserved substrate-binding region (Creemers et al., 1993; Lipkind et al., 1995) and this acts to influence the substrate specificity of the enzyme. The crystal structure of furin reveals that the catalytic cleft/substrate interacting domain not only involves the P1, P4 Arg and P2 Lys, but also extends to the P5 and P6 substrate residues, thus contributing to furin's preference for basic residues at these positions (Henrich et al., 2003). The catalytic domain is followed by the Homo B-domain, which appears to structurally stabilize the catalytic domain and further contribute to enzyme specificity by balancing the negative surface charge asymmetry of the substrate-binding region (Steiner, 1998). The Homo B-domain is also thought to influence calcium dependency and the more acidic pH optimum characteristic of some PCs (Zhou et al., 1999). Finally, the most divergent region, the C-terminal

domain, is subdivided into three distinct subgroups: an amphiphathic helix group consisting of PC1/PC3, PC2 and PC4; a Cys-rich domain group of PACE4 and PC6A; and a Cys-rich/TM group consisting of furin, PC6B and PC7 (Zhou et al., 1999).

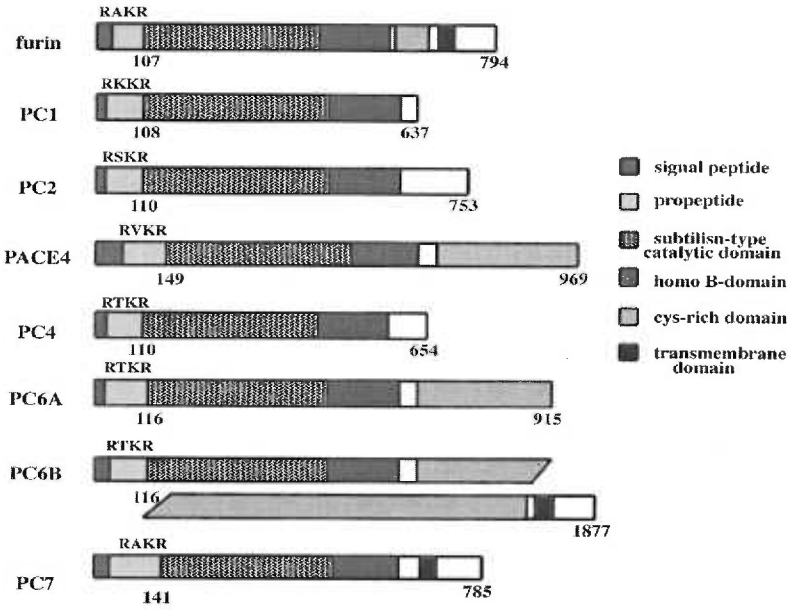

Figure 2: Schematic of PC family members. Primary candidates for BMP-4 cleavage include furin, PC6B and PACE4. Primary cleavage site between prodomain and enzyme is indicated.

Activation of PCs

Furin, the mammalian ortholog of the subtilisin-like serine protease, kexin (Fuller et al., 1989), is the best-characterized PC family member and serves as the primary model

for proteolytic activation and regulation of the other PCs, with the exception of PC2 (discussed below). Furin is synthesized as a preproprotein and the prodomain is autocatalytically cleaved in the endoplasmic reticulum (ER). The cleaved propeptide remains associated with the zymogen to facilitate transport and maintain functional inactivation of the enzyme (Anderson et al., 2002). Autocatalytic cleavage at a second site within the propetide occurs in the mildly acidic, calcium-enriched environment of the TGN, which promotes dissociation of the propeptide and activation of the enzyme.

PC2 is activated in a unique fashion, requiring a co-factor, 7B2, for transport from the ER and activation of the enzyme. *In vitro*, 7B2 binds pro-PC2 after it has been folded (Muller et al., 1997) and association with 7B2 is required for PC2 to exit the ER (Martens et al., 1994). During transport, 7B2 is cleaved at a basic motif near its C-terminus (by furin or a related PC), forming an inhibitory C-terminal fragment and a small, N-terminal peptide that is required to stabilize active PC2 (Lamango et al., 1996). Activation of PC2 requires autocleavage under acidic conditions in a late post-Golgi compartment. Functional inactivation of 7B2 also inactivates PC2, demonstrating the absolute requirement of 7B2 for PC2 function (Seidel et al., 1998; Westphal et al., 1999).

Subcellular localization of PCs regulates their substrate specificity

PCs are regulated by subcellular localization, both to limit access to substrates and to regulate the pH/Ca^{2+} concentration of local environments that may be required for optimal substrate cleavage. Localization and trafficking are best understood for furin. Furin is initially trafficked to the TGN, where it processes precursor proteins such as blood clotting factors and growth factors. These products then move through the

secretory pathway and can be secreted or inserted into the PM (Steiner, 1998). Furin also functions at the PM and can be 'shed' to the extracellular space, to process viral proteins such as the envelope glycoproteins of HIV, bacterial toxins such as anthrax, or secreted proteins such as profibrillin and BMP-1. Furin can further function in the endocytic pathway, where it cleaves endocytosed bacterial toxins (Molloy et al., 1999). Thus, furin is thought to cycle through two distinct recycling loops: one between the TGN and post-TGN compartments, and a second between the cell surface and early endosomes. This cycling process is mediated through a series of phosphorylation/dephosphorylation events, where phosphoryation by casein kinase II (CKII) is thought to retain furin to either of these loops, and where dephosphorylation, by PP2A, allows trafficking between the loops.

Subcellular localization of furin is mediated by cooperative signals found in its cytoplasmic tail, which include an acidic cluster (AC), clathrin-coated pit (CCP) recruitment signals, and tyrosine-containing motifs, while dileucine-like motifs, also in the cytoplasmic tail, may be involved in internalization and budding. Association with distinct cytoplasmic factors, mediated by the modification of these motifs, determines whether furin is directed to the TGN or other subcellular compartments. The sorting protein, phosphofurin acidic cluster protein 1 (PACS-1) binds to the casein kinase II (CKII)-phosphorylated AC of furin, and the tyrosine-containing motif in conjunction with PACS-1 mediate binding to the clathrin-associated adaptor molecule, AP-1. These proteins collectively play a role in directing furin-containing budded vesicles from the TGN to a post-TGN compartment. This post-TGN compartment may represent the functional equivalent of immature secretory granules (ISG) (Molloy et al., 1999), and

may be important in processing of BMP-4 and other proteins that require acidic conditions for cleavage.

Cycling between the PM and early endosomes is mediated in a similar fashion as the TGN-post-TGN loop. Cycling from the PM, however, involves the use of AP-2, rather than AP-1. In this case, clathrin-dependent internalization of cell-surface furin is mediated by binding between one or more CCP recruitment motifs and PACS-1. These interactions mediate binding of AP-2. Once targeted to early sorting or recycling endosomes, CKII phosphorylated furin is recycled back to the PM in a PACS-1 dependent step, most likely by way of the recycling endosome. Shuttling between the TGN/post-TGN and early endosome/PM loops involves specific dephosphorylation of furin by PP2A isoforms and delivery to the TGN. Dephosphorylation by additional, specific PP2A isoforms may also serve to re-direct furin to the PM, although this is yet to be proven (Molloy et al., 1999).

Similar to furin, PC6B is a TM domain protein with a cytoplasmic tail containing multiple sorting signals (De Bie et al., 1996; Xiang et al., 2000) including an AC, tyrosine-containing and dileucine motifs. Like furin, PC6B can be cleaved to release a 'shed' form that may be important for the cleavage of specific extracellular substrates (De Bie et al., 1996). PC6A, a splice isoform of PC6B, is a sulfated, soluble PC sorted to the regulated secretory pathway by way of a signal in its C-terminal tail. PC6A and PC6B contain identical catalytic domains, and it is possible that their distinct subcellular localization allows them to process unique sets of precursor substrates in appropriate convertase/substrate combinations (De Bie et al., 1996; Ulloa et al., 2001). PC7 is unique in its trafficking. Palmitoylation, rather than phosphorylation of the cytoplasmic tail, is

used to influence compartmentalization of the enzyme to the TGN (van de Loo et al., 1997).

Animal models for PC function: knock-out mice

Analysis of mice carrying null mutations in genes encoding various PC family embers has provided clues to the importance of PCs in development. Phenotypic erlaps between PC and growth factor knock-outs give an indication of potential PC strates. In addition, there is evidence for functional redundancy between PC family nbers with the strongest example arising from the complete the lack of phenotype of mice (Roebroek et al., 1998). Although lack of phenotype of PC7$^{-/-}$ mice suggests ne *in vivo* substrates for PC7 can also be cleaved by other co-expressed PCs (van de al., 1997), it is equally plausible that PC7 cleaves substrates that are non-essential development. The pattern of expression and the phenotypes of mice mutant for idate BMP-4 convertases furin, PACE4, and PC6 are summarized below.

C6B is normally expressed in a dynamic fashion throughout development, with n highest at E7.5-8.5 in the extraembryonic primitive endoderm, amnion, soderm and the yolk sac (Constam et al., 1996). This is followed by elevated in the apical ectodermal ridge of limb buds at E9.5-11.5 (Rancourt and 97). The dynamic staining pattern of PC6B in the posterior somites suggests a C6B-mediated cleavages during somitogenesis, a process also known to inv PDGF, Notch and BMPs (Constam et al., 1996). Based on overlapping exp erns, PC6B may play a role in processing of BMP-2 and BMP-7. Mice

mutant for PC6B are reported to die at embryonic day 10.5-11.5 (E10.5-11.5) (Lu and Franzusoff et al, unpublished results in Taylor et al., 2003).

Furin is initially expressed at E6 (Beck et al., 2002). Furin is expressed at low levels in most embryonic cells while at higher level and in dynamically changing patterns later in development (Roebroek et al., 1998). Furin shares overlapping expression patterns with BMP-4, being highly expressed in the heart tube, gut endoderm, lateral plate mesoderm, allantois and notochord plate of E8.5 embryos (Roebroek et al., 1998). Furin$^{-/-}$ embryos develop normally until E8-8.5, but die during early organogenesis, at E10.5-11.5 (Roebroek et al., 1998). Furin null embryos show multiple defects including failure of chorioallantoic fusion, abnormal yolk sac vasculature, reduced numbers of cardiomyocytes, lack of axial rotation, and severe ventral closure abnormalities. Many of these furin$^{-/-}$ phenotypes resemble those seen in mice mutant for TGF-β, BMP or lefty (Dickson et al., 1995; Meno et al., 2001; Winnier et al., 1995; Zhang and Bradley, 1996). For example, TGF-β/BMP signaling is required for specification of cardiac lineages, and the abnormalities in blood vessel formation of the yolk sac are similar to those of the TGF-β knock-out mouse (Dickson et al., 1995).

PACE4$^{-/-}$ mice show incomplete penetrant embryonic lethality (25% lethality at E13.5-E15.5) and suffer primarily from cardiac malformations, situs, and craniofacial defects (Constam and Robertson, 2000; Lowe et al., 2001). The most striking PACE4$^{-/-}$ phenotype involves a reversal in the direction of heart looping (Constam and Robertson, 2000). Left/right axis defects are generally preceded by abnormal expression of the axis determining genes, nodal and lefty, both of which are potential PC substrates. These

genes are normally expressed in an asymmetric fashion, but in PACE4$^{-/-}$ embryos their expression can be absent, reversed, or bilateral.

The phenotype of mice mutant for both furin and PACE4 is consistent with the possibility that these enzymes are required for Nodal activation, even though Nodal is expressed in a region of the embryo that is distinct from where furin and PACE4 are expressed (Beck et al., 2002). Thus, Beck et al. suggest that secreted forms of furin and PACE4 may act at a distance to cleave Nodal, extracellularly. Embryo recombination assays are consistent with this possibility. Compound heterozygous and single mutant ES cells can cleave proNodal added to the culture media, while double mutant ES cells cannot. Thus, Beck et al (Beck et al., 2002) suggest that secreted (shed) furin and PACE4 cleave Nodal *in vivo*. ProBMP-4 is cleaved in these mutant embryos, suggesting that a distinct PC family member may be required for processing proBMP-4.

Analysis of PC substrate specificity

Considerable effort has been invested to determine the sequence specific requirements for PC cleavage. Redundancy exists between consensus sites for the various PCs, and yet knock-out models suggest there is distinct substrate specificity. For example, a great many proteins can be cleaved by furin *in vitro*, but whether they are bon a fide *in vivo* substrates is more difficult to ascertain. One approach for determining *in vivo* substrates involves the use of PC deficient cell lines, generated through knock-out or antisense knock-down strategies. Cell lines, however, do not necessarily mimic the micro-environment in which the substrate and enzymes are normally expressed, and overwhelming the cellular machinery in overexpression systems and mis-expression of

substrate or enzyme may potentially generate misleading results (Taylor et al., 2003; Walker et al., 1994).

Fluorogenic peptides have been used to determine minimal substrate specificity, kinetic parameters and requirements for cleavage *in vitro*. In this way, cleavage rates (k_{cat} and V_{max}) and binding constants (K_m) can be determined. Numerous chromophores have been employed, including 4-methylcoumaryl-7-amide (MCA), peptidyl-MCA, and 7-amino-4-methylcoumarin (AMC), and studies have looked at optimal peptide lengths for these enzymatic studies. Substrate inhibition is a problem associated with longer peptide fragments (hexamers and greater), but not with shorter peptides (tetramers) (Krysan et al., 1999). Substrate inhibition is related to the substrate length and the number of basic residues, and this is as contrasted to the protonation state of acidic residues on the solvent-exposed surface of the enzyme (i.e. pH sensitivity). To minimize these artifacts, peptides must be titrated well below their predicted K_m and optimized for ionic interactions. With these caveats in mind, peptide substrates have been extensively used to define requirements for furin binding and cleavage, and have allowed for comparison between the enzymatic properties of furin, Kex2, and PC1 (Jean et al., 1995; Krysan et al., 1999). Thus, analysis of the kinetics of cleavage of various fluorogenic peptides has been used to define RXK/RR as the optimal consensus motif for cleavage by furin (Molloy et al., 1999). These studies also revealed that residues C-terminal to the cleavage site may be required for optimal cleavage (Krysan et al., 1999) or may be unfavorable for cleavage (Rholam et al., 1995). Furthermore, bulky adducts, such as a chromophore, may actually interfere with substrate binding in the S_1' position. Thus,

more biologically relevant results are thought to result from the use of extended peptides or physiological substrates (Molloy et al., 1992).

The rate of furin cleavage may also depend upon the conformation of substrate. Extending the C-terminus of BRI (the peptide thought responsible for familial British dementia) enhances the rate of cleavage at an upstream site (Kim et al., 1999) suggesting that substrate cleavage is influenced by its location within the proprotein. Other substrates are cleaved optimally at either neutral or more acidic pH (Molloy et al., 1999; Molloy et al., 1992). For example, the P6 position can contribute to pH dependent cleavage, especially in the absence of a P4 Arg. Furin is autocatalytically cleaved in the ER at an optimal furin consensus site (RXR/KR) and the resultant peptide fragment remains in the catalytic cleft as a potent autoinhibitor (Anderson et al., 2002). Exposure of the furin/propeptide complex to mild acid (pH 6.0) and increased calcium results in autocatalytic cleavage of the propeptide at the non-consensus site, RXXXKR (Chiron et al., 1994; Krysan et al., 1999). α1-integrin is also cleaved by furin in a pH-sensitive manner, but contains a His residue in the P6 position (HXXXKR) (Bergeron et al., 2003). Mutation of the P6 His to an Arg enhances α1-integrin cleavage in Chinese-hamster ovary CHO-K1 cells, suggesting that at acidic pH the P6 His residue may mimic a basic residue and therefore compensate for the lack of a basic residue in the P4 position. Such conformational restrictions and pH-influenced context is difficult to model with a tetra- or hexa-peptide fragment. Despite these limitations, pH effects have been shown for an optimal furin consensus hexa-peptide containing a P6 Arg, but not Lys or Ala, (Krysan et al., 1999). These studies suggest that the P6 position can be important in modulating pH-sensitivity of cleavage, most likely mediated through its association with the S6 position

mature activin was also secreted when the activin mature domain was fused to the TGF-β prodomain, indicating some functional redundancy between proregions (Gray and Mason, 1990).

Prodomain substitutions can alter the properties of mature TGF-β family ligands

Vg1 is a member of TGF-β superfamily whose mRNA is localized to the vegetal pole of *Xenopus* eggs and early embryos (Vize and Thomsen, 1994) and is hypothesized to play an early role in mesoderm induction. Injection of proVg1 RNA into oocytes or embryos produces precursor, however cleaved, mature Vg1 protein cannot be detected biochemically or by bioactivity (Dale et al., 1993). The proVg1 signal sequence is not removed, and the protein is not dimerized or secreted. Therefore, in order to study the role for Vg1 in mesoderm induction a series of chimeras were tested to enhance the production of mature Vg1. A chimera consisting of the BMP-4 prodomain fused to the Vg1 cleavage site and mature domain (BVg1) enhanced the processing of proVg1 and resulted in secretion of mature Vg1, but demonstrated weak mesoderm-inducing activity (Dale et al., 1993). Since substitution of the BMP-4 prodomain enhanced processing of Vg1, these data show that the Vg1 cleavage site can be cleaved *in vivo*. A second chimera that fused both the prodomain and cleavage site for proBMP-2 with the mature domain of Vg1 was able to secrete processed, stable, mature Vg1 and was able to dorsalize injected embryos as well as the potent dorsalizing agent, activin (Thomsen and Melton, 1993). Secretion of mature Vg1 was even further enhanced when the activin prodomain and cleavage site were fused to mature Vg1 (Kessler and Melton, 1995).

From these studies, the proregion was shown to be the key determinant in regulating the secretion, processing efficiency, and/or turnover of mature Vg1.

By limiting the processing and secretion of the mature protein, the prodomain region may also play a role in determining the long or short range signaling potential of a secreted protein (Jones et al., 1996). Activin is a secreted TGF-β-related protein that is able to act at long range, while the related protein, *Xenopus* nodal-related protein 2 (Xnr-2), acts only at short range. Replacing the Xnr-2 prodomain and cleavage site with that of activin enabled mature Xnr2 to signal at long range in embryonic tissues, even though it was less active than wild type Xnr-2. These results lead Jones et al to postulate that the prodomain may act to restrict Xnr2 signaling in the early embryo. By contrast, culture media from oocytes expressing the activin/Xnr-2 chimera contained no biologically active Xnr-2 when assayed in animal caps. Athough these data suggest that Xnr2, when expressed from the activin prodomain, is more readily released or stable in embryonic cells than it is in oocytes, these studies also suggest that the prodomain of Xnr-2 may be involved in modulating an activity or function of mature Xnr-2 in a manner that the activin prodomain cannot.

Altering the cleavage site, rather than the entire prodomain, may be sufficient to enhance processing of a secreted factor, by generating a better consensus site or directing cleavage to a more abundant or locally expressed PC. Constam and Robertson (Constam and Robertson, 1999) began to address this possibility when they mutated the cleavage consensus site of nodal to mimic that of activin in the P2' and P3' positions. In cell culture they found that although the modified nodal cleavage site was cleaved more efficiently, mature nodal was still not detected in the culture media. By contrast, when

the dorsalin prodomain was fused to mature nodal it was found to stabilize the mature protein. The dorsalin prodomain remains noncovalently associated with the mature domain following cleavage of wild type or chimeric precursors, and may influence stabilization of the protein. In a reverse experiment, fusion of the nodal prodomain with the mature domain of BMP-4 prevented accumulation of mature BMP-4 into the culture media, possibly by destabilizing mature BMP-4. These studies suggest that individual TGF-β/BMP prodomains can dramatically influence the stability of their processed, mature ligands. More careful cellular and biochemical analysis are need to further refine these hypothesis and to explore the possible role for prodomains in regulating and trafficking these growth factors.

Prodomains act as intramolecular chaperones

One established role for propeptides is the regulation or catalysis of protein folding (Shinde and Inouye, 1993). This activity has been shown for numerous bacterial and eukaryotic proteases (Jacob et al., 2002; Shinde and Inouye, 2000), as well as for members of the TGF-β family of proteins including activin, TGF- β(Gray and Mason, 1990), and Mullerian inhibitory substance (MIS) (Wilson et al., 1993). In the absence of their propeptides these proteins do not fold into functional proteins. After acquiring the proper fold, however, the pro region is proteolytically removed.

The propeptide of subtilisin acts as a single-turnover catalyst to promote folding, and removal of the propeptide is required to prevent unfolding (Shinde and Inouye, 2000). By contrast, the prodomains of several TGF-β family members remain noncovalently attached to their mature domains following cleavage. MIS, for example,

requires the noncovalent association of its prodomain for biological activity after secretion (Wilson et al., 1993), possibly by preventing aggregation of the mature dimer. BMP-7 is also stably associated with its prodomain and this increases the solubility of the protein (Jones et al., 1994). The proregion of TGF-β remains associated with its cleaved mature domain and functions to maintain latency of the growth factor.

Unlike other TGF-β family members, the propeptide of macrophage inhibitory cytokine (MIC)-1 is not required for the folding of secretion of mature MIC-1 (Bauskin et al., 2000). Expression of an MIC construct lacking the propeptide generates a mature dimer that is biologically active (Strelau et al., 2000) and immunogenically identical (Fairlie et al., 2000) to MIC derived from full-length protein. Rather than promoting folding, the pro region of MIC serves a "quality control" function, allowing dimeric precursors to exit the ER while targeting monomeric precursors for proteosomal degradation (Bauskin et al., 2000).

In order to test the requirements for a prodomain to properly fold a mature dimer, a series of MIC/TGF-β chimeras were generated by exchanging portions of the TGF-β and MIC mature domains. In this way it was shown that the major α-helical region of mature TGF-β requires interactions with its propeptide to achieve the correctly folded mature product (Fairlie et al., 2001). Both TGF- β and BMP-7 present at least six mainly hydrophobic residues at the dimer interface, and these make contact with amino acids on the opposite subunit. The prodomains of TGF-β and BMP-7 are thought to facilitate the proper alignment and association between these residues, in part by masking particular regions along the mature peptide that may otherwise form inappropriate interactions during the folding process. Additionally, replacement of this α-helical region of the MIC

mature domain with that of TGF-β generated a proprotein that could be properly folded by the MIC, but not the TGF-β prodomain. This suggests that in addition to the α-helical region, specific interactions with other regions outside this helix may also be essential to align proper prodomain/mature domain interactions.

Novel prodomain roles

A potentially novel role for a propeptide fragment is illustrated by recent studies showing that the *Xenopus* nodal related protein (Xnr)-3 prodomain can regulate BMP signaling *in trans* (Haramoto et al., 2004). In general, the pro regions of the TGF-β-related proteins have been thought to have no intrinsic activity of their own. For example, when expressed alone, the prodomain of BMP-4 does not have BMP-4-like signaling activity (Cui and Christian, unpublished data). The pro regions of Xnr-3 and Xnr-5, however, are able to physically interact with BMP-4 and can block BMP-4 activity when overexpressed in *Xenopus* embryos. This introduces new potential roles for the TGF-β prodomains including the possibility of cross-talk with related, antagonistic proteins.

Multifunctional regulation by the TGF-β prodomain

TGF-β-1 is the best-characterized member of the TGF-β superfamily. Expressed in a biologically latent form, TGF-β-1 requires transient acidification, alkalinization or chaotropic release for *in vitro* activation (Lawrence et al., 1985). Mature TGF-β-1 complexes, *in vivo*, with its prodomain (Miyazono et al., 1988; Wakefield et al., 1988), and *trans* expression of the prodomain, called the latency associated peptide or LAP, is

sufficient to invoke latency to exogenously added TGF- β-1 (Gentry and Nash, 1990). Formation of the TGF-β-1 latent complex requires covalent dimerization of the two prodomain fragments; mutation of either cysteine residue involved in this covalent linkage is sufficient to promote unregulated release of mature TGF-β-1 and an additional 3-5 fold increase in bioactivity. Taken together, these data suggest that prodomain dimerization is necessary for the formation of the TGF-β-1 latency complex (Brunner et al., 1989).

The latency complex also involves association with the latent TGF-β-1 binding protein (LTBP) (Miyazono et al., 1988; Wakefield et al., 1988), a member of the LTBP/fibrillin protein family (Ramirez and Pereira, 1999), a family of proteins containing multiple epidermal growth factor (EGF)-like repeats. One of the EGF-like repeats acts to link LTBP to LAP, while the remaining repeats serve to localize LTBP to the extracellular matrix (ECM) (Unsold et al., 2001). Interestingly, BMP-7, which also remains noncovalently attached to its prodomain following secretion (Israel et al., 1992), is also localized to the ECM by association with fibrillin (Charbonneau et al., 2004; Israel et al., 1992). LTBP is released from the ECM by plasmin-mediated proteolysis, from which latent TGF-β-1 can then be released. Since recombinant LAP is also sensitive to plasmin degradation (Lyons et al., 1990), it is possible that LTBP may also act to protect LAP from unregulated plasmin degradation (Taipale et al., 1994).

The TGF-β-1 prodomain has three consensus N-linked glycosylations sites, two of which are phosphorylated and bind mannose-6 phosphate receptor (MPR) (Purchio et al., 1988) and thus, an additional role for LTBP may involve regulating interactions with the MPR. MPR is involved in trafficking lysosomal proteases from the TGN to the

lysosome, although some MPR proteins may be secreted prior to lysosomal transport and thus may play a role in remodeling the ECM (Brauker et al., 1986; Chao et al., 1990; Kornfeld, 1992). One possible role for LTBP may involve masking of the MPR targeting signal on LAP (Taipale et al., 1994), even though in the absence of LTBP latent TGF-β-1 is retained in the cis-Golgi apparatus of certain cell lines (Miyazono et al., 1992), a compartment that precedes targeting of lysosomal proteins in the TGN. MPR is also identical to the receptor for insulin-like growth factor II (IGF-II), which is involved in endocytic trafficking. In cell culture activation of latent TGF-β-1 requires binding to the M6P/IGF II receptor (Dennis and Rifkin, 1991), implicating a possible role for MPR in TGF-β signaling.

Carbohydrate modification of the pro region of TGF-β-1 precursor is required for transport and secretion of the mature polypeptide (Sha et al., 1989) as well as for the establishment of latency (Miyazono and Heldin, 1989). Blocking the early stages of oligosaccharide remodeling prevents secretion of TGF-β-1, whereas blocking later steps, such as those of Golgi mannosidases I and II, results in changes in the mature glycosylated form of TGF-β-1 while still allowing for proteolytic processing and secretion.

Prodomains regulate protein trafficking

There are numerous examples of propeptides regulating the intracellular trafficking of proteins. Glycosylation of protease prodomains can target protein trafficking to the lysosome (Kornfeld and Mellman, 1989; Metcalf and Fusek, 1993), and information in the prodomain of PCs can direct trafficking to the regulated or constitutive

secretory pathways. Fusion of the PC2 propeptide to α1-antitrypsin is sufficient to cause this constitutively secreted protein to undergo membrane association and low-pH-dependent aggregation, similar to that of wild type PC2. These events are thought to precede sorting into the regulated secretory pathway (Jan et al., 1998). Another example for prodomain trafficking involves a secreted hydrophobic mini-protein, TxVI, whose pro region facilitates ER export by 'hitchhiking' on sorting receptors (Conticello et al., 2003). Thus, the role for prodomains in the trafficking of secreted proteins is only beginning to be explored.

The prodomain of the brain-derived neurotropic factor (BDNF), a secreted factor involved in cardiovascular development and homeostasis of the nervous system, plays a role in the subcellular localization of mature BDNF in some, but not all cells. Heterozygous expression of a polymorphic form of BDNF containing a Val→Met substitution in the pro-region ($BDNF_{met}$) has been linked to memory impairment and susceptibility to neuropsychiatric disorders (Egan et al., 2003; Hall et al., 2003; Hariri et al., 2003; Momose et al., 2002; Neves-Pereira et al., 2002; Sen et al., 2003; Sklar et al., 2002). In polarized neuronal and neurosecretory cells $BDNF_{met}$ is abnormally trafficked, resulting in inefficient sorting to secretory granules without a corresponding defect in processing. $BDNF_{met}$ is capable of heterodimerizing with wild type $BDNF_{val}$ and this causes abnormal trafficking of the wild type protein and decreases the activity-dependent secretion of the protein (Chen et al., 2004). Expression studies in differentiated PC12 cells show that $BDNF_{met}$ is more highly localized in the cell body than $BDNF_{val}$. When co-expressed, $BDNF_{met}$ caused mistrafficking of $BDNF_{val}$ away from the biosynthetic pathway, leading to a concentrated distribution in the cell body similar to homozygous

expression of $BDNF_{met}$. No such trafficking defect was observed in endothelial or vascular smooth muscle cells, which lack the specialized depolarization-dependent secretion pathway and highly polarized morphology of neuronal cells. These data suggest a trafficking signal localized to the prodomain is required for efficient sorting to the regulated secretory pathway and that this is regulated in a tissue specific fashion.

The role for prodomains in regulating intracellular trafficking of proteins is only beginning to be explored. In addition to the examples shown here, there are examples where BMP protein secretion may be influenced by its prodomain. Mutation in a single residue within the prodomain of the zebrafish homologue for BMP-7, *snailhouse*, results in reduced secretion and/or stability of the pro/mature complex while intracellular levels of the proprotein remained unchanged (Dick et al., 2000). Likewise, the work presented here describes how deletions in the prodomain of BMP-4 can cause misfolding of mature BMP-4, and that regulated cleavage and interactions between the mature and prodomains of BMP-4 influence the subcellular trafficking and stability of mature BMP-4.

Intracellular trafficking can regulate growth factor signaling

Mechanisms of endocytosis

Secreted proteins are synthesized in the ER and most of these proteins are modified in the Golgi before they traffic to the TGN. From the TGN, two distinct pathways act to deliver secreted proteins to the PM. The regulated secretory pathway is unique to highly specialized exocrine, endocrine and neural cells (Kelly, 1985). In this

pathway, proteins are packaged into nascent secretory granules by a dynamic, selective process involving active sorting and/or targeting events. These granules are trafficked to the PM where they are later released in response to stimuli. The second pathway, the constitutive pathway, is believed to be ubiquitous for all cells. Proteins destined for the constitutive pathway are sorted away from secretory granules in the TGN and packaged into small vesicles, most of which are directly targeted to the PM for continuous exocytotic release (Farquhar and Palade, 1981; Palade, 1975). BMPs are trafficked through the constitutive pathway.

Receptor-mediated signaling is regulated, in part, through the localization of receptor complexes to distinct regions of the PM where the local membrane environment can contribute to directing the endocytic process through distinct intracellular pathways. In this way endocytosis can potentiate signaling by localizing receptor complexes with their signaling targets to the early endosome, or attenuate signaling by facilitating receptor sorting to the lysosome for degradation. Endocytosis can also regulate the distribution and activity of secreted molecules by controlling the recycling and degradation of internalized ligands across a cell layer (Seto et al., 2002). Endocytosis, therefore, can determine the length and potency of receptor signaling.

Endocytosed proteins can be targeted to the early endosome, from which proteins are then (re)distributed either directly from the late endosome to the lysosome, or indirectly to the PM and TGN through a sorting or recycling compartment (Figure 3). Portions of the early endosomal membrane are believed to bud off to form the recycling endosome, acquiring expression of Rab4, Rab11, and the SNARE protein, Cellubrevin (Peters et al., 2001). These associated proteins allow the recycling endosome to then fuse

with the plasma membrane and release its contents (Peterson and Emr, 2001). This recycling activity has been proposed to be crucial for the promotion of long range signaling through a tissue by a process of sequential internalization at one cell surface and release at the opposing cell surface (Seto et al., 2002).

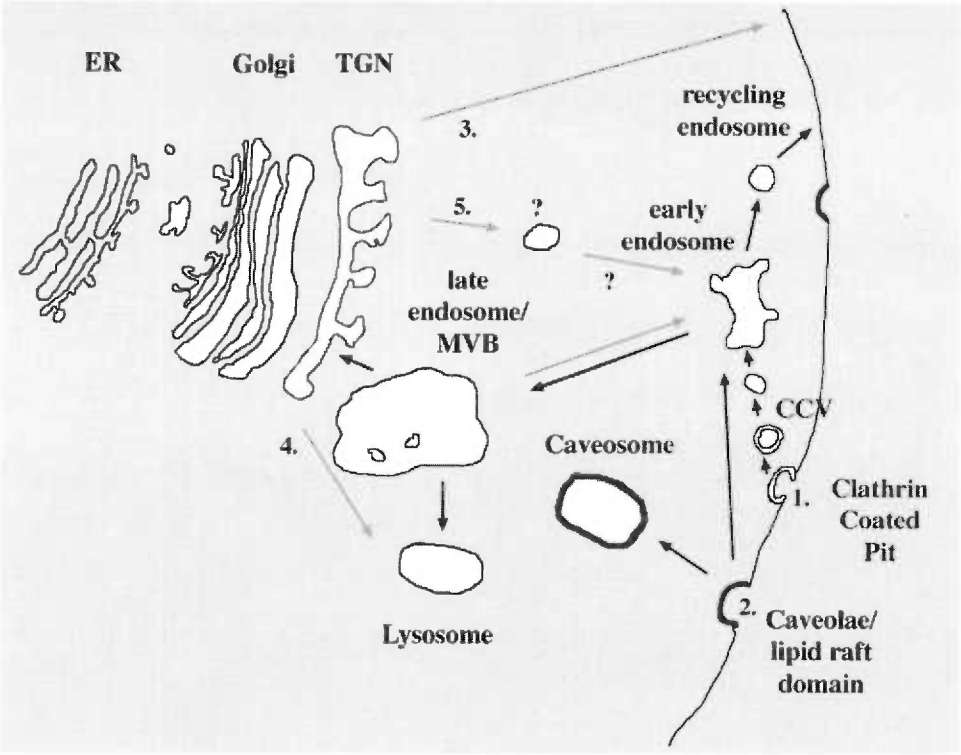

Figure 3. Intracellular trafficking pathways. Receptors and their ligands are endocytosed primarily through 1) Clathrin coated pits (CCP) or 2) Caveolae/lipids raft domains. Endocytosis through CCP allows proteins to be targeted to the early endosome from where these proteins can be recycled to the cell surface via recycling endosomes, or to the lysosome, via the late endosome/MVB (see text for details). Complexes endocytosed through caveolae may be targeted to the Caveosome, although the outcome of such targeting is unknown. Caveolae and lipid raft domains can also target proteins the early endosome for subsequent sorting. 3) The biosynthetic pathway allows proteins to be directly targeted to the PM from the TGN or 4) there is evidence for trafficking to an intermediate or post-TGN compartment. 5) In yeast, proteins can be directly targeted from the TGN to the vacuole (lysosome equivalent) using the AP-3 complex.

Early endosomes can also contribute to the formation of late endosome. In vertebrate systems, two competing models describe the formation of late endosomes: one in which transport vesicles move cargo between the early and late endosomes (Griffiths and Gruenberg, 1991), and the second in which early endosomes mature to form late endosomes (Murphy, 1991). Early and late endosomes are distinguished based on the time required for an endocytosed cargo to reach a specific compartment, or by expression of specific membrane markers, location within the cell, pH, and subcellular morphology. Mammalian early endosomes tend to be localized along the cell periphery and have tubulo-vescular morphology, whereas late endosomes tend to be perinuclear, more acidic, and spherical in shape. Late endosomes can also contain internal vesicles (Hopkins et al., 1990) enriched in PI(3)P and lysobisphosphatidic acid phospholipids (Gillooly et al., 2000; Kobayashi et al., 1998), which contribute to the formation of MVBs.

Endocytosed proteins can be trafficked to the lysosome through the late endosome/MVB. Endocytosed receptors, sorted to the large dense-core vesicles (LDCVs) portion of the MVB undergo vesicle docking and membrane fusion with the lysosome to promote hydrolysis and cargo degradation (Cavalli et al., 2001; Katzmann et al., 2002). Late endosomes and lysosomes are characteristically distinct. Although they both exhibit low pH, perinuclear distribution and expression of specific integral membrane glycoproteins (Piper and Luzio, 2001), late endosomes associate with specific Rab GTPases and two types of MPRs, while lysosomes contain neither protein. Some proteins that are normally sorted to the lysosome also have the capacity to recycle back to the cell surface or other compartments. Transport proteins that carry degradative enzymes to the lysosome, such as the MPR, must be recycled back to the Golgi.

Additionally, yeast α-factor receptor is constitutively endocytosed and targeted for degradation, however, under ligand-binding conditions it is preferentially sorted to the recycling endosome to be recycled to the PM (Chen and Davis, 2000; Chen and Davis, 2002). Less clearly defined, EGFR can also be targeted for degradation or recycling, independent of ligand activation and contingent upon additional cellular factors (Opresko et al., 1995).

Clathrin-mediated endocytosis

Clathrin-mediated endocytosis is the major endocytic pathway for internalizing receptor tyrosine kinases (RTKs) and is used to target proteins to the early endosome and to downregulate many receptors through ubiquitin-dependent sorting processes (Katzmann et al., 2001). Membrane targeting and endocytosis of RTKs is mediated by internalization sorting codes such as dileucine motifs and the tyrosine-based codes NPXY and YXX∅ (where ∅ is any hydrophobic amino acid) (Carpentier et al., 1982; Gorden et al., 1978; Hanover et al., 1984). These signals can be post-translationally modified to regulate both constitutive and ligand-activated endocytosis. For example, phosphorylation or ubiquitination of residues within, or adjacent to, these internalization motifs may increase the rate of receptor internalization following ligand binding (Dietrich et al., 1994; Dittrich et al., 1996; Pitcher et al., 1999; Rotin et al., 2000). When activated, these internalization codes induce rapid clustering of receptor complexes into clathrin coated pits (CCPs) and promote binding to the heterotetrameric adaptor complex, AP-2 (Kirchhausen et al., 1997). The binding of AP-2 in turn promotes recruitment of clathrin, which, in concert with local changes in the phospholipid composition of the membrane,

promotes the formation of the clathrin lattice to drive membrane invagination and vesicle formation. The GTPase, dynamin, then "pinches" off the newly formed vesicle from the PM (Ringstad et al., 1997; Wang et al., 1995) to form a clathrin-coated vesicle (CCV). Epidermal growth factor receptor pathway substrate 15 (Eps15), which is thought to nucleate the internalization complex through protein-protein interactions with Asn-Pro-Phe (NPF)-motif proteins (Chen et al., 1998; Iannolo et al., 1997; Santolini et al., 2000; Wong et al., 1995) and the crosslinking of AP-2 complexes (Iannolo et al., 1997), is also thought essential for clathrin-dependent endocytosis.

After CCV internalization, the clathrin coat is shed and this allows vesicles to fuse with the early endosome (Seto et al., 2002). That early endosomes are formed from internalized endocytic vesicles is demonstrated by various studies overexpressing proteins that block or promote membrane fusion. Rab5, a GTPase localized to early endosomes (Bucci et al., 1992; Chavrier et al., 1990), is essential for early endosome fusion (Gorvel et al., 1991). Dominant negative Rab5(S34N), which preferentially binds GDP, inhibits endocytosis and promotes the formation of very small endosomes, while constitutively active (CA) GTPase-deficient Rab5(Q79L) stimulates endocytosis and forms enlarged early endosomes (Stenmark et al., 1994). These data support a model in which early endosomes are formed from endocytic vesicles, and suggests that late endosomes form downstream of early endosome fusion. At least twenty-two potential effectors of the Rab5 pathway have been isolated and this suggest potential roles for Rab5 ranging from internalization, to early endosome fusion, to movement of endocytic vesicles along microtubules (Seto et al., 2002).

Early endosome antigen-1 (EEA-1) is the only cytosolic effecter necessary for endosome fusion (Christoforidis et al., 1999). EEA-1 contains an N-terminal FYVE domain (Stenmark et al., 1996), which in the presence of Rab-5GTP targets EEA-1 to endosomal membranes enriched in PI(3)P. EEA-1 likely tethers endosomes together to facilitate subsequent fusion events (Christoforidis et al., 1999). The fusion of endosomes, like all membrane fusion events, involves soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes, which promotes a direct interaction between opposing membranes. EEA-1 is also found associated with the SNARE complex protein, Syntaxin13 (McBride et al., 1999). Expression of a dominant negative form of Syntaxin 13 or an EEA-1 peptide can inhibit endosome fusion, showing that EEA-1 may also regulate fusion of early endosomal membranes. Interestingly, CCV-mediated endocytosis can lead to either signal potentiation or attenuation, depending upon the localization of additional factors that regulate subsequent targeting events.

Caveolae-mediated endocytosis

Caveoli-mediated endocytosis involves the formation of small, flask-shaped membrane invaginations enriched in cholesterol, sphingolipids, and caveolin, and these form distinct lipid-rich domains called caveolin-associated lipid-raft domains or caveolea (Anderson and Jacobson, 2002; Simons and Toomre, 2000). These membrane regions play an important role in endocytic trafficking by facilitating protein sorting and assembly of signaling complexes within the condensed packing arrangement of sphingolipids and cholesterol (Sharma et al., 2002). Whether the sphingolipid head group provides specificity for the targeting of these proteins is yet to be determined

(Brown and London, 1998). Caveolae also function in the internalization of lipid-bound toxins, SV40 virus and glycosyl phosphatidylinositol (GPI)-anchored proteins (Anderson et al., 1992; Nichols, 2002; Pelkmans et al., 2001; Sharma et al., 2002). Cholesterol and sphingomyelin are clearly detected at the PM, on recycling endosomes and lysosomes, but not in late endosomes; caveolea are detected at the PM and on endosome-like structures, but not the lysosome. Lipid rafts and caveolea are believed to be involved in vesicular and cholesterol trafficking from the lysosome (Anderson et al., 1992; Razani et al., 2000), but current spectroscopic means may limit their detection in these compartments. Therefore, there is no direct evidence for the involvement of caveolea in the trafficking of cholesterol from the lysosome.

Caveolae-rafts organize into detergent-resistant membranes (DRM) that can be biochemically fractioned from detergent-soluble membranes. There is some evidence that proteins localized to these domains traffic to a distinct subcellular organelle called the caveosome (Nichols, 2002; Pelkmans et al., 2001) by an endosome-independent route. Proteins routed through caveolae can be targeted to the ER (Pelkmans et al., 2001), to the Golgi (Nichols, 2002) or to the lysosome. Caveolea/raft-mediated endocytosis is involved in a variety of signaling pathways (Prevostel et al., 2000; Roy et al., 1999; Smart et al., 1999) and has been shown to play a role in receptor downregulation (Di Guglielmo et al., 2003). Epidermal growth factor (EGF) and TGF-β can be endocytosed by either clathrin-mediated or caveolea-mediated endocytic pathways, but with very distinct and sometimes opposing outcomes. This begins to illustrate the complexity of lipid/membrane interactions with signaling pathways.

Epidermal Growth Factor signaling and endocytosis

Receptor signaling involves ligand/receptor interactions at the PM and at the early endosome. Endocytic trafficking can control the magnitude of growth factor signaling as well as the specificity and duration of the response (Resat et al., 2003). In this way, qualitative differences have been observed from receptor signaling from either the PM or the early endosome.

Receptor internalization can attenuate RTK signaling if the receptor is targeted to the lysosome for degradation, but can also facilitate signaling by bringing together the activated receptor with its downstream components at the early endosome. Activated EGF receptor (EGFR) can be targeted to the lysosome for degradation and down-regulation of signaling. Expression of internalization-defective EGFR induces transformation of cells through constitutive signaling from the receptor complex at the cell surface (Wells et al., 1990), and heterodimers of EGFRs that are internalization compromised exhibit prolonged signaling as compared with EGFR homodimers (Lenferink et al., 1998). By contrast, EGFR internalization can also target the activated receptor complex to early endosomes where interactions with downstream effectors are optimized (Di Guglielmo et al., 1994; Oksvold et al., 2001; Sorkin et al., 2000). At the early endosome, activated EGFR maintains its kinase activity and the receptor becomes increasingly phosphorylated (Cohen and Fava, 1985), activating downstream targets such as MAPK and Shc. Blocking endocytosis with a dominant mutant form of dynamin results in decreased MAPK phosphorylation (Vieira et al., 1996), but hyperphosphorylation of Shc. This suggests that endocytosis of the EGFR is required for activation of only a subset of downstream signaling transducers. These and other studies

suggest that the subcellular localization of an activated receptor complex may selectively activate distinct downstream targets.

Following endosomal targeting, activated receptors can be targeted to the lysosome for degradation, or recycled back to the PM. There is evidence that these sorting decisions may be handled in the MVB, as discussed above (Seto et al., 2002). Receptors targeted for degradation sort to the LDCV of the MVB while other receptors, including inactive EGFR or an activated form of EGFR containing a specific point mutation in the kinase domain (Felder et al., 1990), are targeted to the outer membrane (Felder et al., 1990; Futter et al., 1996) for recycling. Mutations in mice that interfere with EGFR sorting in the MVB lead to tumorogenesis (Ceresa and Schmid, 2000; Di Fiore and Gill, 1999), consistent with constitutive signaling from these internalized receptor complexes. Cbl may mediate sorting events at the MVB (Pai et al., 2000; Yoon et al., 1995) through ubiquitin-mediated sorting of the receptor for lysosomal degradation (Levkowitz et al., 1998; Meisner et al., 1997). Consistent with this possibility, failure to ubiquitinate EGFR drives recycling to the PM (Levkowitz et al., 1998) and sustained signaling.

EGFR is not only internalized through clathrin-mediated endocytosis (Levkowitz et al., 1998) but also by caveolin-mediated endocytosis (Di Guglielmo et al., 2003; Mineo et al., 1996). EGFR can cluster in caveolae invaginations of the membrane and following endocytosis, remains in detergent resistant membrane 'raft' fractions that can be biochemically fractionated and evaluated. Rat hepatocytes were used to study the *in vivo* role of EGF/EGFR in caveolae-raft-mediated trafficking (Pol et al., 2000). During normal adult rat homeostasis, caveolin is mainly concentrated at the plasma membrane

and in recycling endosomes. Injection of EGF, but not pIgA, causes a distribution shift of caveolin towards early/sorting endosomes (Pol et al., 2000), even though it is unknown whether this redistribution is relevant for signal transduction or receptor down-regulation. Future studies are needed to determine whether EGFR associated with caveolae is signaling competent and to evaluate potential roles for the caveosome.

TGF-β signaling and endocytosis

Trafficking of the TGF-β receptor (TβR) complex reveals a very complex, tissue-dependent system of regulation. TβRII has two kinase activities: autophosphorylation and transphosphorylation, the latter of which activates the kinase activity of RI. In mesenchymal cells, the transphosphorylation activity of RII is essential for optimal internalization and receptor down-regulation, while the kinase activity of RI is not required (Anders et al., 1998). By contrast, in epithelial cells RII transphosphorylation is not strictly required for receptor down-regulation (Dore et al., 2001). Smad-2/–3 activation and signaling have been suggested to occur only after endocytic vesicle formation (Penheiter et al., 2002), indicating that endocytosis is necessary for the propagation of post-receptor signaling events. This model remains controversial, as other studies suggest that endocytosis is dispensable for signaling (Lu et al., 2002).

TβR trafficking is not regulated exclusively by ligand stimulation. This suggests that receptor activation is not absolutely required for endocytosis (Ito et al., 2004), although it does not preclude the possibility that receptor activation alters endocytic mechanisms. Recent studies have looked to distinguish between the interdependence of internalization, signaling, and turnover of TβR. In mink lung epithelial (Mv1Lu) cells,

TβR is endocytosed through two independent pathways: clathrin-dependent endocytosis, with sorting to early endosomes, and caveolin-positive vesicles, with sorting to caveolar vesicles (Di Guglielmo et al., 2003). Inhibition of clathrin-dependent endocytosis blocks receptor signaling, whereas cholesterol disruption enhances signaling and Smad2 activation. This suggests that clathrin-mediated endocytosis is required for signaling and that caveolae-mediated targeting signaling limits TGF-β signaling (Figure 4). Consistent

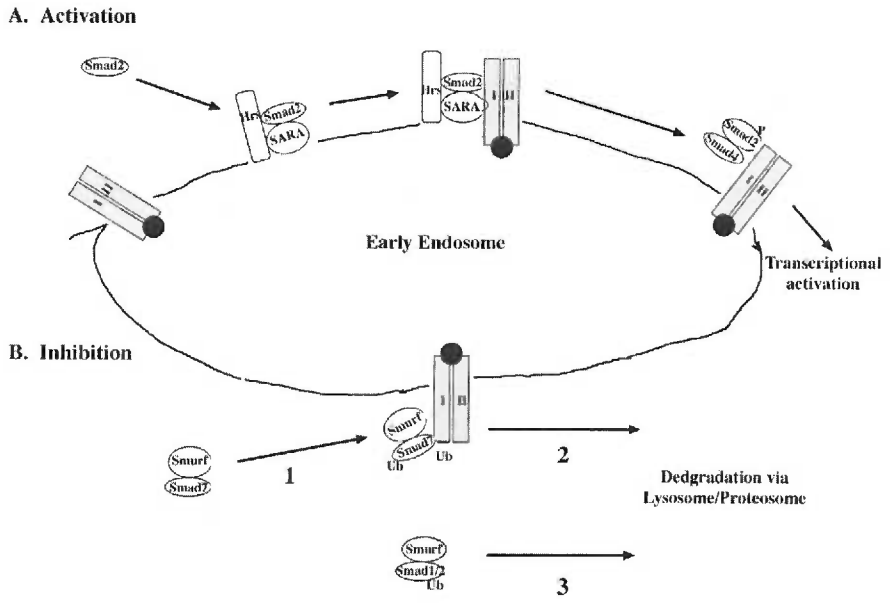

Figure 4. Regulation of TGF-β signaling at the early endosome. **A**. Activation. Following ligand binding the heteromeric receptor complex is endocytosed to the early endosome where it interacts with Smad proteins, whose binding is facilitated by the presence of Hrs and SARA at the endosomal membrane. Phosphorylation of Smad2 by the receptor complex leads to disassociation of SARA and Hrs and promotes the oligomerization of Smad2 with Smad4. **B**. Inhibition. TGF-b signaling is inhibited by Smurf ubiquitin ligases at three levels: 1. Smurf proteins traffic I-Smad7 to the receptor complex where Smad7 blocks R-Smad association. 2. Smurf proteins ubiquitinate the Smad7-receptor complex to promote its degradation. 3. Smurf proteins can ubiquitinate Smad2 (and Smad1) to prevent the formation of active Smad complexes.

with the possibility that clathrin-dependent endocytosis induces TβR signaling, SARA, involved in the up-regulation of TGF-β signaling, segregates to EEA-1/Rab-5 positive vesicles (early endosomes) (Di Guglielmo et al., 2003). There, SARA can bind TβRII and, through its FYVE domain, can also bind PI(3)P at the endosomal membrane to stabilize the receptor complex. SARA also coordinately binds Hrs and Smad2, promoting formation of an active signaling complex (Seto et al., 2002). By contrast, Smad7-Smurf2 associate in lipid raft domains that co-localize with caveolin-1. Thus, targeting TβR to lipid-raft domains results in its ubiquitination and subsequent degradation (Di Guglielmo et al., 2003; Seto et al., 2002). These studies have recently been called into question, however, as another group has shown that TβRs recycle and are degraded in a clathrin-dependent manner and that raft-dependent endocytosis is of minor importance (Mitchell et al., 2004).

Polysaccharide expression also regulates the compartmentalization of TβRI. Following endocytosis, TβRI is targeted to both raft (caveolin-positive) and non-raft (EEA-1 positive) membrane fractions. When co-expressed in the presence of hyaluronan, both receptor and ligand are preferentially targeted to caveolin-positive, lipid raft-associated pools, and thus hyaluronan facilitates increased receptor turnover and attenuation of TGF-β−1 dependent responses. Consistent with these findings, cells stimulated with TGF-β1 in the presence of hyaluronan show decreased nuclear translocation of Smad4 and increased association between TβR and Smad7. These data suggest that hyaluronan-mediated down-regulation of TGF-β1 signaling is a result of

increased segregation of TβR into a lipid raft-caveolar compartment and away from the endosomal signaling compartment (Ito et al., 2004).

Heparin Sulfate Proteoglycans play a crucial role in regulating growth factors

Heparin sulfate proteoglycans (HSPGs) are structurally heterogeneous cell surface molecules that consist of a protein core that is modified by heparan sulfate glycosaminoglycan (HS-GAG). HSPGs are classified according to their protein core structure and are subdivided into three major groups: syndecans, which are integral membrane proteins; glypicans, which are glycosylphosphatidylinositol (GPI)-anchored proteins; and perlecans, which are secreted proteins (Lin and Perrimon, 2000). HSPGs can bind to a diverse array of proteins under physiological conditions and can modulate the actions of extracellular ligands though a variety of mechanisms. HSGPs can link proteins within the ECM, sequester proteins at the cell surface or within secretory vesicles (Bernfield et al., 1999), and protect proteins from endogenous proteases (Gospodarowicz and Cheng, 1986). HSPGs can increase the effective concentration of signaling proteins to a two-dimensional array at the cell surface. Thus, at low concentrations HSPGs can increase the activity of a growth factor by targeting it to its receptor, while at high concentrations HSPGs can reduce signaling activity by competing for the number of surfaces the protein can bind and thus sequester the growth factor (Bernfield et al., 1999). HSPGs can also act as co-receptors and may be required for

receptor internalization and the transport of signaling molecules (Lin and Perrimon, 2000).

HSPG biosynthesis and modification

Numerous genes involved in HSPG biosynthesis have been cloned from genetic screens in *Drosophila*. These include *sugarless* (*sgl*) (Binari et al., 1997; Haerry et al., 1997), *sulfateless* (*sfl*) (Lin and Perrimon, 1999), *tout velu* (*ttv*) (Bellaiche et al., 1998; The et al., 1999), and *pipe* (Sen et al., 1998). *Sgl* encodes a homolog of UDP-D-glucose dehydrogenase (Hempel et al., 1994), which produces one substrate of HSPG biosynthesis, UDP-D glucuronic acid. *Sfl* is a homolog of heparin sulfate N-deacetylase/N-sulfotransferase (Hashimoto et al., 1992) and is involved in HSPG modification. *Ttv* encodes a member of the Exostose (Ext) gene family, implicated in human multiple exostoses (Ext) syndrome (Stickens et al., 1996) and acts as a heparin sulfate polymerase. This family has recently been expanded to include *sister of tout-velu* (*sotv*) and *brother of tout-velu* (*botv*) (Han et al., 2004). *Pipe* encodes a putative heparin sulfate 2-O-sulfotransferase (2-OST) (Sen et al., 1998) that is important for dorso-ventral patterning (Fransson et al., 2004) and has homology to heparan sulfate 2-O-sulfotransferase (HSST) (Kobayashi et al., 1996; Kobayashi et al., 1997). Additional enzymes identified in the HSPG biosynthetic pathway include *Slalom,* a PAPS-synthase, and *Fringe connection*, an UDP-sugar antiporter (Fransson et al., 2004). Mutations in any of these genes disrupt various aspects of growth factor signaling.

HSPG structure and function

Syndecans (syn) are expressed at specific times and tissues during development (Bernfield et al., 1992; Filmus, 2001) and are involved in signaling and cytoplasmic interactions (Bernfield et al., 1999). Syn 1 is expressed very early in development on epithelia and mesenchymal-cell condensations and is associated with tissue morphogenesis. Syn 2 is expressed on mesenchymal tissues, liver and neuronal cells (David et al., 1992) and is required for determining left/right asymmetry in *Xenopus* development (Kramer and Yost, 2002) through the activation of Vg1. Interestingly, activation of Vg1 occurs *in trans* and requires the cytoplasmic, signaling domain of Syn2. Syn3 is largely neural, but is also expressed in some musculoskeletal tissues; Syn 4 is widely distributed throughout development (David et al., 1992). Syndecans are present at and regulate membrane microdomains, and integrates into lipid rafts domains that are associated with signaling molecules (Couchman, 2003). Internalization of syndecans within lipid raft domains occurs both in the presence (syn 1) (Fuki et al., 2000) or absence (syn 4) of caveolin 1 (Tkachenko and Simons, 2002).

Glypicans (gpc) are also expressed in very distinct patterns. Gpc-1 is expressed mainly in the central nervous system (CNS) and skeletal system during development, while it is expressed in additional tissues of the adult (Litwack et al., 1998). Gpc-2 is limited to the developing brain (Ivins et al., 1997) and does not seem present in the adult. Gpc-3, -4, -5, and -6 are expressed widely in the embryo. However, they are expressed in more restricted patterns in the adult and this suggests that they may be involved in promoting tissue morphogenesis (Fransson et al., 2004). GPI-linkages typically direct proteins to the apical surface of polarized cells, however antagonizing signals from the

HS-attachment region are thought capable of overriding the GPI signal (Mertens et al., 1996), allowing for Gpc-1 expression at the basolateral surface. The GPI anchor also targets glypicans to specialized sorting membranes at the PM, such as lipid rich domains and caveolae (Nabi and Le, 2003) where Gpc HS-chains may be involved in the cross-linking events that induce caveolar internalization. Mutations of these genes not only cause developmental defects, but are also often correlated with neoplastic transformation (Midorikawa et al., 2003).

Glypicans are known to modulate BMP-4 activity (Grisaru et al., 2001; Jackson et al., 1997; Paine-Saunders et al., 2000). Although syndecans have not yet been shown to modulate BMP activity, their expression patterns overlap with those of BMP-4 (Fransson et al., 2004), allowing for this possibility. Syndecan HS chains are spaced at a distance from the cell surface, interacting primarily with ECM proteins, while glypican HS-chains are more proximal to the cell, directing proteins to the cell surface. In this way, the spacing of the HS chains from the cell surface rather than the protein core helps to determine the type of interactions HSPGs direct. Syndecans can directly transmit signals to the cytoplasm through their cytoplasmic tails, while glypicans, which do not penetrate the membrane, presumably signal through interactions with auxiliary proteins within lipid raft domains. Both glypican and syndecan ectodomains can be shed from the cell in a regulated fashion while retaining their ligand binding properties (Mertens et al., 1996; Pelkmans and Helenius, 2002).

HSPGs and development: a model of tissue-specific gene activation

HSPGs are expressed in a dynamic fashion throughout development (Teel and Yost, 1996) and the structure of HS chains may be both spatially and temporally regulated to impart tissue-specific preference for one ligand over another (Friedl et al., 1997; Nurcombe et al., 1993). HSPG sulfonation patterns from various tissues show a direct correlation between the length of a N-sulfated region (NS domains) and the degree of 2-O-sulfation, while the rate of 6-O-sulfation is regulated by tissue type (Safaiyan et al., 2000). Thus, 6-O-sulfation appears to be one element of tissue-specific expression. HSPGs act as co-receptors and therefore tissue-restricted expression of HSPGs may dictate, specify and limit receptor/ligand interactions. The fibroblast growth factor receptor (FGFR)-HSPG co-receptor assembly complex for fibroblast growth factor (FGF) illustrates this specificity (Di Guglielmo et al., 1994; Oksvold et al., 2001; Sorkin et al., 2000). Twenty-three FGF molecules have been identified, all of which contain unique HS binding motifs (Allen et al., 2001), and therefore, they are thought to have unique HS specificities *in vivo*. FGF-2 has 'promiscuous' binding, requiring only a 2-O-sulfation residue for binding while FGF-4 requires a more specific, tissue restrictive motif (Allen et al., 2001). Similarly, sonic hedgehog (Shh) is expressed ubiquitously in the cerebellum, but activates proliferation only during the early postnatal period and in the external granule cell layer. Proliferation coincides with increased expression of the glycosyltransferase genes, Exo1 and Exo2, which are thought to potentiate Shh-induced signaling (Rubin et al., 2002). Therefore, the temporal and tissue-specific expression of HSPGs may limit ligand/receptor interactions and modulate signaling during development.

Unlike FGFs (Mali and Joshi, 1993; Steinfeld et al., 1996), BMPs do not appear to require HSPGs as co-receptors. The pattern of HSPG expression, however, can still modulate the activity of BMP-4. Overexpression studies in *Xenopus* embryos show that deletion of the putative HSPG binding domain of BMP-4 has no effect on receptor binding and signaling, but allows the mutant protein to traffic at longer range in *Xenopus* embryos (Ohkawara et al., 2002). Thus, HSPGs can act to restrict the range of BMP signaling. BMPs interact with HSPGs in a tissue-specific fashion. In *Drosophila*, dally enhances Dpp signaling in the eye, antenna, and genitalia, and overexpression of Dpp can suppress *dally* hypomorphic alleles. By contrast, in the wing disc dally *antagonizes* Dpp and *dally* mutants suppress overexpression of *dpp* (Jackson et al., 1997). Therefore, additional, unidentified effector(s) must also regulate these tissue specific effects of Dpp and dally. In mammals, the *dally* homolog, glypican-3 (GPC3), which causes Simpson-Golabi-Behmel dysmorphia syndrome (SGBS), also modulates BMP-4 signaling. Loss of function mutations in GPC3 results in a number of skeletal abnormalities. When $GPC3^{-/-}$ mice are crossed with BMP-4 haploinsufficient mice, the offspring show high penetrance of additional skeletal defects that are not evident with either parental strain and this is consistent with decreased responsiveness to BMP-4 (Paine-Saunders et al., 2000). Therefore, the presence or absence of specific HSPGs and their cofactors can potentiate or suppress BMP activity.

At this time is not known whether BMPs interact solely with glypicans or also with syndecans. Syndecans form clusters in lipid-rich and caveolae domains within the PM. Therefore, interactions between BMPs and syndecans remain an attractive mechanism through which HSPGs may further modulate BMP signaling. In addition to

HSPGs, polysaccharide expression can also regulate signaling. TβR1 is targeted to both lipid raft and non-raft domains in the PM, however, in the presence of hyaluronan, TβR1 is preferentially targeted to caveolin-positive lipid-raft domains, resulting in enhanced receptor turnover (Ito et al., 2004). Hyaluronan also binds to BMP-2 *in vitro* (Kim and Valentini, 2002) and thus may play a role in growth factor targeting to distinct regions of the PM or ECM *in vivo*.

HSPGs and Noggin: establishment of an inverse gradient of BMP inhibitors

Noggin belongs to a class of soluble BMP antagonists that bind BMPs (Smith and Harland, 1992) and prevent them from interacting with their receptor (Zimmerman et al., 1996). Noggin binds cell surface HSPGs and can be directly internalized and degraded along with its bound ligand (Paine-Saunders et al., 2002). Anchoring to HSPGs is proposed to limit Noggin diffusion and thereby help establish an inverse-gradient of BMP activity in the developing embryo (Paine-Saunders et al., 2002). Noggin binds specific N-, 6-O- and 2-O-sulfate residues in HSPG chains (Viviano et al., 2004). The endo 6-O-sulfatase, Qsulf1, which post synthetically removes sulfate residues from the 6-O position of sugars at the PM, promotes release of Noggin from the cell surface and this restores BMP responsiveness to cells. In this way, tissue-specific expression of Q-sulf1 can restore BMP activity in a temporal and spatial manner.

The relationship between HSPGs and BMP signaling is becoming progressively more complex. HSPGs regulate BMP activity gradients by limiting the range of BMP expression and its ability to activate signaling in receiving cells. In addition, HSPGs also regulate BMP activity by regulating the expression range, stability, and release of the

BMP antagonist, Noggin, during development. In this way, activation of BMP-4 targets can be temporally regulated by the tissue specific expression of endo sulfatases (Viviano et al., 2004). It is also possible that specific HSPGs may regulate the degradation or stability of BMP-4, as alluded to in the following chapters, although this is yet to be proven.

Morphogens in development

The term '*morphogen*' refers to a type of signaling molecule that acts directly on cells to induce distinct cellular responses in a concentration-dependent manner. In this way, cells receiving a morphogen signal differentiate in a positional manner, with their distance from the source read along a concentration gradient. Dpp satisfies these conditions. Dpp normally activates the downstream target genes *spalt* (*sal*) and *optomotor blind* (*omb*) at a distance from expressing cell. When cells are made to express the constitutively active form of the Dpp receptor, Thickveins (Tkv*) (Lecuit, 1996; Nellen et al., 1996) *sal* and *omb* are activated in expressing, but not adjacent cells, demonstrating that long range activation is a direct effect of Dpp and not the result of a second signal emanating from expressing cells. Other proteins also characterized as morphogens include hedgehog (Hh) (Mullor et al., 1997; Strigini and Cohen, 1997), sonic hedgehog (Shh) (Briscoe et al., 2001), wingless (Wg) (Neumann and Cohen, 1997; Zecca et al., 1996), Squint (Chen and Schier, 2001) and activin (Gurdon and Bourillot, 2001).

The Drosophila *wing disc: a model for morphogen gradients*

The classic example for morphogen gradients is illustrated by the patterning of the *Drosophila* the wing disc (Tabata and Takei, 2004) (Figure 5). The *Drosophila* adult wing arises from the larval imaginal disc, a two-sided sac consisting of a columnar cell layer, which gives rise to the wing, and a squamous epithelium, known as the peripodial membrane. The wing disc columnar layer is further subdivided into two, non-intermingling compartments called the anterior (A) compartment and the posterior (P) compartment. Expression of the patterning gene, *engrailed* (*en*), specifies the P compartment and induces expression of *Hh*. Hh, expressed in the P compartment, is secreted into the proximal region of the A compartment where is acts as a short-range morphogen. The graded distribution of Hh in the stripe of cells adjacent to the anterior/posterior (AP) compartment boundary patterns the central domain of the wing and induces the expression of *en, patched (ptc)* and *dpp*, at high, middle and low thresholds, respectively. Dpp is induced along the AP boundary and acts as a second morphogen signal (Lecuit, 1996; Nellen et al., 1996) acting at long range in both the A and P compartments to pattern the wing beyond the central domain. Dpp induces expression of *sal* and *omb* at high and lower thresholds, respectively, with *omb* expression extending distal from the Dpp-expressing source. Final patterning of the wing disc involves its subdivision into dorsal (D) and ventral (V) compartments along the dorsoventral (DV) axis, which later becomes the wing margin and is patterned by the morphogen, Wg. Wg induction results from a second signaling cascade initiated by the expression of Apeterour in the dorsal compartment. Apeterour induces expression of *fringe*, which activates the Notch receptor pathway along the DV border, and this results

in activation of Wg along the DV axis. Each morphogen is distributed in a graded fashion extending away from the expression domain (Tabata and Takei, 2004).

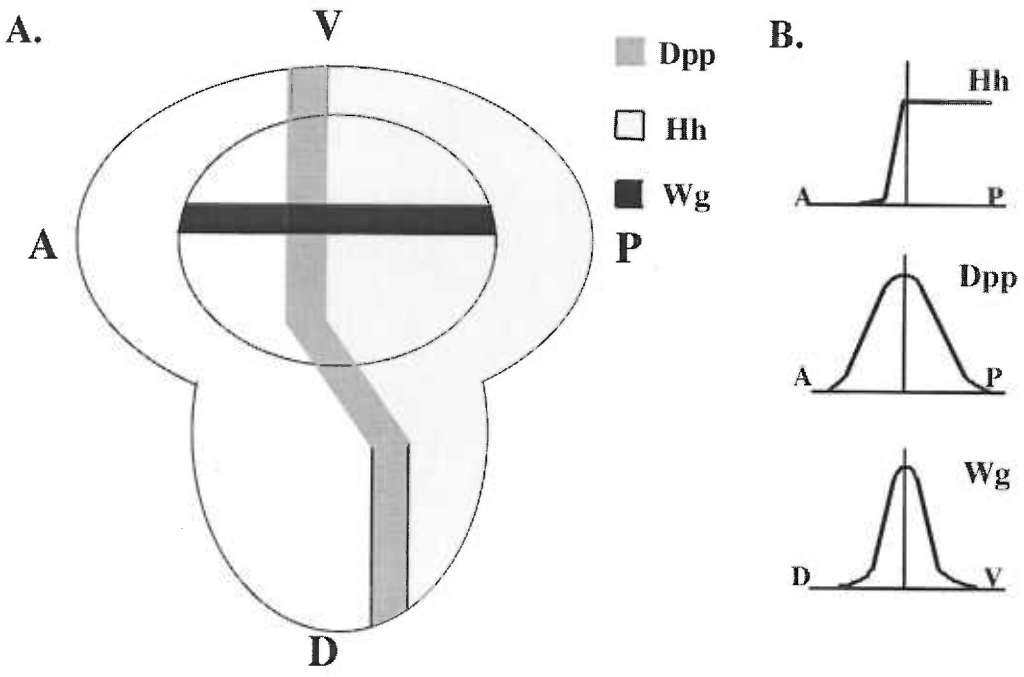

Figure 5. The *Drosophila* wing disc is a model for morphogen gradients. A. Illustration of a wing imaginal disc. Hh is expressed in the posterior portion of the wing. Dpp is expressed in a band of cells along the AP axis. Wg is expressed along the DV axis. B. Range of morphogen transport. Hh is expressed in the posterior compartment, and its range and activity drops off precipitously in the A compartment. Dpp, expressed along the AP axis, is able to act distal from the source and is found in both A and P compartments. Dpp activity in the P compartment is inhibited by the presence of negative regulators. Wg is expressed in a tight band of cells along the DV axis and is transported within a narrow band cells.

Mechanisms for establishing a morphogen gradient

The question of how morphogens activate target gene expression in cells distal from the expression source is controversial. Several mechanisms have been proposed which include (Figure 6) planar transcytosis (Entchev et al., 2000), restricted diffusion (Eldar et al., 2003; Lander et al., 2002), argosomes (Greco et al., 2001), and cytomenes (Gibson et al., 2002; Gibson and Schubiger, 2000). Several of these mechanisms may be acting simultaneously to transfer a given morphogen and certain mechanisms appear better suited for describing the distribution of one morphogen over another (Strigini and Cohen, 2000; Teleman et al., 2001; Vincent and Dubois, 2002).

Planar transcytosis

Planar transcytosis, or transcytosis, involves the transport of a morphogen through a tissue by repeated cycles of endocytosis and re-secretion. Entchev et al (Entchev et al., 2000) first described this mechanism using a temperature sensitive mutant of *Drosophila shibire* (*shi*^{ts}), which encodes dynamin. After inducing mosaic expression of *shi*^{ts} and temporal expression of a Dpp 'wave,' using Dpp fused to the green fluorescent protein (Dpp-GFP), Entchev showed that Dpp-GFP cannot traffic through an endocytosis-defective clone of cells, resulting in accumulation of secreted Dpp-GFP along the front of the clone. Additionally, Dpp was not distributed to cells behind the clonal patch, thus creating a 'shadow' in the endocytic competent cells behind the clonal patch. This showed that long-range distribution of Dpp-GFP requires endocytosis (Entchev et al., 2000). Additional experiments exploited Rab5, a small GTPase required for fusion of

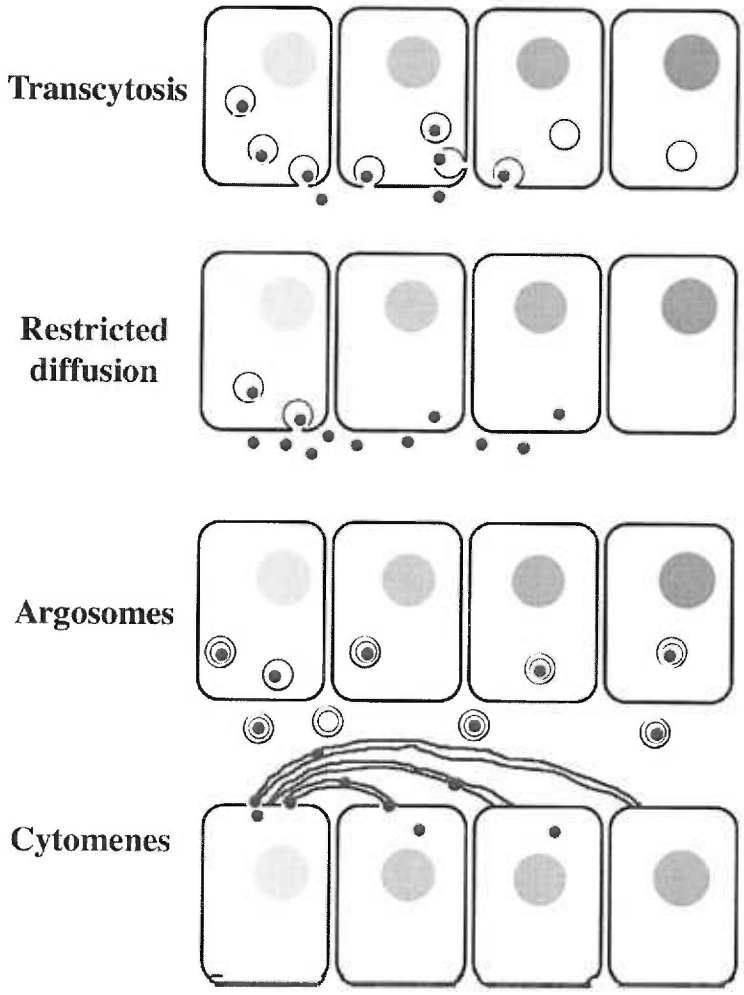

Figure 6. Possible mechanisms for establishing a morphogenic gradient. Transcytosis involves the spread of a morphogen by sequential secretion/endocytosis. Restricted diffusion uses mathematical modeling to allow for receptor uptake/degradation of a secreted ligand and interactions within the ECM that act to limit or promote long range diffusion of a morphogen. Argosomes are a 'vesicle-within-a-vesicle' used to traffic of membrane- or HSPG-associated morphogens. Cytomenes involve filapodia-like cellular extensions that respond to chemoattractive signals from morphogen-expressing cells that deliver the receptor to morphogen expressing cells. The gradient is established through the temporal decay of endocytosed ligand as it traffics along the cellular extension.

endocytic vesicles with early endosomes. When dominant-negative Rab5 was expressed in the wing disc, target gene expression was restricted to cells adjacent to the Dpp-GFP expression source (Entchev et al., 2000). By contrast, when Rab 5 was overexpressed it broadened the Dpp-GFP expression domain. Thus, Dpp-GFP gradient formation is dependent upon trafficking through the endocytic pathway, and, based on studies involving Tkv, is likely regulated by receptor-mediated endocytosis. A second small GTPase, Rab 7, targets endocytic cargo from early-to-late endosomes to the lysosome for degradation. Dominant gain-of-function Rab 7 reduced the range of Dpp signaling, consistent with the possibility that the range of Dpp signaling is restricted by degradation (Entchev et al., 2000). These studies suggest that Dpp can form a gradient by sequential rounds of endocytosis/re-secretion.

Transcytosis does not appear to be the only mechanism driving formation of a Dpp gradient in the wing disc. Studies by Teleman and Cohen (Teleman and Cohen, 2000) show that the bulk of Dpp-GFP in the wing disc is sensitive to proteinase K digestion, indicating that most Dpp is expressed in the extracellular space. Thus, diffusion may also contribute to the formation of a Dpp gradient in the wing.

Restricted diffusion

The idea that morphogens move by simple diffusion was abandoned following experiments where expression of soluble GFP in the *Drosophila* wing disc failed to form a stable gradient. This suggested that free diffusion alone cannot explain the formation of a morphogen gradient (Entchev et al., 2000). Mathematical models were subsequently developed to account for rates of diffusion and compensate for extracellular interactions

with receptors, HSPGs, and extracellular binding proteins, or the restricted diffusion model (Eldar et al., 2003; Lander et al., 2002). As previously described, secreted Dpp cannot traffic through endocytosis-impaired clones; Dpp builds up at the front of such regions and fails to distribute to cells behind the impaired region, creating a 'shadow' (Entchev et al., 2000). Whereas this 'shadow' is explained by the transcytosis model, the mathematical model also predicts this loss of expression by modeling the interaction of Dpp with its receptor at the cell surface (Lander et al., 2002). Blocking endocytosis is predicted to increase cell surface receptor levels, therefore increasing the binding potential of *shi*-mutant cells. Since the gradient shape depends, in part, on cell surface receptor concentration, the gradient becomes steeper with this increase in receptor density. Therefore, restricted diffusion also predicts that morphogen gradients will fall steeply enough through an endocytosis-impaired clone so as to cast a 'shadow' behind the clonal population. Additionally, since the Dpp gradient in the wing is almost fully established within 7 hours of Dpp expression, Lander et al (Lander et al., 2002) suggest that transcytosis would need to occur at an implausibly rapid rate to traffic through such a distance. These data remain a strong source of controversy between the two groups. A recent theoretical and experimental analysis of the restricted diffusion model suggests that extracellular diffusion alone cannot explain the shibire "shadow" results [Kruse et al., 2004]. Thus, endocytosis continues to play an active role in ligand transport beyond the regulation of cell surface receptor levels.

Argosomes

As previously described, soluble GFP fails to form a stable gradient within the wing disc. However, GFP tethered to the outer membrane leaflet by a GPI-linkage can

be detected at a distance of 12-16 cell diameters away for the expressing cells (Greco et al., 2001), which suggests that membrane fragments may traffic across cell layers. The argosome model extends these observations to suggest that a morphogen, presumably with high membrane affinity, can traffic within small vesicles across cell layers (Cadigan, 2002). Cells at the DV boundary produce structures called 'argosomes.' of which approximately 50% co-localize with Wg. This allows for the possibility that Wg may be trafficked across cell layers by argosomes (Greco et al., 2001). Two models have been proposed for the formation of these structures. The first suggests that argosomes are formed predominantly in basolateral endocytic compartments that are morphologically similar to MVB. These MVB-like invaginations result in the formation of a 'vesicle within a vesicle' that becomes the argosome. The second possibility suggests that membranes are internalized by receptor-mediated endocytosis and directly form a 'vesicle within a vesicle' (Cadigan, 2002) that can then traffic across cell layers. Unlike with the transcytosis model, Wg transport in the wing disc can occur in a dynamin-independent fashion (Strigini and Cohen, 2000). Thus, the gradient of Wg expression either occurs by dynamin-independent endocytosis or is formed independent of endocytosis. A second experimental observation showed that when tethered to the PM as a N-terminal fusion to the *Drosophila* Neurotactin (Nrt) type-II transmembrane domain, Wg can signal only to adjacent cells (Zecca et al., 1996). It was argued that anchoring to the PM should not affect argosome-mediated long-range transport (Cadigan, 2002). However, only proteins localized to specific microdomains are predicted to enter argosomes. Thus, GPI-linked proteins can be incorporated into argosomes, while rho-linked proteins cannot (Greco et al., 2001). In addition, Wg may require association with

specific HSPGs to be directed into the appropriate microdomains that develop into argosomes, as treatment of the cell surface with heparatinases prevents Wg entry into these structures (Greco et al., 2001). By contrast, these results can also arise if Wg is incorporated into argosomes intracellularly, in a manner analogous to MVB formation.

Cytomenes

Filipodia-like structures called 'cytomenes,' were observed in the *Drosophila* wing disc, projecting unidirectionally toward local populations of cells expressing Hh or Dpp (Christian, 2000). These structures can be likened to the specialized cellular extensions of nerve cell axons or the filipodia of the sea urchin, archenterons, during gastrulation (Miller et al., 1995). During wing development these processes are proposed to mediate long-range signaling of Hh and Dpp (Ramirez-Weber and Kornberg, 1999). By this model, morphogens, when released from cells, stimulate their cognate receptors only at the sites of cytomene contact. The morphogen gradient is then generated by the intracellular decay of the morphogen as it traffics along the cytomene projection towards the cell body. Hh-expressing cells can induce cytomene formation *in vitro*, with the chemoattractive activity resulting from a downstream, diffusible factor activated by Hh signaling, which is most likely an FGF (Ramirez-Weber and Kornberg, 1999).

Cytomenes are visualized in the lumenal space of the wing disc, *in vivo*, where peripodial cells extend cellular processes across the lumenal space, which separates the peripodial and columnar cell layers and terminating in the columnar, Hh/Dpp expressing cells. These processes are thought to promote morphogen signaling between the two layers (Gibson and Schubiger, 2000). Recent work shows that Dpp expression is

required in the lumenal cavity and is essential for peripodial cell survival (Gibson et al., 2002). Although this demonstrates the need for communication between these two cell layers, it does not definitively show whether this activity occurs through cytomene contact. Cytomenes continue to be a provocative, but controversial mechanism for morphogen signaling.

Morphogen gradients – time or concentration

A final perspective on morphogen gradient formation comes from Pages and Kerridge (Pages and Kerridge, 2000) who question whether a gradient is generated from concentration- or time-dependent factors. Evidence for a concentration-dependent gradient comes from studies with Dpp where at low doses *omb* expression is induced, while at higher doses *sal* expression is induced. This pattern of activation is observed whether Dpp is expressed ectopically (*sal* is expressed centrally; *omb* is expressed distal from the source), at low doses (only *omb* expression is induced), or at high doses (both *omb* and *sal* expression is induced). An alternate model, called the 'sequential cell context' model, emphasizes that it is time the of ligand interaction, not concentration, which dictates cellular responses.

The concentration model is limited in explaining how a cell senses ligand concentrations at saturating levels. Cells can likely respond to signaling events proportional to the number of activated receptors. In a developmental context this assumes that the number of receptors are in excess with respect to the ligand concentration. Contrary to this, both Dpp and Wg downregulate their receptors when

expressed at high levels. Thus, receptor mRNA is expressed weakly when close to ligand-producing cells, but more strongly in cells further away.

Pages and Kerridge (Pages and Kerridge, 2000) argue, therefore, that specificity arises differentially in time and with changing cellular competence. Following an initial signaling event (t_1), an early set of target genes is activated (*omb*). This changes the context of the receiving cell such that when a subsequent signal is given (t_2) these signals act synergistically to allow the expression of novel, later target genes (*omb, sal*). Consequently, the specific response of the cell depends on its prior transcriptional state, or 'priming.' By this model, low receptor numbers should promote long range distribution of a ligand while high receptor number will limit it. What this model does not adequately explain, however, is how, at high ligand concentration, both primary and secondary responses are activated.

HSPGs involvement in the formation of a morphogen gradient

HSPGs clearly play a role in establishing a morphogen gradient. If one accepts the restricted diffusion model, then HSPG interactions are essential to limit or promote the diffusion of a morphogen in a tissue-specific fashion. Extracellular proteins bind specific HS modifications and, in the absence of such modifications, growth factors are free to diffuse to different regions on the cell surface or to adjacent cells. HSPGs can either direct morphogens to their receptors to promote signaling, or can allow them to diffuse freely, to thereby limit signaling. Morphogen binding to cell-associated HSPGs can also function as a temporal sink, sequestering the morphogen until specific desulfatases release the bound protein. This allows for ligands to act in a 'time-released'

fashion such as previously described for Qsulf1, Noggin, and BMP-4 (Viviano et al., 2004).

HSPGs also promote transcytosis or degradation of a given morphogen. Receptors can be endocytosed through caveolae/lipid rafts or clathrin-mediated endocytosis. The method of endocytosis, mediated in part by clustering with specific HSPGs or lipids, serves to target the endocytic vesicle to the recycling endosome or the late endosome-lysosome degradative pathway. Targeting of a morphogen to a specific HSPG, such as syndecan-4, shown to regulate receptor trafficking to recycling endosomes, may then promote the establishment such a gradient. Since BMP-4 is known to bind HSPGs, future studies will attempt to elucidate the role of HSPGs in regulating degradation, localization, and targeting of BMP-4 to establish a short or long range signaling pattern.

Chapter 2

Mechanism by which sequential cleavage of the BMP-4 prodomain regulates mature BMP-4 activity

Catherine Degnin[1], François Jean[2#], Gary Thomas[2], and Jan L. Christian

Department of Cell and Developmental Biology, Biochemistry and Molecular Biology[1], and Vollum Institute[2]
Oregon Health and Science University, School of Medicine

[#]Presently at: Department of Microbiology and Immunology
University of British Columbia
Canada

In the following paper I performed all the experiments except for Figure 8A, which was performed by Dr. Jan Christian. A subset of this work, not including that shown in Figures 5, 7, and 8, has been accepted for publication in Molecular Biology of the Cell.

ABSTRACT

ProBMP-4 is initially cleaved at a consensus furin motif adjacent to the mature ligand domain (the S1 site) and this allows for subsequent cleavage at an upstream motif (the S2 site). Previous studies have shown that S2 cleavage regulates the activity and signaling range of mature BMP-4, but the mechanism by which this occurs is unknown. Here we show that the pro- and mature domains of BMP-4 remain noncovalently associated following S1 cleavage, generating a complex that is targeted for rapid degradation. Degradation requires lysosomal and proteosomal function and is enhanced by interaction with heparin sulfate proteoglycans. Subsequent cleavage at the S2 site liberates mature BMP-4 from the prodomain, thereby stabilizing the protein. We also show that cleavage at the S2, but not the S1 site, is enhanced at reduced pH, consistent with the possibility that the two cleavages occur in distinct subcellular compartment. Finally, preliminary studies suggest that both the S1 and S2 sites are cleaved within the oocyte and require furin for cleavage. Based on these results, we propose a model for how cleavage at the upstream site regulates the activity and signaling range of mature BMP-4 after it has been released from the prodomain.

INTRODUCTION

Bone morphogenetic protein-4 (BMP-4) is a signaling molecule that acts as a morphogen to influence cell fate in a concentration dependent manner. BMP-4 was originally identified as a protein that is capable of inducing ectopic bone formation but more recent studies have shown that it plays many different roles during embryonic development and in adults (Hogan, 1996).

BMP-4 function is essential for normal embryogenesis as illustrated by the fact that mice homozygous for a null allele of BMP-4 form little or no mesoderm and die near the time of gastrulation (Winnier et al., 1995). BMP-4 heterozygous mutant mice are viable but display a variety of birth defects, including reduced numbers of primordial germ cells, polydactyly, and kidney, eye and craniofacial abnormalities (Chang et al., 2001; Dunn et al., 1997; Lawson et al., 1999; Miyazaki et al., 2000). These data indicate that control of BMP-4 gene dosage is essential for normal embryonic patterning.

Excess BMP-4 activity also leads to birth defects. Mice mutant for the BMP antagonists gremlin, noggin and/or chordin show early lethality and/or defects in the spinal cord, forebrain, somites, skeleton and kidney (Brunet et al., 1998; Gong et al., 1999; Khokha et al., 2003; McMahon et al., 1998). In humans, mutations in the noggin gene are responsible for multiple synostoses syndrome, a genetic disease characterized by fusion of the joints (Gong et al., 1999), and abnormally high levels of BMP-4 protein are a key feature of fibrodysplasia ossificans progressiva, a crippling hereditary disorder in which ectopic bone forms throughout the body (Kaplan and Shore, 1998).

The requirement for strict regulation of BMP-4 dosage is met by controlling BMP-4 activity at multiple levels. At the extracellular level, BMP-4 is regulated by binding proteins, such as chordin and noggin, that block activation of cell-surface receptors, and by the protease Tolloid, which cleaves chordin to liberate active BMP-4 (Nakayama et al., 2000). BMP-4 also binds to cell surface heparin sulfate proteoglycans (HSPGs) and these interactions can promote or restrict activity (Ohkawara et al., 2002; Selleck, 2001). At the intracellular level, BMP signaling is negatively regulated in responding cells by Smad6 and Smad7, which block transmission of signals from the membrane to the nucleus, and by the Smurf family of ubiquitin-protein ligases that target BMP receptors and other components of the intracellular signal transduction cascade for proteosomal or lysosomal degradation (Shi et al., 2000).

The bioactivity of BMP-4 is also regulated at the level of proteolytic activation. BMP-4 is synthesized as an inactive precursor that is cleaved following the multibasic motif -RSKR- to yield the active, carboxyl-terminal mature protein dimer (Aono et al., 1995). Specific members of the proprotein convertase (PC) family of endoproteases cleave proBMP-4 (Constam and Robertson, 1999; Cui et al., 1998). In mammals, seven members of this family have been characterized and these exhibit overlapping but distinct substrate specificities. The well-characterized PC, furin, activates proproteins at the carboxyl-terminal side of the preferred consensus sequence -RXR/KR-, but can also cleave following the minimal sequence -RXXR- (Thomas, 2002). Two other family members, PACE4 and PC6 recognize these same consensus motifs (Rockwell et al., 2002).

We have previously shown that proBMP-4 is sequentially cleaved by furin at two sites and that this ordered proteolysis regulates the activity and signaling range of mature BMP-4 (Cui et al., 2001). The initial cleavage event takes place at an optimal furin motif (-RSKR-) at a site (S1) adjacent to the mature ligand, and this is followed by cleavage at a minimal furin motif (-RISR-) at an upstream site (S2) within the prodomain (illustrated in Figure 1A). Our previous studies in *Xenopus* embryos have shown that the first cleavage releases mature BMP-4 while the second cleavage serves a regulatory function. Specifically, ectopically expressed proBMP-4 carrying a point mutation that renders the S2 site noncleavable generates a ligand that shows less activity, signals over a shorter range, and accumulates at lower levels than does BMP-4 cleaved from native precursor (Cui et al., 2001). Analysis of mice harboring this same point mutation demonstrates that cleavage at the S2 site is essential for normal embryonic development (Hackenmiller et al., in preparation). Given that S1 cleavage of native and mutant precursors liberates an identical ligand from the prodomain, it is unclear how subsequent cleavage of the prodomain can alter the activity of this ligand.

In the present study we examine the molecular mechanism by which cleavages within the prodomain regulate the activity of mature BMP-4. Our results show that differential use of the S2 site regulates BMP-4 activity by directing intracellular trafficking of the cleaved ligand to either degradative or secretory/recycling pathways. In addition, our results suggest possible mechanisms by which tissue-specific cleavage at the S2 site might be regulated.

RESULTS

Inability to cleave proBMP-4 at the S2 site targets mature ligand for degradation

Previous studies have shown that failure to cleave proBMP-4 at the S2 site leads to lower steady state levels of mature BMP-4 in *Xenopus* embryos (Cui et al., 2001). The reduced accumulation of mature BMP-4 could result from misfolding of the precursor in the endoplasmic reticulum (ER) and subsequent degradation prior to cleavage, inefficient cleavage of the precursor after it exits the ER, or targeted degradation of mature BMP-4 following cleavage at the S1 site. To distinguish between these possibilities we compared synthesis, processing, and degradation of epitope tagged wild type proBMP-4 with that of a precursor in which the S2 site cannot be cleaved [BMP-4(mS2G)] (illustrated in Figure 1A) in *Xenopus* oocytes.

To determine whether mutation of the S2 site prevents proper folding and thus promotes degradation of proBMP-4(mS2G) prior to exiting the ER, we asked whether this precursor is dimerized and present in post-ER compartments at levels comparable to wild type proBMP-4. RNAs (5 ng) encoding wild type or S2 cleavage mutant proBMP-4 were injected into *Xenopus* oocytes together with [^{35}S]Met/Cys and oocytes were cultured for 20 hours to label newly synthesized proteins. Precursor and mature BMP-4 were immunoprecipitated from lysates using antibodies specific for the myc-tag and were treated with or without deglycosylating agents. Carbohydrates that are transferred onto proteins in the ER are sensitive to Endoglycosidase H (Endo H) digestion. When further modified in the Golgi these moieties become Endo H resistant but remain sensitive to Peptide N-Glycosidase F (PNGase F). Thus, Endo H resistance/PNGase F sensitivity is a hallmark of proteins that are properly folded and able to traffic from the ER. As shown in

Figure 1B, Endo H sensitive (asterisks) and Endo H resistant/PNGase F sensitive (arrowheads) forms of mature BMP-4 cleaved from wild type and cleavage mutant precursors were detected under reducing and non-reducing conditions. This indicates that high mannose, Endo H sensitive carbohydrates are retained at one or more glycosylation site(s) on mature BMP-4, even after it has trafficked through the Golgi. A similar glycosylation pattern is observed for the closely related protein, BMP-2 (Israel et al., 1992), suggesting that a subset of carbohydrates present in the mature domains of BMP-2 and -4 become inaccessible to modifying enzymes in the Golgi once these proteins adopt their fully folded conformation. Significantly less mature BMP-4 accumulated in oocytes expressing proBMP-4(mS2G) than in those expressing wild type precursor, consistent with our previous studies showing that failure to cleave at the S2 site leads to reduced levels of mature BMP-4 in embryos. The majority of wild type and S2 mutant proBMP-4 was present in an Endo H sensitive, monomeric form, but a small amount of dimerized precursor was detected under non-reducing conditions. Relatively equivalent levels of dimerized, Endo H resistant/PNGase F resistant proBMP-4 and proBMP-4(mS2G) were detected [arrows], indicating that the amino acid substitution at the S2 site does not cause misfolding and degradation of the precursor prior to cleavage.

To determine whether the reduced level of mature BMP-4 generated from proBMP-4(mS2G) is due to inefficient cleavage of the precursor at the S1 site, we examined the time course of proBMP processing by pulse-chase analysis. Oocytes were injected with RNA (5 ng) encoding BMP-4 precursors together with [^{35}S]Met/Cys and, after 3 hours, label was chased by incubation in media containing unlabeled methionine and cysteine. BMP-4 precursor and cleaved prodomain were immunoprecipitated from

cell or media fractions at increasing time intervals, using antibodies specific for the HA-tag, and separated on reducing gels. A band corresponding to prodomain cleaved at both the S1 and S2 sites was observed in lysates and media from oocytes made to express wild type proBMP-4, whereas a lower Mr band corresponding to prodomain cleaved only at the S1 site accumulated with equal kinetics in lysates and media from oocytes made to express proBMP-4(mS2G) (Figure 1C). These data demonstrate that the S1 site of proBMP-4(mS2G) is efficiently cleaved.

To directly examine accumulation of mature BMP-4 generated from each precursor, we repeated the pulse-chase experiment, but injected less RNA (0.45 ng) to avoid saturating the system. BMP-4 precursor and mature protein were immunoprecipitated from cell or media fractions at increasing time intervals using antibodies specific for the myc-tag, and separated on non-reducing gels. As shown in Figure 1D, wild type and cleavage mutant precursor proteins dimerized and disappeared from lysates with relatively equivalent kinetics, indicating that both precursors are cleaved with equal efficiency. Mature BMP-4 was readily detected in lysates, and less so in media, from oocytes made to express proBMP-4 but was barely or nondetectable in lysates or media from oocytes made to express proBMP-4(mS2G). Under these same experimental conditions, prodomain cleaved from proBMP-4(mS2G) is barely detectable relative to that cleaved from native precursor (data not shown). Taken together, these data demonstrate that failure to cleave proBMP-4 at the S2 site has no effect on folding of the precursor or cleavage at the S1 site, but leads to rapid degradation of the cleaved prodomain and ligand.

Degradation of mature BMP-4 requires lysosomal and proteosomal function

To test whether degradation of mature BMP-4 requires lysosomal function, we asked whether treatment with the deacidifying agent, chloroquine, rescues accumulation of ligand cleaved from proBMP-4(mS2G). Oocytes were injected with RNA encoding wild type or mutant precursor together with [^{35}S]Met/Cys, cultured for 16 hours to label newly synthesized proteins and then cultured for an additional 3 hours in the presence or absence of chloroquine. Mature BMP-4 was immunoprecipitated from culture media using antibodies specific for the myc-tag. Consistent with our previous data, very little mature BMP-4 was detected in the media of untreated oocytes made to express proBMP-4(mS2G) relative to those expressing proBMP-4 (Figure 2, upper panel). Treatment with chloroquine increased the accumulation of mature BMP-4 generated from proBMP-4(mS2G) by 4 to 15 fold in 5 independent experiments. Chloroquine also increased accumulation of BMP-4 cleaved from wild type precursor in other experiments [data not shown], although to a lesser extent (1.8 to 6 fold).

To test whether proteosomal function is also required for degradation of mature BMP-4, the above experiment was repeated but oocytes were cultured in the presence or absence of 10 μM epoximicin or lactacystin. Levels of mature BMP-4 cleaved from proBMP-4(mS2G) were significantly increased (1.5 to 5.4 fold in four independent experiments) by inhibition of proteosomal function (Figure 2, lower panel). Mature BMP-4 cleaved from wild type precursor showed a more modest increase (1.3 to 2 fold in four independent experiments) under the same conditions. Taken together, our results show that failure to cleave the S2 site leads to rapid degradation of mature BMP-4 and this requires both proteosomal and lysosomal function.

Cleavage at the S2 site disrupts noncovalent association of the prodomain with mature BMP-4

Our observation that cleavage at the S2 site can regulate the activity of mature BMP-4 after it has been excised from the prodomain raises the possibility that these two fragments remain noncovalently associated following cleavage. Co-immunoprecipitation experiments were performed to test this possibility. RNA encoding proBMP-4 or proBMP-4(mS2G) was injected into oocytes and cleavage products were analyzed 24 hours later. Mature BMP-4 was immunoprecipitated from oocyte lysates or media using an anti-myc conjugated antibody. Western blots of immunoprecipitates or total proteins were probed with antibodies specific for HA or myc epitopes. As shown in Figure 3, the intact HA-tagged prodomain generated by cleavage at the S1 site co-immunoprecipitated with mature BMP-4, both inside (upper panels) and outside (lower panels) of the oocyte, whereas the N-terminal prodomain fragment generated by cleavage at the S2 site did not. These data show that the intact prodomain of BMP-4 remains bound to the mature domain following cleavage at the S1 site and that cleavage at the S2 site disrupts this association.

Cleavage at the S2 site, but not the S1 site, is enhanced under acidic conditions

To begin to ask whether cleavage of proBMP-4 at the S1 and S2 sites may occur in distinct subcellular compartments, we analyzed the pH-dependence of the relative rate of cleavage at each site. [^{35}S]proBMP-4 synthesized in oocytes was incubated with recombinant furin *in vitro* under neutral (pH 7) or acidic (pH 6.5) conditions and

cleavage products were analyzed by SDS-PAGE and autoradiography. Under neutral conditions, a band corresponding to the prodomain cleaved at the S1 site alone was apparent within 2 minutes of furin addition, and subsequent cleavage at the S2 site converted this band to the faster migrating species that was barely detectable after 5 minutes (Figure 4A). By contrast, under acidic conditions proBMP-4 was cleaved with equal efficiency at both the S1 and S2 sites within 2-5 minutes, as evidenced by the appearance of bands corresponding to the intact (S1 cleaved) and N-terminal (S1 and S2 cleaved) prodomain fragments. The band intensities of the S1 and S2 cleaved products over a range of substrate concentration (0.8-20.0 nM) and pH (6.5-7.0) were quantified and used to calculate the relative rate of cleavage under neutral and acidic conditions. As shown in Figure 4B, the S1 site was cleaved with equal efficiency at neutral and acidic pH, demonstrating that the acidic environment does not enhance furin activity in general. By contrast, the S2 site was cleaved more efficiently under acidic conditions. Lowering the pH to 6.0 did not lead to further enhancement of cleavage at the S2 site nor did it affect the rate of cleavage at the S1 site (data not shown). Consistent with the above data showing that cleavage at the S1 site is insensitive to pH, no difference was seen in the rate of cleavage of the S1 site of BMP-4(mS2G) under acidic or neutral conditions (Figure 4C).

To ask whether enhanced cleavage of the S2 site at acidic pH is dependent upon the presence of a minimal furin cleavage motif, we analyzed cleavage of proBMP-4(mS2K) in which the upstream cleavage site is converted to an optimal furin motif (RI<u>S</u>R→RI<u>K</u>R). We had previously shown that both sites of this precursor are cleaved rapidly and with equal efficiency, rather than sequentially (Cui et al., 2001). As shown in

Figure 4D, no difference was seen in the rate of cleavage of the optimal furin motifs under neutral and acidic conditions.

To test whether enhanced cleavage of the S2 site at acidic pH requires prior cleavage at the S1 site, we analyzed cleavage of a precursor in which the furin motif at the S1 site had been disrupted [RSKR→GSKR; BMP-4(mS1G)]. As shown in Figure 4E, a band corresponding to the prodomain cleaved at the S2 site alone was first detected at 10 minutes after furin addition under neutral conditions but was apparent within 5 minutes under acidic conditions. Thus, enhanced cleavage of the S2 site at acidic pH can occur independent of cleavage at the S1 site.

Taken together, our results demonstrate that sequential cleavage of proBMP-4 is driven by the presence of optimal and minimal furin motifs at the S1 and S2 sites, respectively, and that cleavage of the minimal furin motif at the S2 is enhanced at acidic pH. Given that organelles of the secretory pathway become increasingly acidic from the ER to the trans-Golgi network (TGN) to secretory granules, our results are consistent with the possibility that cleavage at the S1 site occurs in a proximal, less acidic subcellular compartment (most likely the TGN, where furin is first active) and that the prodomain/ligand complex then traffics to a more acidic, post-TGN compartment in which the S2 site becomes accessible for cleavage.

Fluorogenic peptides do not mimic in vivo cleavage of BMP-4

Fluorogenic peptides are extensively used to determine kinetic parameters for enzymatic reactions. Internally quenched fluorogenic substrates (IQFS) comprising the −11 to +2 sequence of the S1 site or the −12 to +3 sequence of the S2 site of proBMP-4

were generated containing an aminobenzoic acid emitting group and a nitro-tyrosine absorbing group. While monitoring absorbance, IQFS (2.5uM) were equilibrated to the appropriate pH, and then furin was added with continuous mixing and monitoring. The change in fluorescence over the course of the reaction was used to calculate the time to reach 1/2 $V_{max, obs}$ using Graphpad Prism. S1 IQFS was rapidly cleaved at pH 7, reaching $1/2V_{max, obs}$ in 184 sec, while the S2 IQF peptide was cleaved 4.2-fold less readily, reaching 1/2 $V_{max, pbs}$ in 769 sec. Under more acidic conditions (pH 6.2) S1 IQFS was cleaved more slowly, reaching 1/2 $V_{max, obs}$ in 886 sec whereas the 1/2 $V_{max,obs}$ of the S2 IQFS was dramatically reduced 7.8-fold to 6874 sec (Figure 5C). Although a single concentration cleavage reaction does not yield Km and Kcat values, it is clear from these data that the reaction is markedly slowed for both S1 and S2 IQFS under acidic conditions. S2 is cleaved approximately 4-fold slower than S1 at neutral pH, and approximately 8-fold slower at pH 6.2 (Figure 5C). This does not mimic our observations with the full-length protein, which shows the S1 site is unaffected by the reduction in pH while the S2 site is enhanced. Therefore, we continued our cleavage characterization studies using full-length proBMP-4.

A conserved P6 histidine at the S2 site inhibits cleavage at neutral pH

ProBMP-4 contains an evolutionarily conserved P6 histidine residue at the S2 site (underlined in Figure 6A), raising the possibility that this amino acid functions as a pH sensor. Specifically, at neutral pH the deprotonated histidine may mask the S2 site whereas at acidic pH the additional charge might induce a conformational change that enhances accessibility to the S2 site. To test this hypothesis, we analyzed pH-dependence

of cleavage of precursors in which the P6 histidine was substituted with a basic residue [HVRISR→RVRISR; BMP-4(mH-R)], to mimic the protonated state, or with a neutral residue [HVRISR→TVRISR; [BMP-4(mH-T)], to mimic the uncharged, deprotonated state. If our hypothesis is correct, substitution with a basic residue should generate a precursor that is efficiently cleaved at the S2 site under both acidic and neutral conditions, whereas substitution with a neutral residue should generate a precursor that is inefficiently cleaved at both neutral and acidic pH. Precursor proteins were synthesized in oocytes and subjected to *in vitro* cleavage with recombinant furin at neutral and acidic pH as described above. Consistent with our prediction, cleavage of the S2 site was enhanced at both acidic and neutral pH by substitution of the P6 histidine with arginine [BMP-4(mH-R)] (Figure 6B). Contrary to our prediction, however, substitution of the P6 histidine with threonine [BMP-4(mH-T)] enhanced, rather than inhibited cleavage at both pH values (Figure 6C). These results are consistent with a simpler model in which the deprotonated P6 histidine adopts a conformation that negatively regulates cleavage of the S2 site at neutral pH and protonation of this histidine at acidic pH, or substitution with other amino acids that disrupt the inhibitory conformation, enhance S2 cleavage.

BMP-4 is cleaved within the oocyte

Our *in vitro* data suggest that proBMP-4 is sequentially cleaved within two distinct subcellular compartments. Consistent with the hypothesis that proBMP-4 is cleaved during transit through the biosynthetic pathway rather than in the extracellular space following secretion, cleavage products are detected in oocyte lysates as well as in the media (Figure 1). To determine whether mature BMP-4 in oocyte lysates is located

inside the oocyte or is bound to the cell surface following secretion, we asked whether it is susceptible to tryptic digestion. Oocytes were injected with RNA (5 ng) encoding proBMP-4 and [^{35}S]Met/Cys. 24 hours later, oocytes were treated 20-minutes with or without trypsin (100ug/ml) on ice (4^{0}C) or at room temperature to remove cell surface proteins. Trypsinized and control oocytes were lysed, immunoprecipitated using myc specific antibodies, and run on SDS-PAGE. As shown in Figure 7A, trypsinization of the cell surface had no affect on the amount of mature BMP-4 derived from wild type or cleavage mutant precursor-proteins found in oocyte lysates. To show that the tight cysteine knot structure of mature BMP-4 is susceptible to tryptic digestion, we immunoprecipitated secreted mature BMP-4 from the culture media using myc-specific antibodies and subjected that to tryptic digestion under the same conditions as in Figure 7A. As shown in Figure 7B, mature BMP-4 is partially sensitive to tryptic digestion at 4^{0}C, but is digested to a proteolytically-resistant core at room temperature (asterisks). As shown in Figure 7C, mature BMP-4 immunoprecipitated from oocyte lysates is also trypsin-sensitive, indicating there is no intrinsic inhibitory modification made by the oocyte which prevents digestion of attached, rather than secreted, mature BMP-4. These data suggest mature BMP-4 is localized within the oocyte rather than at its surface, consistent cleavage occurring intracellularly. These data, however, do not rule out the possibility that mature BMP-4 is cleaved extracellularly and then rapidly endocytosed, thereby protecting it from tryptic digestion.

To definitively ask whether BMP-4 is cleaved intracellularly or extracellularly, we blocked secretion with bafilomycin A_1, and asked whether BMP-4 is cleaved at the S1 and S2 sites. Bafilomycin A_1 is an H^+-type ATPase inhibitor that specifically blocks

acidification of secretory vesicles, thus preventing TGN-to-plasma membrane trafficking (Umata et al., 1990; Yamaguchi et al., 1998). Therefore, if S1 and/or S2 site cleavage occurs extracellularly, the cleaved prodomain will accumulate within bafilomycin A_1 treated lysates. Oocytes were pre-treated for four-hours with increasing concentrations of bafilomycin A_1 and then injected with RNA (5 ng) encoding proBMP-4 and [^{35}S]Met/Cys. Proteins were allowed to expressed eight-hours, then oocytes and media were collected and immunoprecipitated using myc- or HA-specific antibodies. Bafilomycin A_1 blocked secretion of both mature (Figure 7D, bottom left) and prodomain (Figure 7D, bottom right) fragments of BMP-4 in a concentration dependent manner, whereas it did not prevent processing of proBMP-4 within oocyte lysates, as evidenced by the presence of cleavage products in oocyte lysates (top). These data further support the hypothesis that, in the oocyte, BMP-4 is cleaved prior to secretion.

Furin is required for cleavage of proBMP-4 in oocytes

Our previous studies suggested that furin and/or PC6 are required for maturation of BMP-4 (Cui et al., 2001) but it is possible that distinct PCs cleave the S1 and S2 sites *in vivo*. Furin is expressed throughout the embryo, while PACE4 and PC6, which are also capable of cleaving BMP-4 *in vitro* (Cui et al., 1998), are expressed in a limited subset of tissues and in regions overlapping with BMP-4 expression (Constam et al., 1996; Constam and Robertson, 2000; Cui et al., 1998). Using a candidate approach, we developed an *in vivo* cleavage assay to test whether furin, PACE4, or PC6 cleaves BMP-4 *in vivo*. Oocytes were injected with phosphorothioate-modified antisense oligonucleotides (10 ng) targeted to each candidate *Xenopus* PC alone, or in combination

as indicated (Heasman, 2002). Maternal mRNA stores encoding each PC were degraded within 1-hour of injection as determined by Northern blot (Figure 8A). Maternal mRNA-depleted oocytes were further cultured 72-hours, to allow for turnover of maternal proteins, injected with RNA (5 ng) encoding proBMP-4 and [^{35}S]Met/Cys and then cultured an additional 8-hours to allow for protein expression and processing. Control and depleted lysates were immunoprecipitated using HA-specific antibodies, deglycosylated, and run on SDS-PAGE. Figure 8B shows that when PACE4 or PC6 RNAs were depleted either singly or in combination, the amount of cleaved prodomain was unchanged. When furin was depleted, however, little or no BMP-4 cleavage was detected. These preliminary data suggest that furin is necessary for cleavage of proBMP-4 in *Xenopus* oocytes.

Binding to HSPGs facilitates degradation of mature BMP-4

Binding to HSPGs has been shown to restrict BMP-4 diffusion (Ohkawara et al., 2002) and to target TGF-β family ligands for internalization and degradation (Hashimoto et al., 1997). To test whether binding to HSPGs facilitates targeted degradation of mature BMP-4 generated from proBMP-4(mS2G), we deleted three basic residues in the mature domain (illustrated above Figure 9) that have been shown to be necessary and sufficient for association with HSPGs (Ohkawara et al., 2002) and asked if this affected accumulation of mature BMP-4 in a pulse chase assay. Mature BMP-4 cleaved from proBMP-4(mS2G) was undetectable in oocyte lysates and culture media (Figure 9A), whereas deletion of the heparin-binding motif [BMP-4(mS2G)ΔRKK] resulted in significant accumulation of mature BMP-4 in both lysate and media fractions (Figure

Our results suggest that a conserved histidine residue at the P6 position of the S2 site of proBMP-4 mediates pH-dependent cleavage. Specifically, we propose that at neutral pH the deprotonated histidine adopts a conformation that masks the S2 site, and that protonation at acidic pH disrupts this conformation to allow cleavage to occur. A somewhat analogous situation exists in the case of α4 integrin where a P6 histidine compensates for the lack of a basic residue at the P4 position and renders cleavage sensitive to cellular pH (Bergeron et al., 2003). The P6 position is often not included in the consensus motif for cleavage by PCs, but others have shown that the S6 subsite of furin contributes to substrate binding and prefers basic residues (Rockwell et al., 2002). Consistent with this preference, analysis of the crystal structure of furin has revealed an overall negative surface potential for the S6 subsite of the enzyme (Henrich et al., 2003). Furthermore, this binding site resides in a relatively solvent exposed region (Siezen et al., 1994) and specificity for P6 basic residues can be modulated by pH (Krysan et al., 1999).

IQFS are commonly used to study enzyme kinetics in a highly quantitative manner; therefore we tested S1 and S2 IQFS for cleavage under increasingly more acidic conditions. We hypothesized that basic residues N-terminal to the S2 site may contribute electron-withdrawing potential to the P6 histidine, influencing pH-dependent cleavage; therefore we generated peptides to include this region. In preliminary studies we found that cleavage was reduced, rather than enhanced, at reduced pH, but especially at the S2 site. Under acidic conditions, the S2 site of recombinant proBMP-4 was cleaved approximately three-fold faster, whereas the S2 IQFS reaches 1/2Vmax approximately 7.8-fold slower. We suggest that the conformation and accessibility of the S2 site may be influenced by the global structure of the intact proprotein and hence, not replicated by

IQFS. Bhattacharjya et al (Bhattacharjya et al., 2001) found similar discrepancies in the behavior of peptides and a full-length protein. Solution studies measuring the degree of folding for the furin prodomain at neutral and acidic pH show that the pro peptide is partially folded at neutral pH, but as the pH environment is reduced, the conformational stability of the prodomain is decreased and undergoes unfolding. This unfolding is experienced primarily by the C-terminal region containing the furin cleavage sites. When this acid-sensitive propeptide was tested as a 38-residue peptide fragment, these pH-sensitive conformational shifts were no longer observed, supporting the need for interactions between the propeptide and the protein core. We propose a similar mechanism, requiring the BMP-4 prodomain or protein core, may regulate the pH-enhanced cleavage at the S2 site, and thus IQFS cannot properly quantify these interactions.

Sequential cleavage of proBMP-4 provides a mechanism for tissue-specific regulation of BMP activity and signaling range

Furin undergoes two-step proteolytic activation, analogous to that of proBMP-4, which serves to ensure that the zymogen is not activated prematurely (Thomas, 2002). Specifically, the propeptide of furin is autocatalytically excised within the ER at a consensus furin motif, but remains noncovalently bound within the active site to keep the enzyme inactive. This complex is trafficked to the TGN where reduced pH, coupled with increased calcium, enable furin to cleave the bound propeptide at a non-consensus motif, releasing it from the catalytic cleft so that furin can then cleave its substrates *in trans* (Anderson et al., 2002; Anderson et al., 1997).

By contrast with furin, sequential cleavage of proBMP-4 is not required to prevent premature activation but may instead provide a mechanism for tissue-specific regulation of activity and signaling range. BMP-4, and its *Drosophila* ortholog, Decapentaplegic (Dpp), function as either short- or long-range signaling molecules depending on the tissue in which they are expressed (Neumann and Cohen, 1997), but the mechanisms that confer this tissue-specificity are unknown. Constitutive cleavage of the S1 site in all tissues, coupled with selective cleavage of the S2 site in the subset of tissues where BMP-4 signals at long distance, could provide a mechanism for tissue-specific regulation of BMP signaling range. Consistent with this possibility, studies in *Xenopus* embryos (Cui et al., 2001), together with the current data, demonstrate that cleavage at the S2 site generates a stable ligand that possesses long-range signaling properties whereas cleavage at the S1 site alone generates an identical ligand that is targeted for rapid degradation and thus can signal only to adjacent cells. Our analysis of mice carrying a targeted mutation that prevents cleavage of the proBMP-4 S2 site provide further support for the hypothesis that tissue-specific use of the S2 site regulates the activity of endogenous BMP-4 [Hackenmiller et al, in preparation].

Potential mechanisms for tissue-specific regulation of S2 cleavage

The current results suggest several mechanisms through which tissue-specific cleavage of the S2 site might be achieved. First, our data showing that cleavage at the S2 site is enhanced at low pH raise the possibility that cleavage of the S2 site only occurs in tissues in which BMP-4 is sorted from the TGN into an acidic, endosomal transport pathway. Proteins destined for secretion can be targeted to the endosomal system for

delivery to the cell surface or can be shunted directly from the TGN to the plasma membrane (Bonifacino and Traub, 2003). S1 cleaved BMP-4 could potentially be sorted to endosomes prior to secretion in a subset of cells, where the acidic environment would allow for cleavage at the S2 site. In other cell types, S1 cleaved BMP-4 might be delivered directly to the plasma membrane from the TGN, thereby bypassing the acidic compartment where S2 cleavage occurs. Consistent with this possibility, we find that blocking secretion of BMP-4 with Bafilomycin A_1 does not affect cleavage of proBMP-4, thus showing that proBMP-4 is cleaved within the secretory system of *Xenopus* oocytes.

Selective cleavage of the S2 site of proBMP-4 could also be mediated by tissue-specific expression of a convertase that resides in a post-TGN compartment and cleaves the S2, but not the S1 site. Furin is broadly expressed and resides predominantly in the TGN (Thomas, 2002) whereas PC6B is expressed in only a few tissues (Nakagawa et al., 1993; Seidah et al., 1994) and is localized to a distinct, post-TGN compartment (Xiang et al., 2000). Likewise, PACE4 is also co-expressed with BMP-4 in some but not all tissues and is a candidate for cleaving BMP-4 in vivo (Akamatsu et al., 1999; Constam et al., 1996). Thus, it is possible that the S1 site is constitutively cleaved by furin, whereas the S2 site is cleaved only in tissues that co-express PC6B. The idea that different PCs might cleave a single substrate is not unprecedented. Activation of proinsulin, for example, requires sequential cleavage by PC1 and then PC2 (Zhou et al., 1999). Furthermore, PC1 and/or PC2 mediated cleavage of proopiomelanocortin or proglucagon generates distinct end products that are expressed in tissue-specific patterns, reflecting the differential distribution of PC1 and PC2. Our preliminary studies showing that furin is required to cleave proBMP-4 in *Xenopus* oocytes do not support the hypothesis that the S1 and S2

sites are cleaved by distinct proteases. We require specific antibodies against the *Xenopus* PCs, however, to definitively show depletion of these endogenous proteins. While furin depletion is sufficient to block cleavage in oocytes, this does not mean it is sufficient in all cell types. PC4 is expressed exclusively in male and female germ cells and is responsible for cleaving the pituitary adenylate cyclase-activating polypeptide (PACAP) in these cells (Taylor et al., 2003). By contrast, PACAP requires both PC1 and PC2 for proper cleavage in neuronal cells, thus demonstrating both functional redundancy and tissue-restrictive cleavage by PCs. Similar tissue specific effects may be used to cleave proBMP-4 during development. Beck et al (Beck et al., 2002) observed that BMP-4 is cleaved in $Spc1^{-/-}$;$Spc4^{-/-}$ ($fur^{-/-}$;$PACE4^{-/-}$) mutant mouse embryos during gastrulation, but it is possible that this is not the case at other developmental stages or tissues.

Ectodomain shedding is a process whereby the extracellular domain of a TM protein is proteolytically cleaved to generate a soluble protein. Furin is "shed" within the secretory system (Denault et al., 2002), and this generates bioactive, secreted furin capable of cleaving extracellular proteins such as profibrillin-1 (Ashworth et al., 1999; Wallis et al., 2003), nodal (Beck et al., 2002) and bacterial precursor proteins. Cleaved forms of furin have been detected in the epidermis (Paine-Saunders et al., 2000) and glioma cells (Dubois et al., 1995), although its role in development has not been defined. This allows for the possibility that BMP-4 may be cleaved in the extracellular environment. Contrary to this, we find that BMP-4 is cleaved intracellularly.

Cleavages within the prodomain direct intracellular trafficking and degradation of BMP-4.

We have shown that failure to cleave proBMP-4 at the S2 site promotes rapid degradation of the mature ligand and this requires both lysosomal and proteosomal function. Proteosome inhibitors have been shown to impair endosomal sorting and degradation of a number of cell surface receptors and their bound ligands. These receptors are not direct targets of the proteosome, however, but are degraded by lysosomal hydrolysis (Longva et al., 2002; Melman et al., 2002; Rocca et al., 2001; van Kerkhof et al., 2001). In these cases, the proteosome appears to play a broader function in regulating the trafficking of receptors into the degradation pathway (Hicke and Dunn, 2003). By analogy, proteosomal involvement in degrading mature BMP-4 may reflect its role in lysosomal sorting rather than degradation.

Our studies do not address whether mature BMP-4 is targeted for degradation within the biosynthetic or endocytic pathway. Newly synthesized transmembrane proteins can be delivered directly from the TGN to the lysosome for degradation without ever reaching the cell surface. In yeast, for example, GAP1 is delivered directly to the lysosome under certain environmental conditions (Soetens et al., 2001) and the *Drosophila* axon guidance molecule, Robo, undergoes regulated transport from the secretory pathway to the lysosome as a mechanism to decrease cell surface activity (Keleman et al., 2002). It is possible that motifs within the BMP-4 prodomain bind to a sorting receptor in the secretory pathway that preferentially targets the prodomain/ligand complex for degradation. Dissociation of the prodomain following cleavage at the S2 site would thus enable free mature BMP-4 to bypass the lysosome and be secreted. We detect

minimal amounts of mature BMP-4 at the oocytes surface from either wild type or cleavage mutant forms of proBMP-4, which is consistent with this possibility. In this way, when cleavage at the S2 site is blocked the ligand is directly targeted for degradation and only minimal amounts are secreted at the cell surface and into the extracellular space. Limiting the release of mature BMP-4, then, limits the range and intensity of signaling. More commonly, ligands are secreted, bind their cognate transmembrane receptors and are sorted to either recycling or lysosomal compartments following endocytosis (Seto et al., 2002). Both pathways require ubiquitination of the cytoplasmic tail of transmembrane proteins as well as protein sorting complexes that specifically recognize the ubiquitinated cargo (Babst, 2004).

Targeted degradation of BMP-4 may be mediated by binding to its receptor since BMPs are known to undergo receptor mediated endocytosis and degradation (Jortikka et al., 1997). Furthermore, the Smurf family of ubiquitin-protein ligases has been shown to be recruited to the cytoplasmic tail of activated BMP and TGF-β receptors, thereby targeting them for degradation in a proteosome and lysosome dependent manner (Kavsak et al., 2000; Murakami et al., 2003; Podos et al., 2001). Our data are consistent with the possibility that the prodomain of BMP-4 enhances the affinity of the ligand for its receptor such that both are targeted for lysosomal degradation. By contrast, mature BMP-4 that is released from the prodomain following cleavage at the S2 site may more readily dissociate from the receptor, enabling it to bypass the lysosome. An analogous situation exists in the case of EGF and TGFα, which differ in their ability to target a common receptor for degradation. EGF remains bound to the receptor following internalization,

and is proteolytically degraded in the lysosome, whereas TGFα rapidly dissociates, resulting in recycling of the receptor to the cell surface (Sorkin and Waters, 1993).

Recent studies have revealed a role for receptor-mediated endocytosis and lysosomal degradation in shaping the BMP/Dpp concentration gradient in flies (Entchev et al., 2000; Gonzalez-Gaitan, 2003). These studies suggest that Dpp is transported across the embryonic wing disc by consecutive rounds of endocytosis, active intracellular transport and exocytosis. When clones of cells are generated that are mutant for either the Dpp receptor or for components of the endocytic machinery, Dpp is not internalized and cannot spread across the patch of endocytosis-defective cells. Overexpression of Rab5, which promotes early endocytic trafficking, broadens the Dpp gradient whereas overexpression of a constitutively active form of Rab7, which enhances trafficking from endosomes to the lysosome, reduces the spread of Dpp. A requirement for endocytosis in ligand transport is highly controversial, however, and conflicting evidence suggests that the extracellular Dpp gradient forms instead by free diffusion (Lander et al., 2002). Regardless of whether Dpp moves through the intracellular or extracellular space, the role of lysosomal degradation in shaping the gradient of Dpp and other morphogens is well established (Seto et al., 2002). Our results are consistent with a model in which cleavage at the S2 site occurs in selected tissues, such as the wing disc, preferentially directing the ligand away from the lysosome and enabling a long-range concentration gradient to form. In other tissues, where cleavage at the S2 site does not occur, the ligand would be sorted to the lysosome and signal only at short range.

Role of HSPGs in targeting BMP-4 for degradation

Our data showing that deletion of the heparin-binding motif in BMP-4 stabilizes the mature ligand raise the possibility that transmembrane HSPGs contribute to internalization and/or lysosomal targeting of mature BMP-4. Consistent with this possibility, binding to HSPGs has been shown to accelerate endocytosis and lysosomal degradation of Activin A (Hashimoto et al., 1997). Furthermore, addition of excess free heparin to the outside of cultured cells inhibits association of exogenous BMP-2 with its signaling receptor and suppresses intracellular accumulation of this ligand (Takada et al., 2003). These data suggest that HSPGs promote receptor binding and internalization of BMPs. In *Drosophila*, mutations in HSPG core proteins or in the enzymes responsible for HSPG biosynthesis lead to a reduction in Dpp signaling and loss of cell surface Dpp protein, suggesting that HSPGs sequester and concentrate Dpp at the cell surface (Tabata and Takei, 2004). Cells mutant for HSPG core proteins also show an inability to downregulate Dpp signaling, however, consistent with an additional role in clearing Dpp from the system (Fujise et al., 2003). Future studies will be required to determine whether HSPGs play a general role in promoting BMP-4 degradation or whether they contribute to preferential targeting of S1 cleaved BMP-4 to the lysosome.

MATERIAL AND METHODS

cDNA constructs

The FLAG epitope tag in the prodomain of wild type and mutant forms of proBMP-4FLAG-Myc (Cui et al., 2001) was replaced with an HA epitope tag using PCR.

Mutation of the P6 histidine at the S2 site, and deletion of the three amino acid HSPG binding motif in the mature domain of BMP-4 was accomplished using the PCR-based splicing by overlap extension technique (Horton et al., 1990). Regions of cDNAs generated by PCR were sequenced.

Oocyte isolation and analysis of proteins

Ovaries were surgically removed from mature female frogs and oocytes dissociated with 0.2U/ml Liberase Blendzyme 3 (Roche) in OR-2. Stage VI oocytes were cultured overnight at 18^0 C in ND-96 (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, pH 7.5) with 1.5% horse serum and 50μg/ml gentamycin. Oocytes were injected with *in vitro* synthesized RNAs (Moon and Christian, 1989) together with 700nCi [^{35}S]Met/Cys. For pulse-chase analysis, 5mM Met/Cys was added to the culture media to initiate the chase. After further culture, 6–10 oocytes were harvested and proteins immunoprecipitated from clarified lysates (Xiong et al., 1997) or culture media by incubation overnight at 4^0C with antibodies specific for HA (12CA5) or myc (9E10) epitope tags and Protein A Sepharose 4B beads. Beads were washed, boiled in 0.5% SDS with or without 1% β-mercaptoethanol, and immunoprecipitated proteins were treated with or without Endo H or PNGase F. Radiolabeled proteins were separated by electrophoresis on 11.5% polyacrylamide gels (SDS-PAGE), fixed in 7.5% acetic acid/15% methanol, enhanced using Amplify (Amersham) and visualized by autoradiography.

Inhibition of lysosomal or proteosomal function

Ooyctes were co-injected with 5ng RNA and 700nCi [^{35}S]Met/Cys, cultured for 16 hours, washed and incubated for 3 hours in the absence or presence of chloroquine. For proteosomal inhibition, oocytes were co-injected with 2ng RNA and 700nCi [^{35}S]Met/Cys, labeled for 3 hours, transferred to media containing 5mM cold methionine/cysteine for 12 hours and then incubated in the absence or presence of 10μM epoximicin or10μM lactacystin for four hours. Mature BMP-4 was immunoprecipitated from the culture media using myc-specific antibodies as above.

Co-immunoprecipitation and Western blot analysis

Oocytes were injected with 5ng synthetic RNA and cultured overnight before resuspending in 30 ul HB (Xiong et al., 1997) with 10mM PMSF and lysing in 600μl NETT (20mM Tris, pH 8.0, 100mM NaCl, 1mM EDTA, 0.2% Triton X-100) for 1 hour on ice. Proteins were precipitated from one third of the culture media using 10% tricholoracetic acid (TCA), washed once with acetone, once with ethanol, and resuspended in NETT. BMP-4 was immunoprecipitated from clarified lysates or media by overnight incubation with myc-conjugated beads (Eimon and Harland, 2002). Conjugates were washed 3 times in NETT and twice in PBS. Proteins were deglycosylated with PNGase F, separated by SDS-PAGE and transferred onto PVDF membrane, which was probed with HA- or myc-specific antibodies in 5% milk/TBS-T (10mM Tris, pH 8.0, 150mM NaCl, 0.1% Tween-20). Immunoreactive proteins were detected using chemiluminescence.

In vitro digestion with furin

Radiolabeled precursor proteins were generated by co-injecting oocytes with 5ng of RNA encoding wild type or mutant proBMP-4, 700 nCi [^{35}S]Met/Cys, and 0.5ng of RNA encoding the furin inhibitor, α_1-PDX (Anderson et al., 1993). Precursor proteins were immunoprecipitated from oocyte lysates 16 hours post-injection using myc-specific antibodies, and 5000 cpm of each precursor was resuspended in furin digestion buffer (Anderson et al., 1997) adjusted to the appropriate pH with HEPES. 120nM recombinant furin (Jean et al., 1998) was added to start the reaction. Aliquots were removed at various time intervals and added directly to denaturation buffer (0.5% SDS, 1% β-mercaptoethanol), boiled, and deglycosylated with PNGase F prior to electrophoresis.

To determine relative rates of cleavage, [^{35}S]Met/Cys-labeled proBMP-4 was synthesized in rabbit reticulocyte lysates and immunoprecipitated using myc-specific antibodies. Protein concentration was determined from incorporated cpms, using an input of 0.91μM labeled methionine and assuming 5μM methionine in the reticulocyte lysates. ProBMP-4 [0.8nM to 20nM] was incubated with 2.2nM recombinant furin at pH 7.0, 6.5, and 6.0 and aliquots removed at various intervals and analyzed by SDS-PAGE and autoradiography. Cleavage products were visualized using a Molecular Dynamics phosphorimager and band intensities quantified using the IP lab gel program. Cleavage is ordered and therefore the reaction proceeds as:

$$\text{Precursor} \xrightarrow[S_1]{k_1} \text{prolinker} + \text{mature} \xrightarrow[S_2]{k_2} \text{pro} + \text{linker} + \text{mature}$$

where the linker is the 35 amino acid fragment between the S1 and S2 sites and pro is the N-terminal prodomain fragment lacking the linker. The following assumptions are made

for burst rate kinetics: 1) each step is irreversible; 2) $k_1 > k_2$; 3) initially, [prolinker] = [mature] = [i – precursor] where i is the initial concentration at t_0; 4) at later times [mature] = [prolinker] + [pro]; and 5] [pro] = [linker]. The concentration of S1 and S2 cleaved prodomain was calculated using the equation:

[S1] cleaved = [prolinker] + [pro]; [S2] cleaved = [pro]

$V_{0[S1]}$ and $V_{0[S2]}$ were obtained from Michaelis-Menton plots of [S1]/min and [S2]/min, respectively. Data was plotted and analyzed using Kalidograph to obtain relative rates.

Fluorogenic peptide analysis

IQFS were kindly generated by Hans Peter Bachlinger (Shriners Hospital, Portland, OR). The reaction rate for cleaving S1 or S2 at specific pH was determined by monitoring the increase in fluorescence over real time using a Shimadzu UV 1601 UV-Vis spectrophotometer. The resulting curve was fit to a single-exponential rise to maximum function and 1/2 V_{max} calculated using Prizma graph. The reaction rate was determined by adding 380pM furin to 2.5uM IQF peptide substrate in 100mM HEPES, 0.5% Triton X-100, 1mM CaCl2, with continuous mixing. Cleavage was monitored at excitation/emission wavelengths of 320/400 nm and the resulting curve was fit the plot:

Y=B[1]*(G1/B[2])/(1+G1/B[2]) where G1= 2.5uM peptide

Cell surface trypsinization

Ooyctes were co-injected with 5 ng RNA encoding proBMP-4 or proBMP-4(mS2G) and 700nCi [^{35}S]Met/Cys and allowed to express proteins overnight. Media was collected and immunoprecipitated with myc-specific antibodies. Oocytes were washed two times

in 1x-ND96, and equal numbers of oocytes were either lysed (as above) and immunoprecipitated with myc-specific antibodies or placed directly in 100ul 1x PBS. Trypsin (100ug/ml) was or was not added to expressing oocytes, and these were incubated 20 minutes on ice or at room temperature. Trypsin was inactivated by addition of 500ug/ml soybean trypsin inhibitor for 10 minutes prior to lysis and BMP-4 was immunoprecipitated with myc-specific antibodies. Control immunoprecipitated media and lysates were subjected to trypsinization as above. Samples were run on SDS-PAGE.

Blocking secretion with Bafilomycin A_1

Oocytes were pre-treated 4-hours with or without 5uM or 10uM bafilomycin A_1 as described (Yamaguchi et al., 1998), then injected with 5ng RNA encoding proBMP-4 and 700nCi [^{35}S]Met/Cys and cultured for a further 8 hours in the continued presence of absence of bafilomycin A_1. Oocytes and media were collected and proteins were immunoprecipitated using myc- or HA-specific antibodies.

In vivo cleavage assay

Synthetic phosphorothioate-modified antisense oligonucleotides specific for furin, PACE4, and PC6 were purchased from Invitrogen and tested for specific depletion of target RNAs by Northern blot (Heasman, 2002). Oocytes were injected with 10ng of oligo specific for each PC alone or in combination, as indicated. A subset of oocytes were harvested 1 hour post injection and analyzed for depletion of target RNA by Northern blot as described (Christian et al., 1990). 72 hours post-injection oocytes were co-injected with 2ng RNA encoding proBMP-4 and 700nCi [^{35}S]Met/Cys. Proteins were

allowed to express and process for 8 hours. Oocytes were collected, lysed, and proteins precipitated using HA-specific antibodies as above. Proteins were deglycosylated and run on SDS-PAGE.

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FIGURE LEGENDS

Figure 1. Failure to cleave proBMP-4 at the S2 site targets mature BMP-4 for degradation. **(A)** Schematic illustration of myc- and HA-epitope tagged wild type and cleavage mutant forms of proBMP-4. **(B-D)** RNA encoding wild type or cleavage mutant proBMP-4 was injected into oocytes together with [^{35}S]Met/Cys and cleavage products analyzed by SDS-PAGE. The position of precursor, S1 or S2 cleaved prodomain and mature BMP-4 is illustrated schematically to the right of each gel. **(B)** Oocyte lysates were immunoprecipitated with a myc-specific antibody and duplicate samples treated with or without Endo H or PNGase F prior to SDS-PAGE under reducing or non-reducing conditions as indicated. Bands corresponding to Endo H sensitive (asterisks) and Endo H resistant (arrowheads) mature BMP-4 or Endo H resistant/PNGase F resistant proBMP-4 dimers (arrows) are indicated. **(C-D)** Oocytes were pulse labeled for three hours and then transferred to media containing cold methionine and cysteine. At increasing time intervals, lysates and conditioned media were subjected to immunoprecipitation using antibodies specific for the HA- **(C)** or myc- **(D)** epitope and cleavage products analyzed by SDS-PAGE under reducing **(C)** or non-reducing **(D)** conditions.

Figure 2. Degradation of BMP-4 requires lysosomal and proteosomal function. RNA encoding wild type or cleavage mutant proBMP-4 was injected into oocytes together with [^{35}S]Met/Cys. Oocytes were cultured in the presence or absence of a lysosomal

[chloroquine (chloroq)] or proteosomal (epoximicin or lactacystin) inhibitor for varying amounts of time. Mature BMP-4 was immunoprecipitated from the media using myc-specific antibodies and analyzed by SDS-PAGE and autoradiography.

Figure 3. Cleavage at the S2 site disrupts noncovalent association between the S1-cleaved prodomain and mature BMP-4. Epitope tagged wild type or S2 cleavage mutant proBMP-4 was expressed in *Xenopus* oocytes. Cleavage products present in lysates or media were analyzed by probing immunoblots (IB) of total protein or α-myc immunoprecipitates (IP myc) with antibodies specific for HA or myc epitopes as indicated. The position of S1 or S2 cleaved prodomain and mature BMP-4 is illustrated schematically to the right.

Figure 4. Cleavage at the S2, but not the S1 site is enhanced at acidic pH. Radiolabeled wild type (**A**) or cleavage mutant (**C-E**) forms of proBMP-4 (illustrated schematically above figure) were incubated with recombinant furin at neutral or acidic pH. Aliquots were removed and analyzed by SDS-PAGE at the indicated times. The position of precursor, S1 and S2 cleaved prodomain is illustrated schematically to the right of gels in C-E and is marked by arrowheads in A. (**B**) ProBMP-4 was synthesized *in vitro* and cleaved by recombinant furin over a range of concentrations and pH to generate relative rates of cleavage (arbitrary units) for the S1 (white bars) and S2 (black bars) sites.

Figure 5. Fluorogenic peptides do not mimic in vivo cleavage rates. (**A**) Sequence of fluorogenic peptides containing an N-terminal aminobenzoic acid group (AB_z) and C-terminal nitro-tyrosine group [tyr(NO_2)]. (**B-C**) Continuous absorbance for cleavage of S1-(**B**) or S2-(**C**) peptides. $1/2V_{max}$ was calculated from Graphpad Prism using a single-exponential fit curve.

Figure 6. A conserved P6 histidine at the S2 site inhibits cleavage at neutral pH. Radiolabeled wild type (**A**) or P6 histidine mutant (**B-C**) forms of proBMP-4 (illustrated schematically above each panel) were incubated with recombinant furin at neutral or acidic pH. Aliquots were removed and analyzed by SDS-PAGE at the indicated times. The position of precursor, S1 and S2 cleaved prodomain is illustrated schematically to the right of each gel.

Figure 7. proBMP-4 is cleaved intracellularly in *Xenopus* oocytes. (**A-C**) Oocytes were co-injected with 5ng RNA encoding wild type or cleavage mutant proBMP-4 and 700nCi [^{35}S]Met/Cys and cultured overnight. Mature BMP-4 was immunoprecipitated using myc-specific antibodies and evaluated on SDS-PAGE. (**A**) Oocytes were treated 20 minutes with or without 100ug/ml trypsin, at the indicated temperature, before inactivating with 500ug/ml trypsin inhibitor. (**B-C**). Oocyte media (**B**) or lysates (**C**) were first immunoprecipitated with myc-specific antibodies before treating with trypsin as described above.

Figure 8. Furin is necessary for cleavage of proBMP-4. **(A)** Schematic for experiment. **(B-C)** Oocytes were injected with 10ng of antisense oligonucleotde specific for furin, PC6 or PACE4, as indicated. **(B)** 10 injected or uninjected oocytes were harvested 1-hour post injection and expression of furin, PC6 or PACE4 was examined by northern blot analysis. **(C)** 72-hours after injection of antisense oligonucleotides, RNA [2ng] encoding proBMP-4 RNA and 700nCi [^{35}S]Met/Cys was injected. Oocytes were lysed 8-hours later, and proBMP-4 and cleaved prodomain was immunoprecipitated using HA-specific antibodies. Cleavage of the S1 and S2 sites was analyzed by SDS-PAGE.

Figure 9. Deletion of a heparin-binding motif in mature BMP-4 inhibits degradation. RNA encoding BMP-4 precursors containing **(A, C)** or lacking **(B, D)** the RKK motif that mediates binding to HSPGs (underlined in schematic diagram above figure) was injected into oocytes together with [^{35}S]Met/Cys. Oocytes were pulse labeled for three hours and then transferred to media containing cold methionine and cysteine. BMP-4 was immunoprecipitated from lysates and media at the indicated times post-chase using anti-myc antibodies. The position of mature BMP-4 dimers and proBMP-4 monomers or dimers is indicated to the right.

Figure 10. Model for regulation of BMP-4 activity by sequential cleavage.

See text for details.

Figure 1.

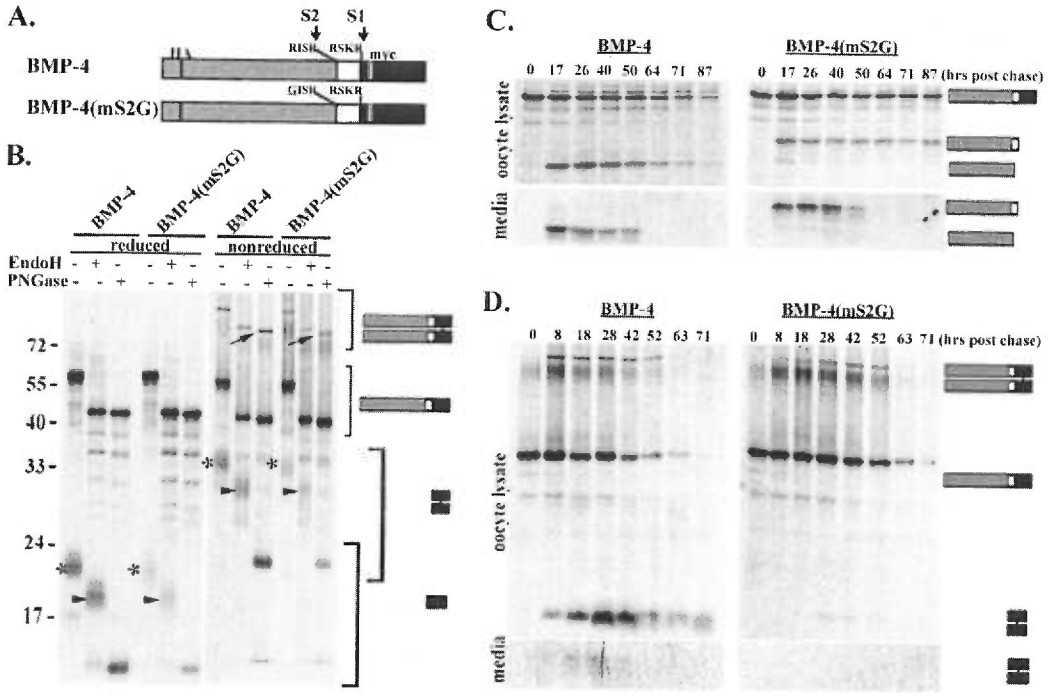


Figure 2.

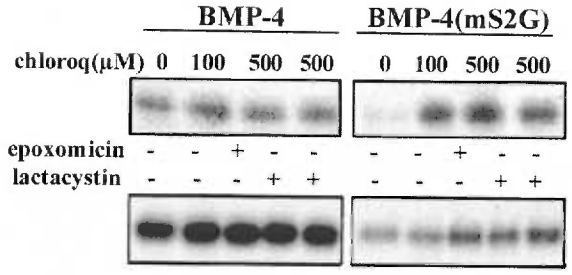


Figure 3.

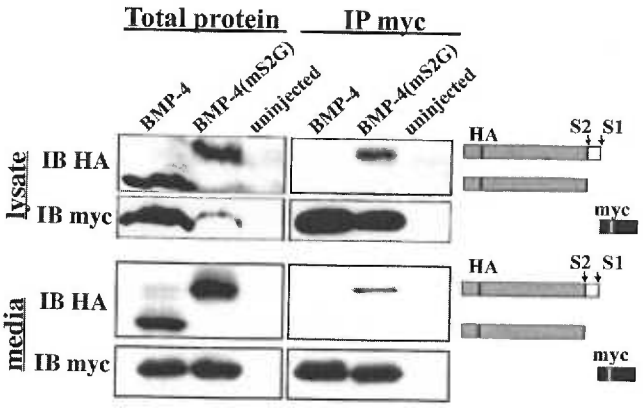


Figure 4.

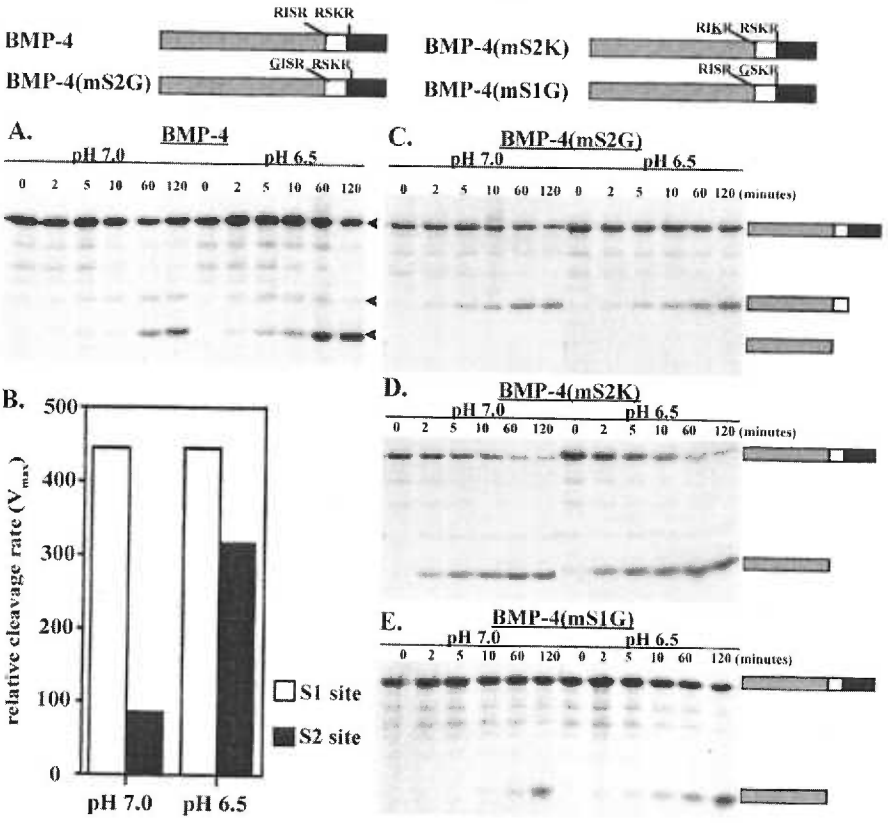


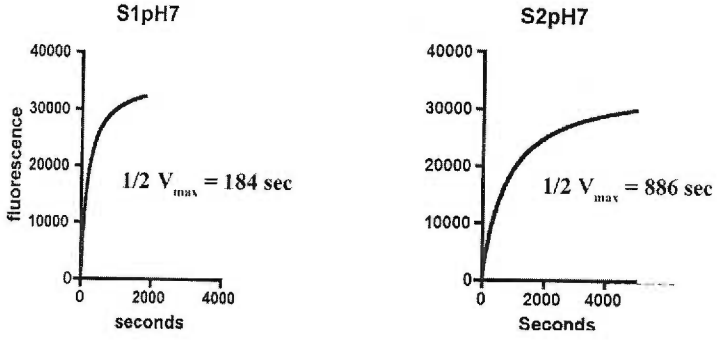
Figure 5

A.

S1: AB_z-R-G-H-A-L-T-R-**R**-S-**K**-**R** ↓ -S-P-K-Tyr(NO_2)-G

S2: AB_z-K-T-Y-Q-G-K-H-V-**R**-I-S-**R** -S-L-L-Tyr(NO_2)-G

B.



C.

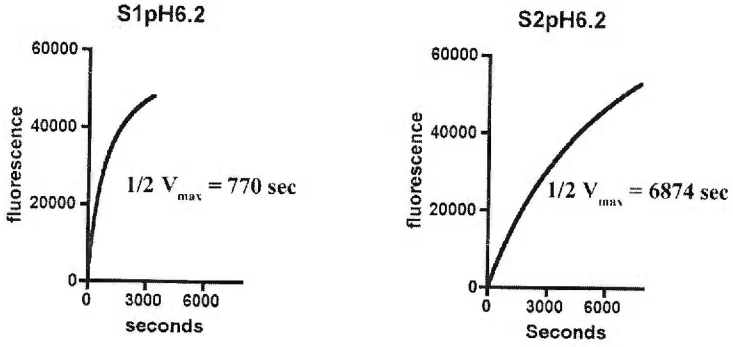


Figure 6

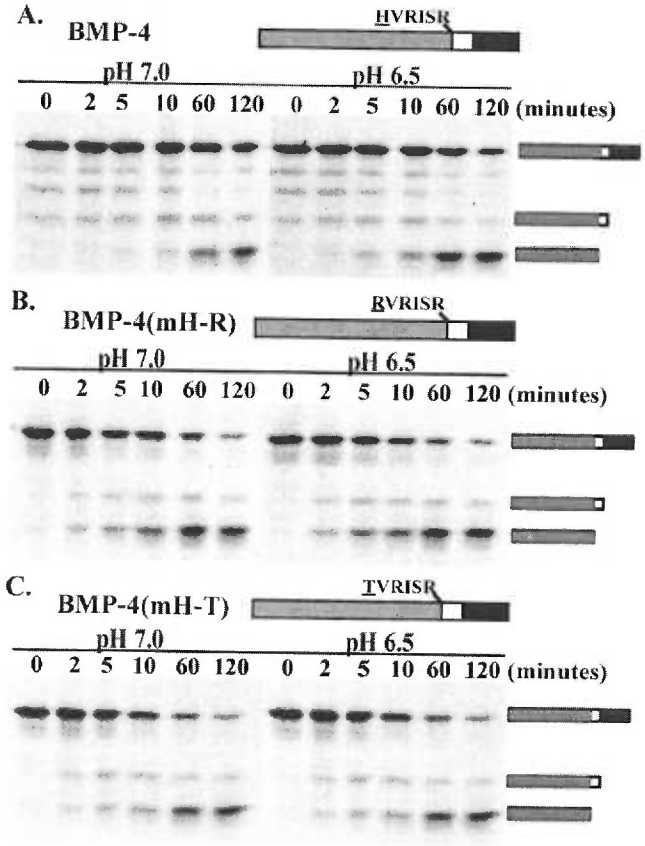

A.
BMP-4
HVRISR
pH 7.0
pH 6.5
0 2 5 10 60 120 0 2 5 10 60 120 (minutes)
B.
BMP-4(mH-R)
RVRISR
pH 7.0
pH 6.5
0 2 5 10 60 120 0 2 5 10 60 120 (minutes)
C.
BMP-4(mH-T)
TVRISR
pH 7.0
pH 6.5
0 2 5 10 60 120 0 2 5 10 60 120 (minutes)

Figure 7

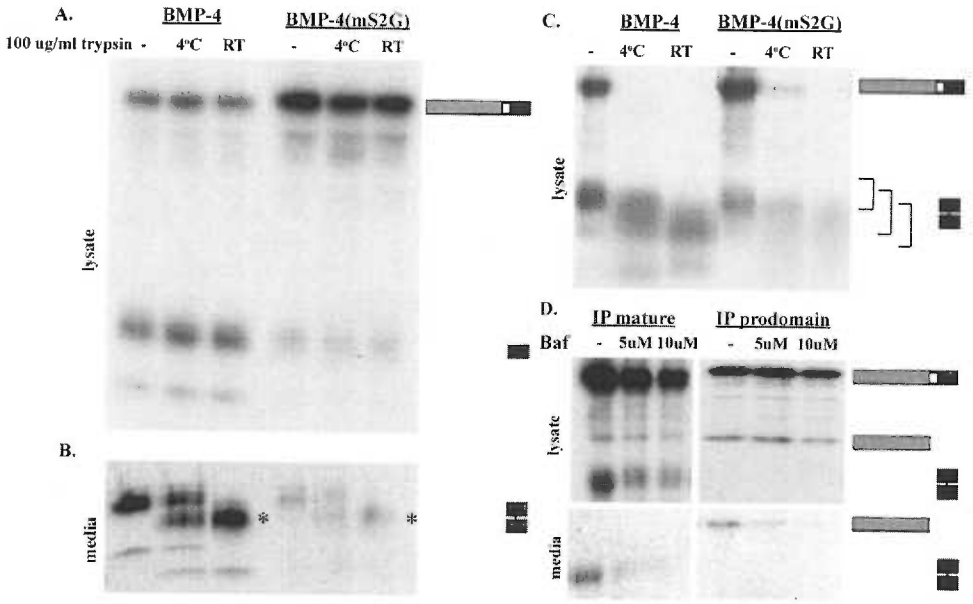


Figure 8:

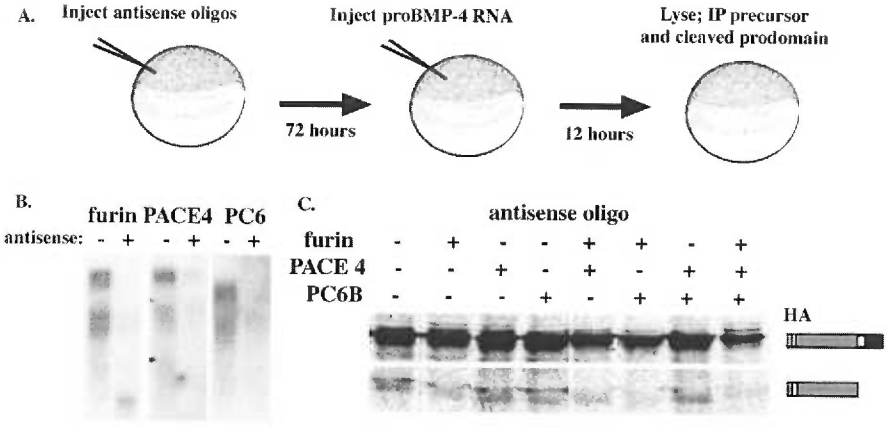


Figure 9.

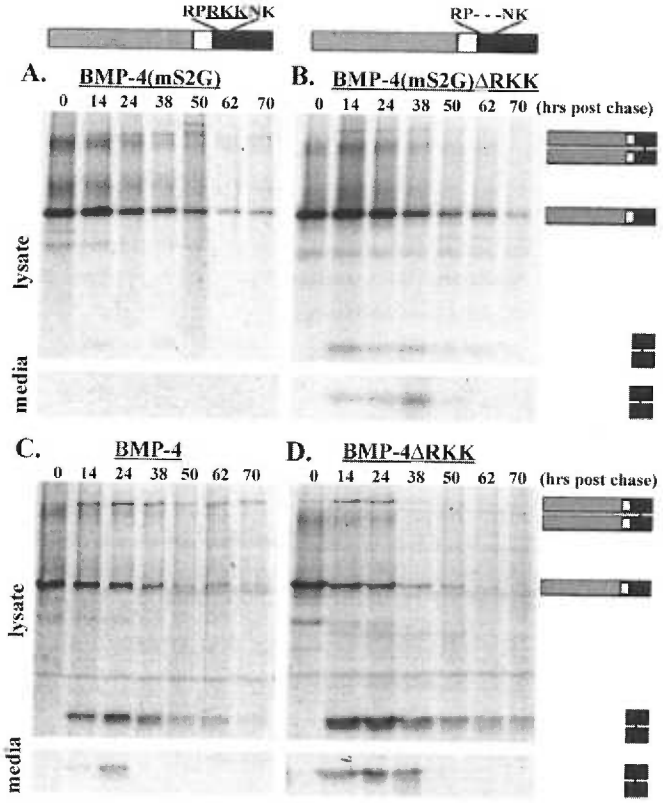
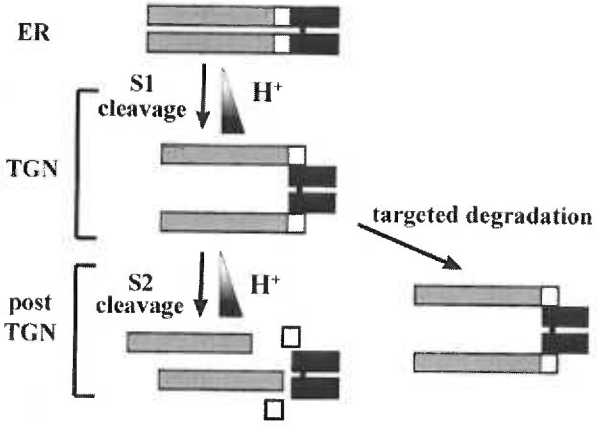


Figure 10.



Chapter 3

Ordered cleavage of proBMP-4 modulates bioactivity, as well as the levels of mature BMP-4 produced, and this may be mediated through sequences N-terminal to the mature domain

Catherine R. Degnin[1,2], Yanzhen Cui[1], and Jan L. Christian[1]

Department of Cell and Developmental Biology[1], Biochemistry and Molecular Biology[2]
Oregon Health and Science University, School of Medicine

In the following paper I was responsible for all the data except for that shown in Figure 2. These experiments were performed by Dr. Yanzhen Cui.

ABSTRACT

ProBMP-4 undergoes ordered cleavage where cleavage occurs first at the S1 site, breaking the covalent linkage between the prodomain and the mature ligand, while allowing for continued, noncovalent, association of these two domains. This is followed by cleavage at the S2 site, within the prodomain, which disrupts the noncovalent association between the prodomain and the mature ligand, and thus protects mature BMP-4 from degradation. To further characterize the role for S2 cleavage, we have begun to study a series of cleavage mutants: one that cleaves only at the S2 site [BMP-4(mS1G)], a second, where the sequence separating the two cleavage sites is removed but the S1 site is retained [BMP-4(ΔS2L)], and a third which has enhanced cleavage at the S2 site [BMP-4(mS2K)]. We find that blocking cleavage at the S1 site still allows for cleavage at the S2 site, but generates a stable, N-terminally extended form of mature BMP-4. We find that the region separating the two cleavage sites is required for proper folding of proBMP-4, and that enhancing cleavage at the S2 site does not grossly affect the stability of mature BMP-4.

INTRODUCTION

Bone morphogenetic protein-4 (BMP-4) is a signaling molecule that acts as a morphogen to influence cell fates in a concentration dependent manner. BMPs, originally isolated from bone extracts, were named for their ability to induce ectopic bone formation, although they have since been shown to play multiple roles in embryonic development and in adult homeostasis (Hogan, 1996). BMP-4 acts at multiple stages of development in a dose-dependent fashion, and hence BMP-4 activity and signaling range is very tightly regulated.

Ordered cleavage of proBMP-4 at two multibasic furin motifs regulates the activity and signaling range of mature BMP-4 (Cui et al., 2001). Cleavage occurs first at an optimal furin motif (RSKR) adjacent to the mature domain (S1 site), and this is followed by cleavage at a minimal furin motif (RISR) within the prodomain (S2 site). Our previous studies show that the first cleavage releases mature BMP-4, but, if the S2 site is not cleaved, this ligand is rapidly degraded (Degnin et al, in press) and signals only at short range. Second site cleavage generates a more active, stable ligand that signals at long range. Our recent studies show that the pro/mature domains remain noncovalently attached following cleavage at the S1 site and that association with the prodomain targets the entire pro/mature complex for rapid degradation (Degnin et al, in press). Cleavage at the S2 site disrupts this interaction, releasing the mature domain, which enhances its stability and promotes longer range signaling. In contrast to these studies where cleavage at the S2 site regulates the stability of mature BMP-4, a precursor in which the S2 site is

mutated to an optimal furin consensus motif (-R-S-K-R) generates mature BMP-4 with enhanced activity and signaling range, although the steady state protein levels are unchanged (Cui et al., 2001).

BMP-4 and its closest homologue, BMP-2, share significant sequence identity within their mature domains as well as N-terminal to the identified cleavage site, extending to a conserved minimal furin consensus motif (-R-I-S-R-) (Wozney et al., 1988). Thus, BMP-2 is likely to be cleaved in a sequential fashion, similar to BMP-4, although this has yet to be demonstrated. Previous studies have shown that mutation of the optimal furin consensus site (S1) to a non-consensus site in BMP-4 (Hawley et al., 1995) or BMP-2 (Suzuki et al., 1997b) generates a dominant-negative cleavage mutant (cm) form of the precursor that is able to inhibit signaling by endogenous BMP-4 and BMP-2. In both studies, the S1 site was mutated from an optimal consensus sequence (-R-X-K-R-) to a non-cleavable motif (-G-V-D-G- or -G-Q-Q-G-) and then assayed in *Xenopus* embryos for their ability to either induce expression of BMP target genes or to block function of endogenous BMPs. BMP-4 functions in ventral regions of the embryo to specify epidermal fates, while it is inhibited in dorsal regions, to allow for neural specification (Hawley et al., 1995). Loss-of-BMP-function, by the use of a truncated receptor or inhibition of downstream signaling events, causes dorsalization of the embryo and expanded neural induction (Frisch and Wright, 1998). Consistent with these roles of BMP-4, ectopic expression of wild type proBMP-2 or proBMP-4 in *Xenopus* embryos induced globin and Xbra expression, whereas expression of the cm-proproteins failed to induce these markers. Likewise, expression of wild type proBMP-2 or proBMP-4 failed to induce neural or cement gland (XAG1) markers, while the corresponding cm-

proproteins activated these transcripts as effectively as a dominant-negative form of the BMP receptor, tBR (Graff et al., 1994). In addition, cmBMP-2 was able to induce a secondary axis and could revert the ventralization of embryos co-injected with wild type proBMP-2, suggesting that cmBMP is able to block signaling from either endogenous or ectopically expressed forms of proBMP. The cleavage mutant forms of proBMP-4/-2 are hypothesized to block BMP activity by forming dimers with wild type proBMP-4/-2, thereby preventing proper processing and secretion of wild type proteins (Hawley et al., 1995; Suzuki et al., 1997b), as has been shown for BMP-7 (Suzuki et al., 1997b) and activin (Wittbrodt and Rosa, 1994). Consistent with this, Hawley et al (Hawley et al., 1995) were unable to detect cleaved prodomain in oocytes made to express cmBMP-4. Others, however, have reported that the single, S1 site cleavage mutants retain weak ventralizing activity and mutation of both sites is required to completely abolish activity (Nishimatsu and Thomsen, 1998).

Ordered cleavage of proBMP-4 results in the proteolytic release of a 35-amino acid 'linker' domain separating the two cleavage sites. This region is evolutionarily conserved between BMP-4 and BMP-2 homologs and with the *Drosophila* ortholog, Decapentaplegic, Dpp (Cui et al., 2001), but its function is unknown. What is not known is whether sequential cleavage of proBMP-4 at two sites functions strictly to promote release of its prodomain (Degnin et al, in press), or whether the linker domain generated by second site cleavage plays additional roles in regulating BMP-4 activity. To further characterize the roles for the linker domain and S2 cleavage we have begun to study a series of cleavage mutants: one that cleaves only at the S2 site [BMP-4(mS1G)], a second, where the linker domain separating the two cleavage sites is removed but the S1

site is retained [BMP-4(ΔS2L)], and a third in which cleavage at the S2 site is optimized [BMP-4(mS2K)].

RESULTS

Preventing cleavage of the S1 site does not block in vivo cleavage at the S2 site.

Our previous studies showed that the S2 site is cleaved, *in vitro*, in a pH-dependent fashion, and that cleavage of the S2 site is independent of S1 cleavage (Degnin et al, in press). This suggested that reduced pH is sufficient for S2 site cleavage *in vitro*. Since blocking cleavage of the S1 site is reported to generate a dominant-negative cleavage mutant form of BMP-4 (Hawley et al., 1995), we hypothesized that *in vivo*, cleavage at the S1 site is necessary for trafficking proBMP-4 to an appropriate post-TGN compartment where the S2 site can then be cleaved. Mutation of the S1 site should, therefore, prevent cleavage of proBMP-4.

To begin to examine the role of S1 cleavage, we characterized a mutant form of BMP-4, BMP-4(mS1G), in which the S1 site cannot be cleaved (schematic illustration in Figure 1A). We first asked whether mutation of the S1 site prevented proper folding of the precursor and exit from the ER by assaying whether the S1-cleavage mutant precursor was dimerized and present in post ER-compartments. RNAs (5 ng) encoding HA- and myc- tagged wild type or S1-cleavage mutant forms of proBMP-4 were injected into *Xenopus* oocytes together with [^{35}S]Met/Cys, and oocytes were cultured 20-hours to label newly synthesized proteins. Precursor and mature BMP-4 were immunoprecipitated from oocyte lysates using antibodies specific for the myc-tag, and then treated with or without deglycosylating agents. Proteins within the ER are sensitive to digestion with

Endoglycosidase H (Endo H), but become Endo H resistant when further modified in the Golgi network. Conversely, all N-linked glycosylated proteins are susceptible to deglycosylation with Peptide N-Glycosidase F (PNGase F). Therefore, we used Endo H resistance/PNGase F sensitivity as a hallmark of proteins that are properly folded and able to traffic from the ER. As shown in Figure 1B (top panel), Endo H sensitive (arrow) and Endo H resistant/PNGase F sensitive (arrowheads) forms of proBMP-4 and proBMP-4(mS1G) dimers were detected in oocyte lysates and this suggests that blocking cleavage at the S1 site does not prevent the precursor from dimerizing or trafficking out of the ER. Since previous studies suggested that blocking cleavage at the S1 site prevented cleavage of proBMP-4, we predicted that proBMP-4(mS1G) would not be cleaved at the S2 site. Contrary to this expectation, we observed two-myc-immunoreactive bands corresponding to mature BMP-4 in lysates of oocytes made to express BMP4(mS1G). The two bands do not result from differential N-glycosylation since they are present even following Endo H and PNGase F treatment (asterisks). The relative molecular mobility (Mr) of mature BMP-4 dimers generated by cleavage at the S2 site alone is predicted to be ~6kDa larger than that generated by cleavage at both sites, therefore we predict that the lower molecular weight band may correspond to linker-attached mature BMP-4. The additional myc-reactive mature BMP-4 band may result from 1) cryptic activation of an upstream cleavage site, which should also generate an additional prodomain species, 2) tight noncovalent association with an unrelated band, mediated through the attached linker domain, 3) novel covalent modification(s) within the linker domain, or 4) changes in the apparent Mr resulting from alternate disulfide bond arrangements, and these may be resolved under reducing conditions. To begin to distinguish between these possibilities

we examined prodomain fragments generated by cleavage of proBMP-4(mS1G) to ask whether an additional cleavage site is activated when the S1 site is not cleaved. Duplicate samples were immunoprecipitated using HA-specific antibodies and subjected to deglycosylation as above. Following deglycosylation, a single band corresponding to the S2-cleaved prodomain was observed in lysates of oocytes made to express the wild type or S1-cleavage mutant precursor (Figure 1B, bottom panel, arrow). Thus, the additional Mr species of mature BMP-4 generated from proBMP-4(mS1G) is not generated by cleavage at an upstream site within the prodomain. Therefore, these data show that mutation of the S1 site not only allows proBMP-4 to fold, dimerize and exit the ER, but also allows the S2 site to be cleaved, *in vivo*, to produce an altered form(s) of mature BMP-4. These results were reproducibly obtained in 2 of 2 experiments.

Pulse chase analysis was used to determine whether blocking cleavage at the S1 site influences the rate of cleavage and/or the stability of the cleavage products from proBMP-4(mS1G). Oocytes were injected with RNA (0.45 ng) encoding wild type or S1 cleavage mutant forms of BMP-4 together with [^{35}S]Met/Cys. After 3-hours label was chased by incubation in media containing unlabeled methionine and cysteine. HA-tagged BMP-4 precursor and cleaved prodomain peptides were immunoprecipitated from oocyte lysates or media at increasing time intervals using antibodies specific for the HA-tag and then separated on reducing gels. As in Figure 1B, a single 31kDa protein band corresponding to prodomain cleaved at both the S1 and S2 sites (BMP-4) or the S2 site only [BMP-4(mS1G)] was observed in oocyte lysates and culture media (Figure 1C). Prodomain fragments accumulated at comparable rates, although more prodomain was released into the culture media when the S1 site was not cleaved. Thus, the inability to

was chased using unlabeled methionine/cysteine and oocytes were collected at various timepoints. Myc-tagged BMP-4 precursor and mature domains were immunoprecipitated from oocyte lysates using myc-specific antibodies and then separated by SDS-PAGE under non-reducing (Figure 1E, left) and reducing (right) conditions. Mature-plus-linker BMP-4 derived from proBMP-4(mS1G) generated two distinct bands under non-reducing conditions (arrows) and these appeared to persist under reducing conditions (arrowheads). These data support the hypothesis that two distinct species of mature-plus-linker are generated in *Xenopus* oocytes. In addition, the slower Mr protein is expressed prior to the faster Mr protein species, consistent with the possibility that the slower Mr protein may convert into the faster Mr species. By contrast, when deglycosyated samples were run under non-reducing and reducing conditions the two bands detected under non-reducing conditions appeared to resolve into a single band, although the faster Mr species may be migrating along the dye front under the given gel conditions and thus is not detected (data not shown). Therefore, these data do not clearly rule out the possibility that the two form of mature-plus-linker represent distinct conformations of the same protein.

Having demonstrated that proBMP-4(mS1G) is cleaved *in vivo*, we next asked whether the ligand(s) generated from such cleavage retain bioactivity. In a single experiment, 2-cell *Xenopus* embryos were injected with RNA encoding wild type or S1G mutant precursor, and phenotypes were scored. Embryos injected with wild type proBMP-4 RNA were ventralized; but surprisingly, embryos injected with proBMP-4(mS1G) RNA were also ventralized, indicating that active ligand was produced (Christian, data not shown).

The linker domain is required for BMP-4 activity

The linker domains of BMP-4 and BMP-2 are highly conserved between species (Figure 2A). To determine whether the linker domain is required for protein function, a deletion mutant form of proBMP-4, lacking the 35-amino acids comprising the linker domain as well as the S2 site, was generated (Figure 2B). This precursor, proBMP-4(ΔS2L), contains an optimal S1 site (RSKR), which, if cleaved, produces wild type mature BMP-4. To test whether deletion of the linker domain affects the biological activity of BMP-4, a previous post-doctoral fellow in the lab, Yanzhen Cui, injected 2-cell *Xenopus* embryos with RNA (100pg) encoding proBMP-4 or proBMP-4(ΔS2L). Embryos were cultured to stage 8 (blastula), at which point the ectodermal layer (animal cap) was removed, and then further cultured until sibling embryos reached stage 10.5 (Figure 2C). mRNA isolated from animal caps and whole embryos was analyzed by RT-PCR for the mesoderm marker, *Xenopus* Brachyury (Xbra, a downstream BMP-4 target gene) and EF1α, which was used as a loading control. Xbra expression was detected in animal caps isolated from embryos expressing wild type proBMP-4 but not proBMP-4(ΔS2L) (Figure 2D, Cui and Christian, unpublished data).

Similar to the prodomains of other TGF-β family members, the prodomain of BMP-4 has been shown to act as a chaperone that can function *in trans* to direct proper folding, dimerization and processing of BMP-4 (Gray and Mason, 1990). Preliminary studies by Cui and Christian suggest that co-injection of mRNA expressing the intact pro-linker region can partially rescue Xbra induction in embryos expressing proBMP-4(ΔS2L) (data not shown). These data show that the linker peptide is required to generate

a bioactive BMP-4 signaling molecule and suggests that the linker may act *in trans*. Clean biochemical studies are needed to solidify these preliminary results.

The linker domain is required for dimerization and exit from the ER

Since the prodomain is likely to be required for folding of proBMP-4, it is possible that the linker region is essential for this function. If this is true, then deletion of the linker region should prevent proBMP-4 from exiting the ER and thus prevent formation of bioactive mature BMP-4. A second, equally plausible explanation for the inability of proBMP-4(ΔS2L) to activate BMP-4 signaling is that the linker domain may normally remain associated with mature BMP-4 following cleavage and may therefore be required to stabilize the protein. In this case, proBMP-4(ΔS2L) would be proteolytically cleaved, but mature BMP-4 would be rapidly degraded.

To begin to distinguish between these possibilities we first asked whether deletion of the linker region prevents cleavage of the S1 site *in vitro*. ProBMP-4(ΔS2L) was generated in reticulocyte lysates and then subjected to *in vitro* cleavage using recombinant furin. As shown in Figure 3A, proBMP-4 is cleaved at both the S1 and S2 sites, whereas proBMP-4(ΔS2L) is cleaved only at the S1 site. Cleavage of the linker mutant is efficient and independent of pH (data not shown), consistent with previous kinetic studies that characterized S1 site cleavage (Degnin et al, in press).

To determine whether deletion of the linker domain prevents proBMP-4 from folding properly and exiting the ER, we asked whether Endo H resistant/PNGase F sensitive forms of proBMP-4(ΔS2L) could be detected. RNAs (5 ng) encoding myc-tagged wild type or linker mutant proBMP-4 and [^{35}S]Met/Cys were injected into

oocytes. Precursor and mature BMP-4 was immunoprecipitated from lysates using antibodies against the myc-tag and treated with or without deglycosylating agents. In lysates of oocytes made to express native BMP-4, Endo H sensitive (Figure 3B, arrow) as well as Endo H resistant/PNGase F sensitive (arrowhead) forms of the dimerized precursor can be detected, indicative of proBMP-4 dimers trafficking through post-ER compartments. These proBMP-4 dimers migrate as a tight protein band, with an additional diffuse band that migrates slightly faster (asterisks). By contrast, in oocytes made to express proBMP-4(ΔS2L), the tight band is absent and only the diffuse band is observed. This protein is Endo H sensitive (arrow), but is resistant to further digestion by PNGase F (arrowhead), suggesting that this proprotein dimer does not exit the ER. Consistent with this possibility, mature BMP-4 is not detected in lysates from proBMP-4(ΔS2L) expressing oocytes. To further demonstrate that proBMP-4(ΔS2L) is not cleaved *in vivo*, we asked whether deletion of the linker domain permitted formation of a prodomain fragment. Oocyte lysates were immunoprecipitated with antibodies against the HA-tag and further treated with or without deglycosylating agents. The predicted S2-cleaved prodomain product from oocytes made to express proBMP-4 was detected following treatment with PNGase F, but not Endo H, indicating this protein was highly modified in post ER compartments (Figure 3B, bottom). By contrast, prodomain fragments were not detected in oocyte lysates made to express the linker mutant. Together these data suggest that the linker is required for proBMP-4 to properly fold and exit the ER.

These deglycosylation data are consistent with the possibility that correctly folded proBMP-4 dimers migrate as a tight, higher Mr band that is modified while trafficking

through the Golgi, and that incorrectly folded dimers migrate as a broad, lower Mr band consisting of protein dimers that do not exit the ER. These data are also consistent with the possibility that the higher Mr band results from the noncovalent association of a protein, most likely a chaperone that may be essential for BMP folding. If this were true, then the tightly migrating band would not be sensitive to reducing agents. Therefore, to distinguish between these possibilities we used a 2-D gel approach to separate proBMP-4 dimers from any large molecular weight associated proteins. Oocytes were injected with RNA (5 ng) encoding myc-tagged wild type and linker mutant forms of proBMP-4 and [^{35}S]Met/Cys, and lysates were collected 24-hours after injection. Precursor and mature BMP-4 were immunoprecipitated from lysates with antibodies against the myc-tag and deglycosylated with PNGase F. Immunoprecipitated proteins were run on non-reducing gels (Figure 3C, first dimension, non reduced; NR). Duplicate lanes were excised and soaked in β-mercaptoethanol before overlaying onto reducing gels (second dimension; reduced). In the second dimension, protein monomers will run along a diagonal path while any disulfide-linked multimers will migrate off the diagonal. Under non-reducing conditions (NR), proBMP-4 cleavage yielded mature BMP-4 dimers, as well as monomer (dot), dimer (arrow, asterisks), and aggregate (angled arrow) forms of proBMP-4. Duplicate lanes run under reducing conditions (Figure 3C, reduced) showed a clear diagonal, with mature BMP-4 (arrowhead) migrating off the diagonal (indicated by gray line). Reduced, immunoprecipitated proteins did not migrate with a greater Mr than proBMP-4 monomers, suggesting that proBMP-4 does not associate with a large molecular weight protein or chaperone under these conditions. Both the tight band (reduced, asterisks) and the broadly smeared band (reduced, arrow) reduced down to

monomers of similar Mr in the second dimension, consistent with the possibility that the diffuse band represented a misfolding and/or mismatching of cysteine residues involved in dimer formation. 2-D gel analysis of the linker mutant (Figure 3D) showed that, consistent with Figure 3B, mature BMP-4 is not produced, and there is a complete absence of the tight band representing properly folded dimerized precursor. The broad, diffuse dimer band (arrow; NR) migrated at the same Mr as precursor monomers under reducing conditions and showed multiple degradation products. Together, these data suggest the linker domain is required for proper folding of proBMP-4, as deletion of this region allows for dimerization but prevents export from the ER.

Enhanced biological activity of BMP-4(mS2K) is not due to increased expression of mature BMP-4

Previous work in *Xenopus* embryos showed that optimizing the furin cleavage motif at the S2 site [BMP-4(mS2K)] had no effect on the steady state levels of mature BMP-4, but rather enhanced the activity and signaling range of the processed product (Cui et al., 2001). One possible explanation for these results is that optimizing the S2 cleavage motif may enhance the rate of cleavage at the S2 site, allow BMP-4 to act earlier in embryogenesis. If this were true, then premature activation of mature BMP-4 in embryos by a single cell division could expand the range of tissue specified by BMP signaling. To test for this possibility, we compared the rate of cleavage of proBMP-4 and proBMP-4(mS2K) using pulse-chase analysis. *Xenopus* oocytes were injected with minimal amounts of RNA (0.45 ng) encoding HA- and myc-tagged wild type and S2K cleavage mutant forms of proBMP-4 and [^{35}S]Met/Cys. Ooyctes were labeled for three-

hours and then transferred to media containing methionine/cysteine. Lysates and media were collected at increasing time intervals and immunoprecipitated with antibodies against the HA-tag in the prodomain. An early (8-hour) time point was taken to determine the earliest point of prodomain generation. As illustrated in Figure 4A, prodomain isolated from oocytes made to express proBMP-4 was detected within 8-hours post-chase, peaked by 28-hours, and later became undetectable. When cleavage at the S2 site was optimized, prodomain was generated and disappeared at a rate comparable rate to wild type cleavage, although less prodomain was reproducibly observed in 2 of 2 experiments. No prodomain was detected in the culture media of oocytes made to express wild type or mutant precursor. These data suggest that when cleavage at the S2 site is optimized, *in vivo,* the prodomain may be degraded more rapidly than from wild type cleavage.

To determine the effect enhanced prodomain-turnover may have on mature BMP-4, duplicate lysates and media were immunoprecipitated with antibodies against the myc-tag and analyzed on non-reducing gels. As shown in Figure 4B, equivalent levels of wild type and optimized S2 cleavage forms of proBMP-4 were synthesized, dimerized, and cleaved, although slightly less mature BMP-4 accumulated when cleavage of the S2 site was optimized. Similarly, mature BMP-4 was barely detected in the culture media when cleavage of the S2 site was optimized and this suggests that long range signaling is not a result of enhanced secretion of mature BMP-4. These data suggest that mature BMP-4 levels are not substantially altered by optimized S2 cleavage but that other mechanisms are likely employed to regulate enhanced activity when cleavage at the S2 site is optimized.

DISCUSSION

Ordered cleavage of proBMP-4

ProBMP-4 must dimerize and fold in order to exit the ER and traffic to the TGN where cleavage at the S1 site occurs. Although we originally hypothesized that S1 cleavage was required for proper trafficking of proBMP-4 to a post-TGN compartment where the S2 site is cleaved, we now show that S2 cleavage can occur independent of S1 cleavage, *in vivo*. When the S2 site is not cleaved, rapid degradation of mature BMP-4 is promoted. However, this is not true when the S1 site is not cleaved since mature-plus-linker BMP-4 accumulates to levels comparable to those of wild type mature BMP-4. Optimizing cleavage at the S2 site has minimal affects on the stability or secretion of mature BMP-4 but increases its activity and signaling range, suggesting that the bioactivity of this protein is enhanced by an unknown mechanism. It is possible that the linker domain may mediate some of these diverse affects of cleavage, where modulation of BMP activity may be related to the promoted release or altered association of the linker and mature domains of BMP-4. We found that the linker domain is required for proper folding and subsequent trafficking of proBMP-4, but what is not known is whether the linker peptide remains associated with the mature domain or is released following cleavage of the S2 site. Resolution of this question may begin to elucidate additional roles for the linker domain and address the possibility of whether the cleaved linker acts to stabilize the mature domain or target subcellular localization.

The S1 and S2 sites are independently cleaved in vivo.

We hypothesized that BMP-4 cleavage would be regulated in a manner analogous to that of furin, where cleavage at the first site allowed for a conformational change necessary for rendering the protein 'competent' to traffic to a second compartment where the second site would be cleaved (Molloy et al., 1999;Anderson et al., 2002). This prediction was based, in part, on work from Hawley et al (Hawley et al.,1995) who showed that mutating the S1 site from RSKR to GVDG generated a dominant-negative form of proBMP-4 that could not be cleaved and had no bioactivity. Contrary to our expectations, introduction of a more conservative mutation at the S1 site (RSKR→GSKR) allowed for efficient cleavage at the S2 site. This data is consistent with work published by Nishimatsu and Thomsen (Nishimatsu and Thomsen, 1998), who showed that mutating the S1 site fromRSKR to KSTK did not block all bioactivity, and that further mutation of the S2 site (RISR→KISK) was required to generate an inactive form of proBMP-4. Therefore, it is possible that the S1 site mutation generated by Hawley et al. (Hawley et al., 1995) caused misfolding of proBMP-4 and that this, rather than preventing cleavage of the S1 site, lead to the observed dominant-negative phenotype. By contrast, the more conservative S1 site mutations generated in the current studies and those conducted by Nishimatsu et al. (Nishimatsu et al.,1998) may not cause misfolding and thus would allow for proper cleavage at the S2 site. To explore this possibility the bioactivity of cleaved proBMP-4(mS1G) in *Xenopus* embryos needs to be repeated over a range of concentrations.

When the S1 site is not cleaved, the covalently attached linker-mature domain is relatively stable but may not be readily secreted. If the linker domain has trafficking

potential it could act to sequester linker-mature BMP-4 in a distinct subcellular compartment that may or may not have signaling potential. Conversely, it is possible that when the S1 site cannot be cleaved a 'quality control' mechanism is activated, which sorts mis-processed BMP-4 into a distinct subcellular compartment from which it is not subsequently trafficked. Evidence for the existence of such a quality control mechanism outside the ER has been shown for a specific mutant of intestinal sucrase-isomaltase (Ouwendijk et al., 1996) and a temperature-sensitive mutant of a G protein of vesicular stomatitis virsus (Chen and Huang, 1986), both of which exit the ER but are blocked in a pre-Golgi compartment rather than sorting to the cell surface.

Possible mechanisms for enhancing the activity of BMP-4 when the S2 site is optimized

We have shown that deletion of the linker domain prevents proper folding of proBMP-4. However, it is unlikely that this is the sole function for the linker domain. When cleavage of the S1 and S2 sites are altered the activity, stability, and trafficking of mature BMP-4 is changed. Whereas blocking cleavage at the S1 site promotes rapid degradation of mature BMP-4, it is unclear how optimized cleavage of the S2 site enhances the activity and signaling range of mature BMP-4 without a corresponding increase in stability. One possibility is that promoting cleavage at the S2 site leads to early activation of proBMP-4 in the embryo, and that this leads to an expansion in its signaling domain, although this would be difficult to prove. A second possibility is that promoting cleavage of the S2 site further accelerates the dissociation of the pro-ligand complex to further protect mature BMP-4 from degradation. However, since we detected less, rather than more mature BMP-4 from oocytes and embryos made to express proBMP-4 where the S2 site is optimized, this is not likely to be true. A third possibility

involves possible roles for the linker domain. It is not known whether or not the linker domain remains associated with mature BMP-4 following cleavage at the S2 site. However if this were true then it is possible that the linker domain could target the subcellular localization of mature BMP-4. That a small, soluble peptide can direct intracellular trafficking is not totally unheard of. The highly hydrophobic conotoxin, TxVI, is dependent upon its propeptide for exit from the ER and trafficking to the Golgi by-way-of a 'hitchhiker' mechanism. Here, the pro-region of TxVI binds unprocessed sortilin (a member of the Vps10p family of sorting proteins involved in TGN-to-endosome trafficking) in the ER and remains associated until sortilin is proteolytically activated in the Golgi (Conticello et al., 2003). By a similar mechanism, linker domain may promote targeting of mature BMP-4 to the recycling endosome or to distinct subcellular regions that promote its signaling activity. In this way, the bioactivity of mature BMP-4 could be enhanced without a change in its relative stability. It is equally possible that cleavage at the S2 site also dissociates the linker domain from the mature domain and that the linker domain plays another, unknown role in regulating mature BMP-4 activity. Further studies are needed to distinguish between these possibilities.

Potential roles for the linker domain: the linker as a chaperone.

An additional role for 'linker' peptides has been ascribed to lactase-phlorlizn hydrolase (LPH), an enzyme of the small intestine that undergoes sequential cleavage. In the ER, the signal peptide of LPH is cleaved to produce pro-LPH. Following glycosylation in the Golgi, proLPH is proteolytically cleaved to form LPHα, the chaperone domain, and LPH$\beta_{initial}$, the enzymatic domain. Finally, at the cell surface, an

additional 134aa 'linker domain' is cleaved from $LPH\beta_{initial}$ to form $LPH\beta_{final}$ (Jacob et al., 1996; Naim et al., 1987; Wuthrich et al., 1996). Pro-LPH is transport-competent, correctly sorted and enzymatically active. Similar to TGF-β proteins, when only the mature product, $LPH\beta_{final}$, is expressed, the protein does not exit the ER and is catalytically inactive. Expression of $LPH\beta_{initial}$, however, generates a fully functional, properly targeted enzyme under permissive (20^0C), but not restrictive (37^0C), temperatures. Recent studies show that the 'linker' portion of $LPH\beta_{initial}$ facilitates binding to the LPHα chaperone domain, but also can bind the chaperone proteins, BiP and calnexin, in the absence of LPHα. Under permissive temperatures these chaperones are able to inefficiently direct proper folding of LPHβ, however, under physiologic (restrictive) temperatures, BiP and calnexin are unable to facilitate folding of $LPH\beta_{initial}$ (Jacob et al., 2002). Interestingly, final processing of $LPH\beta_{initial}$ to $LPH\beta_{final}$ can be blocked with no apparent change in enzymatic activity or specificity. Thus, the linker domain is essential for the chaperone domain to bind to and properly fold LPH_{final}.

Similar to LPH, our data are consistent with the possibility that the linker domain facilitates the noncovalent association between mature BMP-4 and its prodomain. We also propose a chaperone role for the linker domain, since it is required for proper folding and to exit the ER. Furthermore, the linker domain may be able to restore mature BMP-4 activity when expressed *in trans*. Whether the linker serves exclusively as a chaperone, promotes association with a cellular chaperone or plays a trafficking role is yet to be elucidated.

Covalent modifications can regulate subcellular localization and secretion

Perhaps the most provocative model for the regulation of mature BMP-4 activity and expression arises from potential parallels with the regulation of Apo E expression, modification and secretion. Apo E is retained at the cell surface of macrophages (Lucas and Mazzone, 1996) hepatocytes and steroidogenic cells. Up to 8% is retained at the surface of expressing cell lines, but only a portion is actually released from the cell surface (Zhao and Mazzone, 2000). There exist two pools of Apo E: a low molecular weight form that is retained at the cell surface, and this is endocytosed to a recycling compartment where it is trafficked to a post-Golgi compartment and is modified with sialic acid, a modification which is required for final release of ApoE from the cell surface (Huang et al., 2004). Only the modified form of Apo E is released, while a substantial portion of unmodified Apo E is targeted for degradation (Duan et al., 1997; Mazzone et al., 1992).

Our data are consistent with the possibility that proBMP-4 is modified by mechanisms other than N-linked glycosylation. N-terminally extended mature BMP-4 resulting from cleavage at the S2 site migrates as two isoforms: the slower migrating form that can be secreted and the faster migrating form that may or may not be secreted, and with the larger form appearing to convert into the smaller form. Interestingly, O-linked glycosylation can act as a sorting motif in yeast (Proszynski et al., 2004), where the presence of O-linked glycosylation can alter protein targeting from the Golgi to the vacuole. The sizes of the two mature BMP-4 isoforms need to be carefully characterized and tested for carbohydrate as well as lipid modification, noting that some lipid modifications can increase the Mr of a protein on SDS-PAGE rather than retard it. Our data are also consistent with the possibility that the two species of mature BMP-4

represent alternate disulfide bond arrangements of the mature dimer. When proteins are separated by SDS-PAGE they adopt a rod-like structure that allows proteins to be separated according to their molecular weight. Disulfide-linked proteins, however, can adopt additional secondary structures and alternately bridged proteins can migrate with different Mr under non-reducing conditions, and this may explain the presence of two-distinct mature bands from proBMP-4(mS1G) cleavage. If this were true, then it is possible that mS1G may inhibit endogenous BMP-4 by acting as a dominant-negative confomer of BMP-4 (Weber et al., 2001). Consistent with this possibility, under reducing conditions deglycosylated mature BMP-4 derived from proBMP-4(mS1G) cleavage appeared to migrate as a single band in two experiments. These two possibilities need to be carefully examined and will provide significant insight into the mechanism whereby blocking cleavage at the S1 site can alter signaling without affecting S2 site cleavage.

The extended form of mature BMP-4 generated by cleavage at the S2 site alone may allow us to begin to study the role of the linker domain in regulating BMP-4 activity. When the linker domain remains covalently attached to the prodomain following cleavage at the S12 site alone, the fragment is rapidly degraded. However, when the linker domain is covalently attached to the mature domain following cleavage at the S2 site alone, this fragment appears to be stable, although it may undergo posttranslational modification. Since the cleaved linker cannot be detected *in vivo*, these constructs may aid in identification and characterization of additional roles for the linker peptide. Finally, these studies support evidence from *Xenopus* embryos that suggest optimizing cleavage at the S2 site has minimal affects on mature BMP-4 levels (Cui et al., 2001).

Further studies, perhaps involving the role of the linker domain, may begin to elucidate the mechanism by which the activity of this protein is enhanced.

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 (Nieuwkoop and Faber, 1967). Capped synthetic RNA was produced by *in vitro* transcription. Embryonic explants were isolated and cultured as described (Cui et al., 1998).

RT-PCR analysis

RT-PCR analysis of RNA samples was performed as described previously (Cui et al., 1998). The sequences of Xbra and EF-1α (Kengaku and Okamoto, 1995) primers have been published previously. The 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 isolation and analysis of proteins

Ovaries were surgically removed from mature female frogs and oocytes were dissociated with 0.2U/ml Liberase Blendzyme 3 (Roche) and Stage VI oocytes were cultured overnight as described (Degnin et al, in press). Oocytes were injected with *in vitro* synthesized RNAs (Moon and Christian, 1989) in the presence or absence of 700nCi [^{35}S]Met/Cys. Pulse-chase and deglycosylation analyses were performed as described (Degnin et al, in press).

2-D analysis

Oocytes were co-injected with 5ng RNA and 700nCi [^{35}S]Met/Cys, and cultured 20 hours. Lysates were isolated and immunoprecipitated with myc-specific antibodies as above. Duplicate sets of immunoprecipitated proteins were run on SDS-PAGE under non-reducing conditions. Duplicate lanes of the gel were either fixed, enhanced, dried and visualized by autoradiography, as above, or excised and reduced 30-minutes in Laemmli buffer (50mM Tris-HCl (pH 6.8), 380mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol). Reduced lanes were then subjected to SDS-PAGE under reducing conditions and proteins were visualized as above.

In vitro digestion with furin

Wild type and linker-mutant forms of proBMP-4 were synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]Met/Cys as described. Equal protein counts were subjected to cleavage by 2.2nM recombinant furin at pH 7.0 and 6.5 (data not shown). Aliquots were taken at the times indicated, denatured immediately in Laemmli buffer, and then analyzed by SDS-PAGE as above.

FIGURE LEGENDS

Figure 1. Cleavage of the S1 site of proBMP-4 is not required for cleavage at the minimal S2 site. **(A)** Schematic illustration of myc- and HA-epitope tagged wild type and cleavage mutant forms of proBMP-4. **(B-E)** RNA encoding wild type or cleavage mutant proBMP-4 was injected into oocytes together with [^{35}S]Met/Cys and cleavage products were analyzed by SDS-PAGE. The positions of precursor, S1 and S2 cleaved prodomain, and mature or mature-plus-linker are illustrated schematically to the right or left of each gel. **(B)** Oocyte lysates were immunoprecipitated with a myc- or HA-specific antibody and samples were treated with or without Endo H or PNGase F prior to SDS-PAGE under non-reducing or reducing conditions as indicated. Bands corresponding to Endo H sensitive (arrow), Endo H resistant/PNGase F sensitive (arrowhead), proBMP-4 dimers are indicated; fully-deglycosylated mature BMP-4 (asterisks) is indicated and mature doublet bands resulting from proBMP-4(mS1G) cleavage are further indicated by a gray bracket **(C-E)** Oocytes were pulse labeled for three hours and then transferred to media containing cold methionine and cysteine. At increasing time intervals, lysates and conditioned media were subjected to immunoprecipitation using antibodies specific for the HA- **(C)** or myc- **(D)** epitope and cleavage products were analyzed by SDS-PAGE under reducing **(C)** or non-reducing **(D)** conditions. **(E)** Oocyte lysates were immunoprecipitated with myc-specific antibodies and separated by SDS-PAGE under non-reducing (left) and reducing (right) conditions. The positions of the two species of mature-plus-linker dimers (NR, arrows) and monomers (reducing, arrowheads) are indicated schematically.

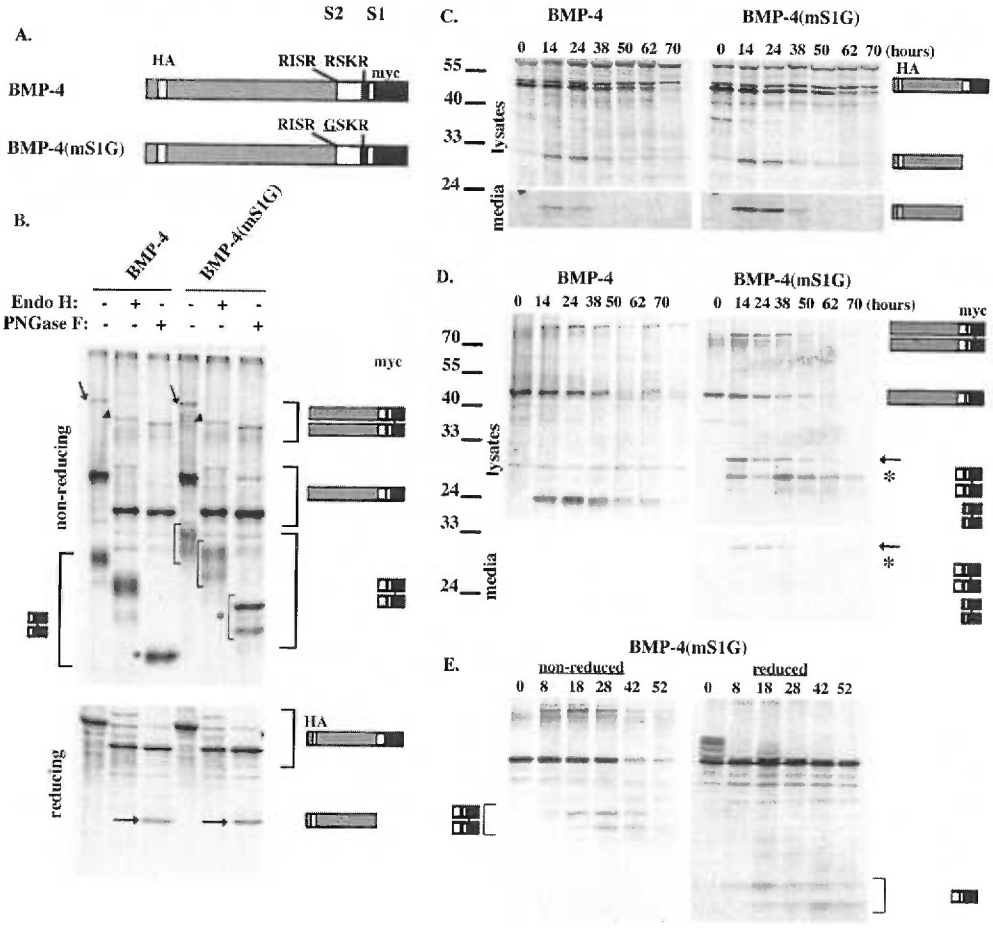
Figure 2. The linker domain of proBMP-4 is essential for BMP-4 activity. **(A)** Evolutionary conservation of BMP-4 and BMP-2 linker domain. Conserved residues are in black; changes are in gray. **(B)** Schematic of experimental procedure. RNAs encoding wild type and linker-deleted forms of proBMP-4 were injected into two-cell embryos and animal caps were dissected at the blastula stage (stage 8) and cultured until sibling embryos reached stage 10.5. RNA isolated from animal caps and whole embryos was subjected to PCR. **(C)** RNA was analyzed for expression of the mesodermal marker, Xbra, and EF-1α, as a control for RNA loading, in the presence of absence [RT(-)] of reverse transcriptase.

Figure 3. The linker domain of proBMP-4 is required for proper folding. Schematic of myc- and HA-epitope tagged wild type and linker mutant forms of proBMP-4 is illustrated at top. **(A)** Radiolabeled wild type or linker mutant forms of proBMP-4 were synthesized in rabbit reticulocyte lysates and incubated with recombinant furin at neutral pH. Aliquots were removed and analyzed by SDS-PAGE at the indicated times. The position of precursor, S1 and S2 cleaved prodomains are illustrated schematically to the right. **(B-C)** RNA encoding wild type or linker mutant forms of proBMP-4 was injected into oocytes together with [^{35}S]Met/Cy.; Lysates were harvested 24 hours post injection and cleavage products were analyzed by SDS-PAGE. The position of precursor, S2 cleaved prodomain and mature BMP-4 are illustrated schematically to the right **(B)** or top **(C)** of each gel. **(B)** Oocyte lysates were immunoprecipitated with a myc-specific (top) or HA-specific (bottom) antibody and samples were treated with or without Endo H or

PNGase F prior to SDS-PAGE under non-reducing or reducing conditions, as indicated. Bands corresponding to Endo H sensitive (arrow) or Endo H resistant/PNGase F sensitive (arrowhead) dimers are indicated. Diffuse bands representing possibly misfolded dimers are labeled (asterisks). **(C-D)** 2-D gel analysis. Oocyte lysates were immunoprecipitated with a myc-specific antibody and duplicate samples were separated under non-reducing conditions (NR). Duplicate gel lanes were excised, gel fragments were reduced, and then separated under reducing conditions, as indicated. Monomers migrate along the diagonal, indicated by gray line. **(C)** ProBMP-4 aggregates (angled arrow), dimers (arrow) and misfolded dimers (asterisks), and mature BMP-4 (arrowhead) are indicated. **(D)** ProBMP-4(ΔS2L) aggregates (angled arrow) and misfolded dimers (arrow) are indicated.

Figure 4. Optimized cleavage of the S2 site does not enhance the production of mature BMP-4. Schematic illustration of myc- and HA-epitope tagged wild type and cleavage mutant forms of proBMP-4 are shown above representative gels. Oocytes were injected with RNA encoding wild type or cleavage mutant forms of BMP-4 in the presence of [35S]Met/Cys, as indicated. **(A-B)** Oocytes were pulse labeled three hours and then transferred to media containing cold methionine and cysteine. BMP-4 was immunoprecipitated from lysates and media at the indicated times post chase using HA- **(A)** or myc- **(B)** specific antibodies and run on SDS-PAGE under reducing **(A)** or non-reducing **(B)** conditions. The position of precursor, S2 cleaved precursor and mature BMP-4 are illustrated schematically to the right of each figure.

Figure 1: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S2 S1
HA
RISR RSKR
myc
BMP-4
RISR GSKR
BMP-4(mS1G)
B.
BMP-4
BMP-4(mS1G)
Endo H: - + - - + -
PNGase F: - - + - - +
myc
non-reducing
reducing
HA
C.
BMP-4
BMP-4(mS1G)
0 14 24 38 50 62 70 (hours)
HA
55
40
33
24
lysates
media
D.
BMP-4
BMP-4(mS1G)
0 14 24 38 50 62 70 (hours)
myc
70
55
40
33
24
lysates
media
E.
BMP-4(mS1G)
non-reduced
reduced
0 8 18 28 42 52

Figure 2:

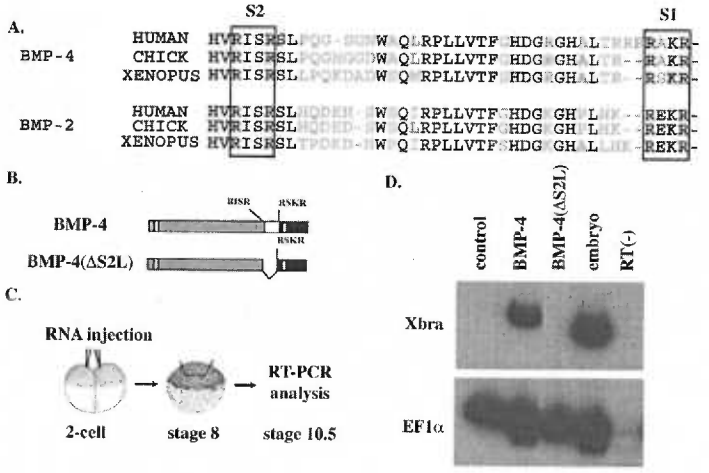


Figure 3:

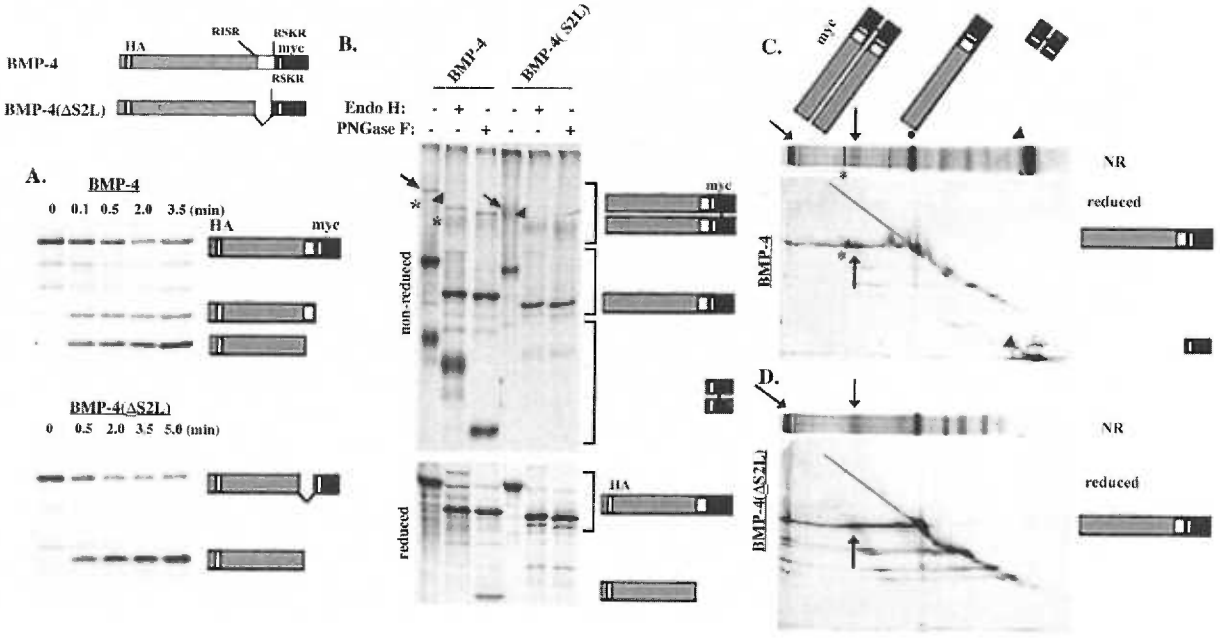
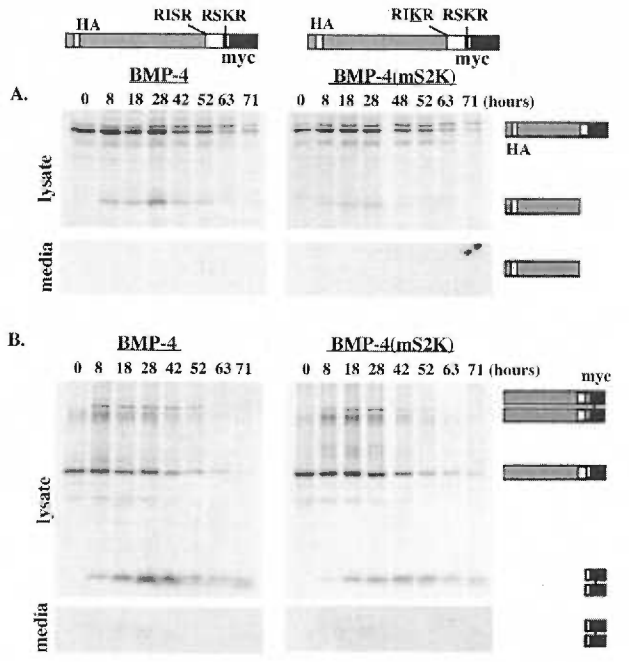


Figure 4:


HA
RISR RSKR
myc
BMP-4
A.
0 8 18 28 42 52 63 71
lysate
media
HA
RIKR RSKR
myc
BMP-4(mS2K)
0 8 18 28 48 52 63 71 (hours)
B.
BMP-4
0 8 18 28 42 52 63 71
BMP-4(mS2K)
0 8 18 28 42 52 63 71 (hours)
myc
lysate
media

Chapter 4

Conclusions

Summary

The bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF- β) superfamily and are important developmental regulators whose activity and signaling range is tightly regulated (Miyazono, 2000; Wilson et al., 1997). Too much, as well as too little BMP signaling can lead to severe developmental defects (Dudley et al., 1995; Kingsley, 1994; Luo et al., 1995; Storm et al., 1994; Winnier et al., 1995; Zhang and Bradley, 1996). Therefore, BMP activity is regulated at multiple levels within an organism. BMP-4, like other TGF-β proteins, consists of an extensive prodomain that comprises approximately two-thirds of the total proprotein. Similar to other TGF-β family proteins, this prodomain region can act to regulate folding and secretion of the mature dimer (Gray and Mason, 1990). Additional roles have been ascribed to specific TGF-β family prodomains. The prodomain of TGF-β plays a role in the establishment of latency (Miyazono et al., 1988; Wakefield et al., 1988); the prodomains of Xnr-3/-5 act *in trans* to repress BMP activity (Haramoto et al., 2004); and the prodomain of BMP-7 and TGF-β serve to anchor mature protein to the ECM (Charbonneau et al., 2004; Israel et al., 1992; Unsold et al., 2001). BMP-4 is the first

example where regulated cleavage of the prodomain acts to modulate the activity and signaling range for a signaling molecule (Cui et al., 2001).

Previous work has shown that proBMP-4 is proteolytically cleaved at two sites within the prodomain: first at a furin consensus site called S1 (-R-S-K-R) and second at an internal, minimal consensus site called S2 (-R-S-I-R-), which is cleaved in a regulated fashion (Cui et al., 2001). Cleavage at the S1 site generates mature BMP-4 while cleavage at the S2 site stabilizes mature BMP-4 and allows for long range signaling. Unexpectedly, optimizing cleavage at the second site does not further stabilize the mature protein as compared to wild type cleavage, however it enhances signaling activity and range. These data suggest a direct role for cleavage in modulating the bioactivity of mature BMP-4. The studies in this thesis were designed to begin to determine the mechanism through which regulated cleavage at this upstream (S2) site regulates the activity and signaling range of the secreted ligand.

Xenopus oocytes are extensively used to biochemically study eukaryotic proteins. Oocytes faithfully translate, process, posttranslationally modify and secrete biologically active proteins from microinjected mRNAs (Colman and Morser, 1979; Gurdon et al., 1971). However, the use of *Xenopus* oocytes is also limiting. Oocytes are optically opaque, which prevents the use of live-imaging to follow protein trafficking, and are slow to endocytose, making drug and inhibitor studies difficult to regulate. In addition, *Xenopus laveis* are tetraploid, eliminating their usefulness for genetic studies. Therefore, while oocytes were extremely useful for our initial biochemical characterization of cleavage, further model refinements may require the use of more amenable organisms.

The following sections will summarize our work, outline the questions left unanswered, and suggest some approaches may be pursued to elucidate those questions.

Blocking cleavage at the S2 site promotes degradation of the mature domain.

Previous studies demonstrated that blocking cleavage at the S2 site decreased the activity and signaling range of mature BMP-4 in *Xenopus* embryos and led to reduced steady-state levels of mature BMP-4 (Cui et al., 2001). Our studies in *Xenopus* oocytes are consistent with these results, showing a marked decrease in mature BMP-4 levels when cleavage at the S2 site was blocked. These data are consistent with the possibility that signaling range and activity may directly result from decreased protein levels and secretion. Our present data supports the possibility that targeted degradation involves both lysosomal and proteosomal function, and shows that targeted degradation of mature BMP-4 is a saturable event. Increasing the synthesis of precursor overwhelms the degradative pathway and results in abundant mature BMP-4 production, which suggests that a limited number of receptors may be involved in subcellular targeting and/or degradation of mature BMP-4. These data, however, do not distinguish whether mature BMP-4 is targeted for degradation while localized to the biosynthetic or endocytic pathways.

Embryos injected with proBMP-4(mS2G) synthesize signaling competent BMP-4. Mature BMP-4 derived from proBMP-4(mS2G) cleavage may be secreted to activate cell surface receptors, or consistent with our observation that no mature BMP-4 is detected in the culture media when limiting amounts of proBMP-4(mS1G) are expressed, may activate receptors within the biosynthetic pathway, signaling only to expressing cells.

Endocytosis is required for the activation of some receptor complexes (Seto et al., 2002). Whereas there is no direct evidence for TGF-β family receptor activation within the biosynthetic pathway, the juxtaposition of receptor and ligand within a common vesicle allows for this possibility. In support of this possibility, activation of target genes from embryos injected with proBMP-4(mS2G) is primarily (60%) restricted to expressing cells (Cui et al., 2001). In these embryos it is possible that the pro/mature complex may both signal and be targeted for degradation from within the biosynthetic pathway.

The pro/mature complex may also be secreted and able to active receptors at the cell surface. For this to be true, the noncovalent association between the prodomain and the mature domain would act to target the pro/mature/receptor complex for degradation via the endocytic pathway. Analogous to TGF-β (Miyazono, 2000; Ramirez and Pereira, 1999; Wakefield et al., 1988) and BMP-7 (Charbonneau et al., 2004; Jones et al., 1994)], association with the prodomain at the cell surface could alter the BMP-4 interactions in the ECM or modulate the activity of the mature domain. Consistent with these possibilities, overexpression of proBMP-4(mS2G) in oocytes resulted in secretion of pro/mature complexes into the culture media (Degnin et al, in press). Unfortunately, these data also lead to a circular argument, where overexpression can saturate intracellular targeting events and therefore result in secretion of proteins that would otherwise be trafficked to the lysosome (Stevens et al., 1986).

Dominant-negative inhibitors of endocytosis such as the dynamin mutants, dyn1(K44A) (specific for apical surfaces) and dyn2(K44A) (specific for basolateral surfaces), contain mutations within their GTPase domains that block receptor-mediated endocytosis (Altschuler et al., 1998). Overexpression of dyn(K44A) potently inhibits

endocytosis of transferrin and EGF receptors, while having no effect on Golgi-to-lysosome transport of cathepsin D (Damke et al., 1994). To directly ask whether blocking cleavage at the S2 site promotes degradation through the biosynthetic pathway requires the development of stable cell lines expressing low levels of wild-type or cleavage mutant forms of BMP-4 as well as an inducible form of dyn(K44A). These lines would need to express the appropriate PC (furin) and BMP receptors, and also be able to appropriately regulate proBMP-4 processing and degradation. If the pro/mature complex is targeted for degradation via the biosynthetic pathway, then mature BMP-4 derived from proBMP-4(mS2G), but not proBMP-4, should be degraded when dyn(K44A) is induced. Conversely, if degradation is mediated exclusively through the endocytic pathway, then mature BMP-4 derived from wild type and cleavage mutant precursors should be equivalently protected from degradation when endocytosis is blocked. One potential problem with these studies is that disrupting dynamin function would block recycling of furin, which has been shown to be essential for cleavage of some substrates.

Furin most likely cleavage both the S1 and S2 sites of proBMP-4 in Xenopus oocytes

To begin to understand how cleavage of the S2 site may be regulated, we asked whether the S1 and S2 sites were cleaved by distinct PCs in *Xenopus* oocytes and whether they were cleaved inside of outside of cells. Several PCs, including furin (Molloy et al., 1999), PC6B (De Bie et al., 1996) and PACE4 (Beck et al., 2002), can cleave their substrates outside, as well as inside, the cell. Since these PCs were our primary candidates for proBMP-4 cleavage, we demonstrated that both the S1 and S2

sites were cleaved within the oocyte. We next developed an *in vivo* cleavage assay in *Xenopus* oocytes to ask whether depletion of these candidate PCs could block cleavage of proBMP-4. In our preliminary studies furin, but not PC6B or PACE4, was required for proBMP-4 cleavage. Subsequent to these data we found that the S2 site can be cleaved, *in vivo,* independent of the S1 site. Consequently, future depletion assays need to monitor for the size of mature BMP-4 produced in order to control for depletion of a PC that may cleave at the S2, but not S1 site. Based these preliminary results, it is possible that furin is sufficient for cleaving proBMP-4 in oocytes, but that other PCs may be necessary for cleavage at different stages of development (Beck et al., 2002). The tissue-specific cleavage of PACAP is similarly regulated: in germ cells PACAP is cleaved exclusively by PC4 (Li et al., 2000), while in neuronal cells PACAP requires both PC1/3 and PC2 for complete cleavage (Taylor et al., 2003).

Cleavage at the S2 site is regulated

For cleavage to modulate BMP-4 activity during development, it needs to be regulated in a tissue-specific or developmental fashion *in vivo*. Our biochemical studies show that the S2 site is cleaved by furin in a pH-dependent fashion and that this pH-dependency requires an evolutionarily conserved P6 His residue. Mutation of the P6 His relieves the pH-restriction for cleavage of the S2 site, allowing for efficient cleavage at either neutral or acid pH. These data suggest that regulated cleavage of the S2 site may involve conformational constraints regulated by the P6 His and may also involve interactions beyond the primary amino acid sequence of the cleavage site (Chapter 2).

Similar restrictions have been observed for cleavage of α-4 integrin (Bergeron et al., 2003) and the minimal site of furin (Bhattacharjya et al., 2001).

Analysis of the S2G-cleavage mutant mouse (Hackenmiller-Paradis et al, in preparation) revealed a tissue-specific requirement for cleavage at the S2 site, where blocking cleavage of the S2 site generated a limited, tissue-specific phenotype that is distinct from that of the BMP-4 heterozygous knock-out mouse. These data suggest that in most tissues mature BMP-4 may be labile and required only for short-range signaling. Based on the limited phenotype of the cleavage mutant mouse and our *Xenopus* embryo studies (Cui et al., 2001) we predict that cleavage of the S2 site is not required in these unaffected mouse tissues. We are attempting to generate a mouse-BMP-4 prodomain antibody that will allow for characterization of prodomain cleavage patterns from wild type and cleavage mutant mouse tissues. We expect that cleavage will occur primarily at the S1 site in most, but not all, wild type mouse tissues. We also predict that the range of BMP-4 signaling, as visualized by phospho-Smad1 expression, will be restricted in the affected tissues of the cleavage mutant mouse, consistent with the hypothesis that preventing cleavage of the S2 site generates a short-range signaling molecule. These mouse studies will provide *in vivo* evidence for our model of regulated S2 cleavage.

Cleavage at the S2 site may regulate bioactivity as well as targeted degradation of mature BMP-4

Contrary to blocking cleavage at the S2 site, optimizing cleavage at S2 appears to affect the bioactivity of cleavage of the S2 site. *In vitro*, optimizing cleavage at the S2 site greatly enhances the rate of mature BMP-4. *In vivo*, this does not translate into

increased accumulation of mature BMP-4 in embryos (Cui et al., 2001) or oocytes (Chapter 3). Injected embryos and oocytes appear to accumulate mature BMP-4 at the same rate from wild type or S2K mutant precursors, suggesting that premature activation of the protein may not be regulating its activity and that optimized cleavage at the S2 site may generate a genuine hypermorph, although this is yet to be determined. By contrast, our *in vivo* techniques may not be sensitive enough to detect early cleavage of the precursor. Pages and Kerridge (Pages and Kerridge, 2000) proposed that the formation of a morphogen gradient is based on time, rather than concentration, of a given morphogen. This model predicts that early cleavage of proBMP-4 could expand the range and enhance the signaling from the same level of mature ligand, within the context of the developmental organism. To begin to test the effects of optimized cleavage at the S2 site, a conditional BMP-4(mS2K) knock-in mouse is currently under construction.

It is also possible that enhanced mature BMP-4 activity, when cleavage of the S2 site is optimized, involves novel roles for the linker domain. The linker domain is essential for protein folding and exit from the ER (Chapter 3), but whether it has additional roles is yet unexplored. It is possible that the linker domain remains noncovalently associated with mature BMP-4 following cleavage at the S2 site, and that this interaction serves to alter the targeting of mature BMP-4 within the ECM (to enhance signaling) or to recycling endosomes (to promote long range trafficking). By this model, optimized cleavage of the S2 site might enhance the formation of this noncovalently associated linker-mature protein to allow it the act at longer range. At this time we have no means to determine the fate of the linker domain. This is in part due to the size of the linker domain (30 amino acids) and because our attempts to generate a linker-specific

antibody have been unsuccessful. The role of the linker domain, however, may be crucial in resolving how optimizing cleavage at the S2 site creates a hypermorph of mature BMP-4.

The linker domain may play a trafficking role for BMP-4

Early work in *Xenopus* embryos showed that blocking cleavage of the BMP-4/-2 S1 site(s) generated a dominant-negative inhibitor of endogenous BMP signaling (Hawley et al., 1995; Suzuki et al., 1997a). Inhibition was thought to result from a complete block in BMP-4/-2 cleavage, although this has never been shown. Our present work shows that blocking cleavage of the S1 site allows for cleavage of the S2 site (Chapter 3).

In oocytes, mature BMP-4 dimers resulting from proBMP-4(mS1G) cleavage migrate as two distinct proteins, varying in size by approximately 3kDa. It is possible that these two bands may represent alternately disulfide-bonded, misfolded isoforms of mature BMP-4. However, dimerization and folding of proBMP-4 occurs in the ER, prior to trafficking to the Golgi and TGN where the S1 site should be cleave. Therefore, for this model to be true, the point mutation in the S1 site would not only prevent cleavage of the S1 site, but would also allow misfolded proBMP-4 to escape the cellular quality control mechanisms that would normally direct the refolding or degradation of misfolded proteins.

It is equally possible that under optimal gel conditions two species of mature BMP-4 generated from proBMP-4(mS1G) cleavage will also be resolved under reducing conditions. If this is true, then it becomes possible that the two species of mature BMP-4 represent different post-translational modifications of the same protein. Since these

alternate forms are not detected when the S1 site is cleaved, it is likely these modifications reside within the linker domain. Most sugar modifications are added in the ER or Golgi networks, although many other covalent modifications occur in post-TGN compartments, the cytoplasm, or the extracellular space. Our results suggest that the putative linker domain modification is not an N-linked glycosylation and that this modification is transient, since pulse chase analysis suggests that the larger Mr protein (Chapter 3, Figure 1D, arrow) may be converted to the lower Mr protein (Chapter 3, Figure 1D, asterisks). Our data also suggests that this modification may influence mature BMP-4 protein trafficking in a manner distinct from that of mature BMP-4 without the linker domain. Possible modifications include, but are not limited, to O-linked glycosylation (O'Donnell, 2002), sialylation (Huang et al., 2004), fucosylation (Bruckner et al., 2000), and palmitoylation (Chamoun et al., 2001). The absence or presence of these modifications may regulate the trafficking and stability of the associated protein.

To begin to determine the role for S1 cleavage we have generated a cleavage mutant where cleavage at the S1site is blocked, while cleavage at the S2 site is optimized [BMP-4(mS1G.S2K)]. If cleavage of the S1 site is required solely to promote cleavage at the S2 site *in vivo*, then embryos injected with RNA expressing proBMP-4(mS1G.S2K) should retain some BMP-4 activity. If, instead, the covalent association of the linker domain alters the activity or subcellular targeting of the mature protein, then both BMP-4(mS1G) and BMP-4(mS2G.S2K) should behave in a similar fashion. When the linker domain remains covalently attached to the prodomain the protein (complex) is highly labile. However, when attached to the mature domain the protein appears to be stable.

Therefore, these mutants may prove invaluable in assessing novel roles for the linker domain of proBMP-4.

Possible roles for HSPGs in BMP-4 regulation

The role of HSPGs in BMP signaling is complex. The N-terminal domains of BMP-2 (Ruppert et al., 1996) and BMP-4 (Ohkawara et al., 2002) mediate binding to HSPGs but are neither required for receptor binding nor activation. These regions do, however, modulate biological activity. When the HSPG binding motif of BMP-4 is deleted [BMP4(ΔRKK)], mature BMP-4 accumulates in the media of oocytes made to express either wild type or S2G-cleavage mutant forms of proBMP-4 (Degnin et al, in press). This is consistent with the increased distribution observed in *Xenopus* embryos (Ohkawara et al., 2002). These results are consistent with HSPGs acting to anchor growth factors within the extracellular matrix (ECM), and the possibility that release from the ECM protects mature BMP-4 from proteolytic degradation by ECM proteases. In contrast, deletion of the HSPG binding motif did not lead to increased protein levels from *Xenopus* embryos (Ohkawara et al., 2002). In addition to increased mature BMP-4 in the culture media from oocytes, there is also significantly more mature BMP-4 found in oocyte lysates when the HSPG binding motif is removed, irrespective of whether the S2 site is cleaved and independent of decreased binding to the oocyte surface. These results are consistent with the possibility that HSPGs may facilitate endocytosis and/or targeting for degradation from either the biosynthetic or endocytic pathway.

Little is known about the effect HSPGs have on BMP-4 signaling during development. HSPGs modulate cellular responsiveness to BMPs (Paine-Saunders et al.,

2000), and yet, unlike fibroblast growth factors (FGFs) (Mali and Joshi, 1993; Steinfeld et al., 1996), are not required as co-receptors. HSPGs are expressed in a dynamic fashion during development (Teel and Yost, 1996). There is evidence that the structure of heparin sulfate chains may be both spatially and temporally regulated to impart tissue-specific preference for certain ligands over others (Friedl et al., 1997; Nurcombe et al., 1993). Thus, it is possible that the pattern of HSPG expression modulates of the activity of BMP-4 by different mechanisms. This is observed with two alternatively expressed isoforms of follistatin: one that can activate activin signaling, and the other that can target activin for degradation following binding to HSPGs (Hashimoto et al., 2000). Other novel, tissue-specific effects are observed for the Drosophila glypican mutant, division abnormally delayed (*dally*). In most tissues (eye, antenna, genitalia), dally enhances Dpp signaling and overexpression of Dpp can suppress *dally* hypomorphic alleles. However, in other tissues such as the wing, dally antagonizes Dpp, and *dally* mutants suppress overexpression of *dpp* (Jackson et al., 1997). Additional, yet unidentified, effector(s) are likely required in either the wing disk or the eye to modulate the Dpp/Dally interaction, and to generate these phenotypic differences. Thus, the presence or absence of specific HSPGs (and their cofactors) can potentiate or suppress BMP activity.

In *Xenopus* embryos, deletion of an eight-amino acid basic stretch in the N-terminal domain of BMP-4 (**KQQRPRKK**) (Δ1BMP-4) had slightly different effects on BMP-4 than did deletion of the three amino acid basic region (**RKK**) (Δ2BMP-4) (Ohkawara et al., 2002). These differences may be explained by decreased expression of the Δ1BMP-4 protein, or they may indicate that the N-terminal domain of BMP-4 has additional interactions with HSPGs (or another protein) through these additional basic

residues. Studies have begun to characterize the precise HSPG sulfonation patterns that mediate growth factor interactions (Irie et al., 2003; Viviano et al., 2004); however no reciprocal studies have characterized the amino acid requirements of growth factors for interacting with specific sulfonated residues. Therefore, although we detect enhanced expression of BMP-4 when RKK is deleted (Chapter 2), it would be of interest whether removal of the entire eight-amino acid stretch can further modulate BMP-4 expression and stability and whether these differences are limited to specific subsets of tissues.

In these studies we have begun to characterize the mechanism for regulated cleavage to modulate mature BMP-4 activity and signaling range. Whereas these studies model how restricting cleavage at the S2 site can cause targeted degradation, and hence limit the range of action of BMP-4, *in vivo* systems are clearly more complex. During embryogenesis the S2 site is likely cleaved with a range of efficiencies. Expression of specific HSPG may augment or potentiate the effects of S2 cleavage and will most likely significantly affect the establishment of a BMP gradient. These two factors, cleavage of the S2 site and expression of HSPGs, may significantly contribute to determining routes on endocytosis and determine whether mature BMP-4 is targeted for degradation (short range) or recycling (long range). An additional layer of complexity also arises from the unknown role of the linker domain. Future studies that can determine whether this peptide is released or associated with fully processed mature BMP-4 will help to further understand the regulation of BMP-4 in development and may provide insights into greater therapeutic uses for BMP-4 in bone repair.

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