Regulation of the Bioavailability of TGF- β and TGF- β -Related Proteins

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The bioavailability of members of the transforming growth factor β (TGF- β) family is controlled by a number of mechanisms. Bona fide TGF- β is sequestered into the matrix in a latent state and must be activated before it can bind to its receptors. Here, we review the molecules and mechanisms that regulate the bioavailability of TGF- β and compare these mechanisms with those used to regulate other TGF- β family members. We also assess the physiological significance of various latent TGF- β activators, as well as other extracellular modulators of TGF- β family signaling, by examining the available *in vivo* data from knockout mouse models and other biological systems.

The bioavailability of transforming growth factor β (TGF- β) family ligands is not only regulated by the release of the growth factor from cells, but it is also controlled by extracellular mechanisms that modulate, inhibit, activate, or enhance the binding of these signaling molecules to their receptors.

This is especially true of the bona fide TGF- β dimer; as when TGF- β is secreted from cells it is tightly bound in a latent complex consisting of its dimeric pro-peptide (referred to as latency-associated peptide [LAP]), and a latent TGF- β -binding protein (LTBP). This tripartite complex of TGF- β , LAP, and LTBP is called the large latent complex (LLC). Within this complex, LAP confers latency to the cytokine and the LTBP functions to direct and sequester the growth factor into the extracellular matrix (ECM) and assists in the conversion of the la-

tent TGF- β to its active form, a process known as activation.

In the last decade, it has become clear that the properties of the TGF- β LLC are critical in modulating the action of the cytokine and that controlled release of TGF- β from the ECM by activation is central to understanding TGF- β signaling in a larger biological context. Here, we review the extracellular regulation of TGF- β and examine the available *in vivo* data to place these regulatory mechanisms in a physiological context.

BIOSYNTHESIS AND LATENCY OF TGF- β

TGF- β Processing, Latency, and Interaction with LTBPs

TGF- β is initially translated as a $\sim$ 50-kDa pro-protein containing both growth factor and

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LAP. This pro-protein is vectorially discharged into the endoplasmic reticulum where it dimerizes and folds (Fig. 1, lower right). Within the endoplasmic reticulum, the dimeric pro-TGF- β is linked to a single LTBP by a pair of disulfide bonds between LTBP and LAP (Miyazono et al. 1991; Gleizes et al. 1996; Saharinen et al. 1996). The pro-peptide (LAP) is subsequently cleaved from the mature cytokine in the *trans*-Golgi by furin or furin-type enzymes (Fig. 1, lower left)

(Dubois et al. 1995; Hyytiainen et al. 2004). The complex of TGF- β and LAP is referred to as the small latent complex (SLC).

One of the remarkable properties of the TGF- β latent complex is the fact that the interaction of LAP with the growth factor is of such high affinity that all TGF- β isoforms are secreted as part of a latent complex, either LLC or SLC. The one known exception is Camurati–Engelmann disease, in which patients have spe-

Figure 1. Scheme for the secretion and extracellular regulation of transforming growth factor β (TGF- β). Starting at the *bottom right* (1), TGF- β and latent TGF- β -binding protein (LTBP) are translated into the endoplasmic reticulum (ER) where pro-TGF- β dimerizes and is then disulfide bonded to LTBP to form a ternary complex. The TGF- β dimer is cleaved from its pro-peptide (latency-associated peptide [LAP]) in the *trans*-Golgi network, but TGF- β and LAP remain strongly associated via noncovalent interactions forming the large latent complex (LLC) (*lower left*) (2). Once secreted (*middle left*) (3), the LTBP may bind various matrix fibers that sequester latent TGF- β until it is released by an activator (*upper left*) (4). The latent complex is then activated (*upper right*) (5), by one of several potential mechanisms, releasing the mature TGF- β . Active TGF- β may bind to cell-surface receptors (*lower right*) (6), although other factors may also bind the active growth factor at this stage and either inhibit or promote receptor binding.

cific mutations in TGF- β 1 LAP or in the TGF- β 1 signal peptide that yield increased levels of secreted, constitutively active TGF- β that may be the cause of the bone thickening observed in this rare condition (Saito et al. 2001; Janssens et al. 2003).

LTBPs may serve a chaperone-like function for pro-TGF- β by enhancing its folding and secretion (Miyazono et al. 1991). Without LTBP, the cysteines in LAP that bind LTBP may form incorrect disulfide bonds, and this complex would be degraded within the cell (Brunner et al. 1989). This may account for the enhanced secretion of TGF- β in the presence of LTBP (Miyazono et al. 1991; Rifkin 2005) and is consistent with the heightened secretion of TGF- β 1 C33S compared with wild-type protein (Annes et al. 2004). However, some cell types, such as the osteosarcoma line UMR-106, produce only the SLC, and the ROS 17/2.8 line produces a mixture of both SLC and LLC (Dallas et al. 1994).

Significant amounts of mature TGF- β are also secreted when HEK-293T cells are transfected with TGF- β expression constructs alone (Walton et al. 2010). Thus, the pro-TGF- β dimer can properly fold and be processed on its own, but covalent interaction with an LTBP may still enhance the rate of folding and secretion.

Unlike the TGF- β LAP interactions, which are noncovalent, the binding of LAP with LTBP is mediated by a pair of disulfide bonds between the most amino-terminal cysteines in LAP and a unique cysteine pair in LTBP. The LTBPs are large multidomain glycoproteins that consist of tandem arrays of calcium-binding epidermal growth factor-like domains (cbEGFs), 8-Cys type domains (which can be subcategorized into TGF- β -binding [TB] domains and hybrid domains), and regions with no clear domain homology that introduce flexibility into these proteins (Fig. 2) (Robertson et al. 2014). LTBPs bind LAP through a pair of cysteines in the third

Figure 2. The multidomain structure of latent transforming growth factor β (TGF- β)-binding proteins (LTBPs). Domain structures of the four LTBPs found in the human genome, along with human fibrillin-1 for comparison. Some regions associated with specific functions are highlighted in LTBP-1. The key at the bottom of the figure describes the different domain types. L in LTBP-1 and -4 denotes the long form. The question marks on domains in LTBP-1L and LTBP-3 denote that it is unclear from their sequences whether these EGF domains bind calcium. EGF, Epidermal growth factor domain; cbEGF, calcium-binding epidermal growth factor domain.

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8-Cys domain (or second TB domain) (Gleizes et al. 1996; Saharinen et al. 1996), but within the LTBP family (LTBP-1, -2, -3, and -4), there is variability with respect to the ability to bond to LAP (Saharinen and Keski-Oja 2000). LTBP-1 and -3 bind well to all three TGF- β isoforms, as shown by cotransfection assays, whereas LTBP-2 does not bind at all, and LTBP-4 binds only to TGF- β 1-LAP and does so inefficiently. LTBP-4-binding to LAP may be enhanced in an amino-terminally extended LTBP-4 splice form, but the nature and significance of this binding are unclear (Kantola et al. 2010).

The binding of LTBP to the TGF- β pro-peptide is primarily determined by the availability at the protein surface of a cysteine pair in the third 8-Cys (second TB) domain. LTBP-1, -3, and -4 each contain a dipeptide insertion found only in TB domains that bind to LAP (Saharinen and Keski-Oja 2000). The dipeptide is missing in the third 8-Cys domain of LTBP-2 and in all other 8-Cys domains of the LTBPs and the structurally related fibrillins. Addition of the dipeptide into the LTBP-2 third 8-Cys domain converts a nonbinding domain into a LAP-binding domain. The initial docking interaction between LAP and LTBP involves a number of acidic and basic residues, the importance of which has been highlighted by mutagenesis studies (Chen et al. 2005; Walton et al. 2010). The higher number of negatively charged residues arranged around the reactive cysteines may explain the more effective binding of the third 8-Cys domains of LTBP-1 and -3 to SLC compared with LTBP-4. Secretion of LTBP-3 is dependent on association with TGF- β (Penttinen et al. 2002), but LTBP-1, -2, and -4 are secreted efficiently without bound TGF- β (Saharinen et al. 1999).

The potential functions of all four LTBPs in vivo have been investigated using LTBP-null mice (Robertson et al. 2015). LTBP-1 and -4 exist as both long (L) and short (S) forms generated by the use of separate promoters and initiation codons. Deleting exons specific to the generation of the long form of LTBP-1 in mice results in cardiac outflow tract defects that cause embryonic lethality, potentially a result of decreased levels of TGF- β (Todorovic et al. 2007, 2011). Interestingly, deleting an exon shared by

both long and short forms of LTBP-1 resulted in only minor craniofacial abnormalities (Drews et al. 2008). The lack of a more severe phenotype appears to be a result of compensatory splicing around the deleted exon (Todorovic and Rifkin 2012), and a study of mice with conditional deletion of exon 8, which prevents expression of both isoforms of LTBP-1, showed similar lethal cardiovascular defects to those seen with LTBP-1L-null mice (Horiguchi et al. 2015). Gene targeting of LTBP-2 on the other hand results in only minor defects, such as disorganization and detachment of the ciliary zonules (Inoue et al. 2014). These results are consistent with LTBP-2's inability to bind LAP and suggest LTBP-2 may be important for microfibril assembly.

LTBP-3-null mice are viable but display bone defects, including osteopetrosis and premature ossification of the skull synchondroses (Dabovic et al. 2002; Colarossi et al. 2005), phenotypes that indicate reduced TGF- β signaling. Interestingly, morpholino gene silencing of LTBP-3 in zebrafish causes cardiovascular defects not seen in the mouse model (Zhou et al. 2011). These defects were phenotypically similar to those caused by inhibition of TGF- β signaling and were rescued by expression of a constitutively active TGF- β receptor, demonstrating the importance of LTBP-3 in facilitating TGF- β action.

Studies of mice with a disrupted *Ltbp4* gene revealed multiple developmental defects including impaired alveolar septation, cardiomyopathy, and rectal prolapse (Sterner-Kock et al. 2002), phenotypes reflected in human patients with LTBP-4 deficiency (Urban et al. 2009). Reduced deposition of extracellular TGF- β and reductions in phospho-Smad2 in various tissues were also seen in this study. However, detailed examination of the lungs of *Ltbp4*-null animals revealed a surprising increase in phospho-Smad2 (Dabovic et al. 2009). Furthermore, breeding these mice onto a *Tgfb2*^{-/-} background rescued the alveolar septation defect (Dabovic et al. 2009), suggesting that this defect was actually caused by increased TGF- β signaling. This rescue effect was specific to TGF- β 2 as a *Tgfb1*^{-/-} or *Tgfb3*^{-/-} background did not rescue the lung defect (Dabovic et al. 2014). To

investigate the importance of the LTBP-4–LAP interaction, a mouse was generated in which the cysteines (Cys 1235 and 1260) in LTBP-4 responsible for putative binding to LAP were mutated to serine to inhibit disulfide bond formation. These mice appeared phenotypically normal (Dabovic et al. 2014), which suggests that sequestering TGF- β via LAP is not a significant function of LTBP-4. Additional evidence shows that the primary role of LTBP-4 appears to be the regulation of elastic fiber assembly through its interactions with fibulin-5 and fibrillin (Noda et al. 2013; Dabovic et al. 2014). This makes LTBP-1 and LTBP-3 the LBTPs of greatest interest when considering the extracellular pool of latent TGF- β .

Latency is dependent solely on the presence of LAP as mutant TGF- β 1 LAP (C33S) that cannot bind LTBP still maintains TGF- β in the latent state (Gentry and Nash 1990; Annes et al. 2004). This SLC is efficiently secreted without LTBP (Yoshinaga et al. 2008). However, the Cys33 residue in LAP plays an important role in TGF- β 1 function in vivo, as mice with the C33S TGF- β 1 mutation develop inflammation that is similar to that observed in TGF- β 1-null mice (although not as severe), implying defective activation of SLC (Yoshinaga et al. 2008). These mutant mice also develop gastrointestinal tumors (Yoshinaga et al. 2008; Shibahara et al. 2012). If the primary function of this cysteine is binding to LTBP, these results reflect the crucial importance of SLC–LTBP interactions for proper extracellular TGF- β function.

Substitution of Cys223 and Cys225 in TGF- β 1 LAP with serines, on the other hand, prevents the dimerization of LAP and results in the secretion of constitutively active TGF- β (Brunner et al. 1989). This mutant form of TGF- β has been used in many transgenic studies that show the destructive potential of the release of constitutively active TGF- β and highlight the importance of latency in proper TGF- β function (Sellheyer et al. 1993; Sanderson et al. 1995). However, although these transgenic studies provide useful information on the effects of a generalized abundance of active TGF- β , they are less informative for examining the more targeted mechanisms of latent TGF- β localization and activation that occur in normal tissues.

Interactions between the LLC and the ECM

Besides promoting effective secretion of latent TGF- β from cells, another major function of LBTPs is localization of latent TGF- β to the ECM (Fig. 1, upper left) (Koli et al. 2005). This feature has many important implications for latent TGF- β activation and TGF- β bioavailability.

The best-studied interactions of an LBTP with the ECM are those of LTBP-1. Initial studies using immunofluorescence to monitor binding of recombinant fragments to matrix showed that LTBP-1 possessed two ECM-binding regions at the amino terminus and one at the carboxyl terminus (Unsold et al. 2001). Further experiments showed specific interactions with fibronectin (Dallas et al. 2005a; Fontana et al. 2005; Kantola et al. 2008) and fibrillin fibers (Isogai et al. 2003; Ono et al. 2009; Massam-Wu et al. 2010), as well as the formation of transglutaminase cross-links with the ECM (Nunes et al. 1997). The specific ECM component to which LTBP-1 is cross-linked was not determined.

The last three carboxy-terminal domains of LTBP-1 interact with the amino terminus of fibrillin (Isogai et al. 2003; Ono et al. 2009), and this region of LTBP-1 contains flexible linkers that may facilitate this interaction (Robertson et al. 2014). The carboxyl termini of LTBP-2 and LTBP-4 are closely homologous with the carboxyl terminus of LTBP-1 and interact with fibrillin in a similar manner (Isogai et al. 2003; Ono et al. 2009). LTBP-3, on the other hand, has strikingly different sequence features at its carboxyl terminus (Robertson et al. 2011, 2014), and a recombinant fragment of this region of LTBP-3 does not interact with the fibrillin amino terminus in solid phase assays, suggesting that the LTBP-3 carboxyl terminus may have distinct properties (Isogai et al. 2003). The interaction of LTBP-1 with fibronectin has not been well characterized, and, in some assays, this interaction is mediated by heparan sulfate proteoglycans (Chen et al. 2007). Fibronectin is essential for the incorporation of LTBP-1 into extracellular fibers in cell culture. Studies conducted by Ono et al. showed LTBP-1 ECM in-

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corporation by murine neonatal fibroblasts (cultured for 13 days) was dependent on fibrillin-1 (Ono et al. 2009). However, more recently, Zilberberg et al. (2012) showed that fibrillin-1 is not essential for incorporation of LTBP-1 into the ECM of aortic smooth muscle cells or primary lung fibroblasts. But fibrillin-1 is still required for matrix incorporation of LTBP-3 and LTBP-4 in these cultures (Zilberberg et al. 2012), suggesting a link between LTBP-3 and the fibrillin matrix, although no direct interaction between these molecules has yet been shown. The assembly of fibrillin fibers is also dependent on fibronectin in most cell cultures (Kinsey et al. 2008; Sabatier et al. 2009), making it difficult to tease apart the relative importance of the fibrillin and fibronectin networks in LTBP incorporation and TGF- β regulation.

The functional significance of TGF- β 's ECM localization is still unclear. Some clues may derive from studies of Marfan syndrome (MFS), a genetic condition caused by mutations in the fibrillin-1 gene resulting in a number of symptoms, including tall stature, arachnodactyly, ectopia lentis, and aortic dilatation. Fibrillin is an important structural component of the ECM (Jensen et al. 2012), but interestingly the reduction of fibrillin-1 levels in MFS also leads to increased levels of active TGF- β (Neptune et al. 2003). Defects in MFS mice, such as impaired alveolar septation, mitral valve prolapse, and aortic dissection, can be prevented by inhibition of TGF- β signaling (Judge et al. 2004; Ng et al. 2004; Habashi et al. 2006, 2011; Cohn et al. 2007). One hypothesis is that an insufficiency of fibrillin-1 fibers leads to a shortage of available sites to anchor latent TGF- β in the matrix, resulting in excess soluble LLC available for activation. An alternative hypothesis is based on the observation that fibronectin contributes to latent TGF- β activation by integrins by providing cell-ECM traction (see below). Therefore, perturbing the strength or structure of the ECM by reducing fibrillin-1 levels in MFS could directly affect latent TGF- β activation via traction, rather than passively releasing more latent cytokine from the matrix. It is also conceivable that perturbations of LTBP–fibrillin interactions are not directly involved in MFS pathology and

that cellular responses to a defective matrix actually cause the increase in TGF- β activity. Additionally, direct inhibition of TGF- β in young mice can actually worsen the aortic phenotype (Cook et al. 2015), whereas loss of LTBP3 in fibrillin-deficient mice protects them from developing aortic aneurysms (Zilberberg et al. 2015). Both studies show that the relationship between fibrillin and TGF- β may be more complex and nuanced than first thought.

Disruptions of the ECM associated with enhanced levels of active TGF- β have been reported in a variety of genetic diseases of connective tissue, including multiple myopathic states (Cohn et al. 2007), fibromuscular dysplasia (Ganesh et al. 2014), and geleophysic and acromicric dysplasias (Le Goff et al. 2011; Le Goff and Cormier-Daire 2012). This may indicate that a primary response to abnormal matrix is the activation of latent TGF- β . One report of particular interest shows dysregulated TGF- β signaling in mouse models of osteogenesis imperfecta (OI), a brittle bone disease usually caused by mutations in type I collagen genes (Grafe et al. 2014). Mouse models of both dominant and recessive forms of OI showed increases in TGF- β signaling, and bone density was significantly improved by treatment with the TGF- β neutralizing antibody 1D11 (Grafe et al. 2014). These observations reinforce the idea of latent TGF- β activation as a process responsive to generalized perturbations in matrix structure and integrity.

Recently, LTBP-1 has been shown to bind and colocalize with extracellular deposits of Notch3 present in the cerebral arterioles of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) patients, and although the precise implications for TGF- β signaling are unclear, this further highlights the potential sensitivity of the LLC to extracellular perturbations (Kast et al. 2014).

Other Sources of Latent TGF- β

As well as LBTPs, latent TGF- β binds covalently to the *trans*-membrane leucine-rich repeat protein glycoprotein-A repetitions predominant

protein (GARP) (Wang et al. 2012). GARP expression appears restricted to regulatory T cells and platelets (Wang et al. 2008; Stockis et al. 2009; Tran et al. 2009), whereas LTBP β s are expressed ubiquitously (Rifkin 2005). GARP-like leucine-rich repeat proteins do not appear until the evolution of bony fish (Robertson and Rifkin 2013), although TGF- β and LTBP β s evolved earlier, as they are found in sea urchins, acorn worms, and lancelets (Robertson et al. 2011; Robertson and Rifkin 2013), suggesting that LTBP β s may have more general functions in TGF- β biology, with GARP activities restricted to T-cell and platelet-based functions. In fact, use of anti-GARP antibodies has been reported to suppress the activity of regulatory T-cells and aid with anticancer immunotherapy (Cuende et al. 2015).

E-selectin-ligand-1 binds intracellular pro-TGF- β (Olofsson et al. 1997); however, its role appears to be that of a negative regulator of TGF- β by inhibiting pro-TGF- β intracellular processing and secretion (Yang et al. 2010). The existence of other extracellular latent TGF- β complexes, independent of LTBP β s, cannot be ruled out. Mice lacking expression of TGF- β 1 and TGF- β 3 (Mu et al. 2008), or TGF- β 2 and TGF- β 3 (Dunker and Kriegelstein 2002) display significant deleterious effects in embryonic development, but double-null mutations of LTBP-3 and LTBP-4 are viable after birth and do not show clear phenotypic similarities with TGF- β -null mutations (Dabovic et al. 2011). As previously mentioned LTBP-1-null mice display lethal defects in embryonic heart development (Todorovic et al. 2007, 2011; Horiguchi et al. 2015), but direct phenotypic similarities with TGF- β -null mice are not clearly apparent. Inactivation of both LTBP-1 and LTBP-3 in the same mice has not yet been reported, but these may be the most informative for testing the overall significance of LTBP β s in TGF- β regulation in vivo.

Latency and ECM Interactions of Other TGF- β Family Members

TGF- β is not alone in its localization to ECM fibers; many other members of the TGF- β family and their pro-peptides form associations

with ECM components. Fibrillin, in particular, interacts directly with the pro-peptides of a number of TGF- β family members, including bone morphogenetic protein 2 (BMP-2), BMP-4, BMP-7, BMP-10, and growth and differentiation factor 5 (GDF-5) (Sengle et al. 2008a). Surface plasmon resonance studies showed that the pro-peptides of these growth factors interact with the amino-terminal regions of fibrillin-1 and -2 with similar affinities (Gregory et al. 2005; Sengle et al. 2008a), and some pro-peptides associate with other regions of the fibrillin molecule. The binding sites for BMP-7 and BMP-5 have been localized more precisely in the fibrillin amino terminus and have been shown to be dependent on the presence of the fibrillin unique amino-terminal (FUN) domain (Sengle et al. 2008a, 2011; Yadin et al. 2013). However, not all pro-domains of the TGF- β family members interact with the amino terminus of fibrillin. For example, the myostatin (GDF-8) pro-domain does not bind this region, but it does associate with the ECM via perlecan domain V glycosaminoglycan (GAG) side chains (Sengle et al. 2011).

Although the interactions between latent growth factors and fibrillin have been clearly shown in vitro, their in vivo significance is unclear. BMP-7 and BMP-4 colocalize with fibrillin-1 in tissue sections (Gregory et al. 2005; Sengle et al. 2008a), suggesting that these interactions occur in vivo, but their importance is unknown. Functional hypotheses might be proposed similar to those suggested for LLC binding to fibrillin; for example, BMP binding to fibrillin may localize and direct growth factor signals to certain regions of the ECM, and so determine specific features of tissue development (Ramirez and Rifkin 2009). TGF- β , BMP-10, and myostatin are latent when complexed with their pro-domains and unable to induce signaling without the help of an activator, for example, BMP-1 can activate BMP-10 by cleaving its pro-domain (Lee and McPherron 2001; Sengle et al. 2011). However, BMP-4, -5, -7, and -9 pro-domain complexes are not latent, and their pro-domains can be displaced from the growth factor allowing these molecules to bind to their receptors without activation

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(Brown et al. 2005; Sengle et al. 2008b, 2011). The fibrillin interaction might confer “latency” to growth factors not rendered latent by their pro-peptides alone, as localization to the ECM may shield the growth factors from cell-surface receptors. Signaling then could be regulated by displacement of the growth factor from fibrillin or contact of fibrillin microfibrils with the cell. Alternatively, fibrillin interaction could promote growth factor activity by preventing inhibitor binding.

Increased BMP activity in fibrillin-1 deficient osteoblasts is consistent with the hypothesis that fibrillin may inhibit BMPs activity (Nistala et al. 2010), but whether this involves a direct mechanism of fibrillin-binding BMP pro-domains was not tested.

Clues to the in vivo mechanisms by which fibrillins regulate BMP activity are also sparse but there are some suggestive data. For example, fibrillin-2-null mice display syndactyly, whereas BMP-7-deficient mice display polydactyly (Arteaga-Solis et al. 2001). Heterozygous *Bmp7*^{+/-} or *Fbn2*^{+/-} mice are normal, but a combined heterozygous deficiency of BMP-7 and fibrillin-2 yields mice with both polydactyly and syndactyly. These enhanced digit abnormalities in double heterozygotes may indicate a form of epistasis. In fibrillin-2 deficient mice transcription of several genes, including *Msx*, was rendered insensitive to implanted BMP-4 beads. These results suggest that fibrillin-2 and BMPs play interrelated roles in specific developmental pathways, but the full significance of their interaction is unclear.

In summary, there are several pieces of in vivo and in vitro evidence connecting the ECM and signaling by other molecules of the TGF- β family, but the precise mechanisms at work are not yet understood.

ACTIVATION OF LATENT TGF- β

The latent TGF- β complex can be considered as a sensor that remains in the ECM until triggered by a specific signal to release TGF- β (Annes et al. 2003); a process referred to as activation (Fig. 1, upper right). A number of different latent TGF- β activators have been described,

ranging from extremes of pH, to proteases, to cell-surface integrins.

ACTIVATION BY PROTEINS

Integrins

Cell-surface integrins are well-established activators of latent TGF- β (Munger et al. 1999; Annes et al. 2002; Mu et al. 2002). Integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ are the best described activators, but other α_v integrins, such as $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$, have also been implicated as interacting with LAP and activating latent TGF- β (Munger et al. 1998; Lu et al. 2002; Ludbrook et al. 2003; Wipff et al. 2007; Wipff and Hinz 2008; Munger and Sheppard 2011; Tatler et al. 2011; Hinz 2013; Sarrazy et al. 2014).

The best-studied mechanism for integrin-mediated activation of latent TGF- β is direct activation by traction between cells and matrix (Fig. 3A). Early studies showed that overexpression of β_6 integrin-activated latent TGF- β 1, even in the presence of protease inhibitors (Munger et al. 1999). Further studies showed that latent TGF- β 1 activation by $\alpha_v\beta_6$ was dependent on covalent attachment of LAP to LTBP-1 (Annes et al. 2004), the presence of specific ECM-binding regions in the hinge domain of LTBP-1 (Annes et al. 2004), and the presence of a fibronectin matrix (Fontana et al. 2005). The integrin-binding RGD (Arg-Gly-Asp) sequence in LAP and the cytoplasmic sequences of β_6 integrin that anchor it to the actin cytoskeleton were also required for TGF- β activation (Munger et al. 1999; Annes et al. 2004). These results are consistent with an activation mechanism whereby the conformation of LAP anchored to the ECM by LTBP and bound to the cell surface by integrins is distorted by traction between matrix and cells, liberating active TGF- β (Annes et al. 2004; Fontana et al. 2005; Wipff et al. 2007). This mechanism has been further elaborated by experiments describing the integrin-dependent release of active TGF- β by contracting myofibroblast cytoskeletons (Wipff et al. 2007), as well as from cell-free matrix in a force-dependent manner by ferromagnetic beads coated with either integrins or anti-LAP antibodies (Buscemi et al. 2011). Indeed, the

Figure 3. Proposed mechanisms of latent transforming growth factor β (TGF- β) activation by integrins. Two mechanisms have been proposed by which integrins activate latent TGF- β . (A) Traction between cells and extracellular matrix (ECM) is transmitted to latency-associated peptide (LAP) via latent TGF- β -binding proteins (LTBPs) bound to the matrix and cell-surface integrins bound to the cell's actin cytoskeleton. This deforms LAP and releases active TGF- β . (B) Integrins bind LAP at the cell surface and this may make LAP more accessible to proteases, such as membrane-type matrix metalloproteinase 1 (MT1-MMP), that cleave LAP and release the active growth factor, by localizing it and recruiting proteases into its vicinity, or by changing the structure of LAP so that it is rendered more protease sensitive.

state of the latent complex may be sensitized for activation by previous modifications of the matrix (Klingberg et al. 2014). The traction mechanism is supported by the crystal structure of TGF- β bound to LAP, which shows that Cys33, required for LTBP attachment, and the RGD motif are on opposite sides of the molecule, with TGF- β sandwiched between two arms of a LAP “straightjacket” (Shi et al. 2011). This straightjacket can be deformed by traction applied to the molecule, as shown by atomic force microscopy experiments (Buscemi et al. 2011).

The mechanism of latent TGF- β activation by $\alpha_v\beta_8$ is more obscure. Latent TGF- β activation by this integrin was reported to be blocked

by the presence of matrix metalloproteinase (MMP) inhibitors and did not require the cytoplasmic domain of $\alpha_v\beta_8$, indicating a traction-independent mechanism (Mu et al. 2002). This suggests an alternative protease-dependent mechanism, perhaps involving recruitment of MMPs by integrins to facilitate LAP cleavage and release of TGF- β (Fig. 3B). Alternatively, binding by the integrin may induce changes in LAP structure that promote protease cleavage. MT1-MMP (membrane-type matrix metalloproteinase 1) was suggested as a potential candidate protease, as it both conferred the ability to activate latent TGF- β , when transfected into the $\alpha_v\beta_8$ -expressing cancer line H1264 and co-

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localizes with integrin $\alpha_v\beta_8$ at substrate contacts (Mu et al. 2002). However, as described below, inactivation of MMPs does not produce a TGF- β , null-like phenotype.

As well as activating latent TGF- β within the LLC, both integrins $\alpha_v\beta_6$ and $\alpha_v\beta_8$ can activate latent TGF- β bound to GARP on the surface of cells expressing this complex (Wang et al. 2012; Edwards et al. 2014). This form of latent TGF- β may play an important role in regulating immune responses, as GARP is primarily expressed by T-cells.

The critical nature of integrin-mediated latent TGF- β activation in vivo was illustrated by a mouse model in which the integrin-binding RGD motif in TGF- β 1-LAP was replaced with an RGE sequence (Yang et al. 2007; Aluwihare et al. 2009). These mice phenocopy TGF- β 1-null mice and display severe multiorgan inflammation and absence of epidermal Langerhans cells. As the mutant TGF- β is secreted normally, this phenocopy shows that the integrin-binding RGD motif in TGF- β 1 LAP is essential for latent TGF- β 1 activation. TGF- β 3 also contains an RGD motif, the importance of which has not been conclusively tested in vivo. However, mice with null mutations in both $\alpha_v\beta_6$ and $\alpha_v\beta_8$ integrins display cleft palate, also observed in TGF- β 3 knockout mice. Pharmacological inhibition of $\alpha_v\beta_6$ in mice lacking $\alpha_v\beta_8$ causes inflammation similar to that seen in TGF- β 1-deficient mice (Yang et al. 2007; Aluwihare et al. 2009). These observations suggest that integrins play a key role in the in vivo activation of both TGF- β 1 and 3. Replacement of the TGF- β 3 coding sequence with a TGF- β 1 sequence into the *Tgfb3* locus partially rescues the palate closure defect seen in TGF- β 3-null mice (Yang and Kaartinen 2007), indicating that TGF- β 1 LAP shares some critical properties with TGF- β 3 LAP, as latent TGF- β 1 can be appropriately activated in place of TGF- β 3 in this context.

In addition to being important for latent TGF- β activation during development, integrins also play a significant role in diseases like fibrosis, in which TGF- β is a major mediator (Leask and Abraham 2004). Conditional deletion of the α_v integrin gene significantly attenuated hepatic, pulmonary, and renal fibrosis in

mouse models (Henderson et al. 2013), and deletion of integrin β_6 helps protect against radiation-induced lung fibrosis (Puthawala et al. 2008). TGF- β is also an important mediator of immune tolerance, and conditional deletion of β_8 integrin in dendritic cells causes severe inflammatory bowel disease and autoimmunity (Travis et al. 2007). Integrin β_8 expressed by effector regulatory T cells is also important for suppressing aberrant T-cell-mediated inflammation (Worthington et al. 2015), consistent with the role of this integrin in activating TGF- β and promoting immune tolerance. Integrin β_8 expressed by lung fibroblasts also plays a critical role in activating TGF- β to regulate dendritic cell trafficking in lung tissue, which can in turn contribute to fibrosis and inflammation (Kitamura et al. 2011). In addition, integrin β_8 expressed on dendritic cells regulates maturation of T_H17 cells, which contribute to autoimmunity (Melton et al. 2010), and integrin β_8 -mediated TGF- β activation also inhibits epithelial proliferation in bronchial tissue (Fjellbirkeland et al. 2003). Therapeutic strategies using monoclonal antibodies to target TGF- β activation by integrins have shown promise in treating fibroinflammatory airway disease in animal models (Minagawa et al. 2014).

As well as playing roles in fibrosis and inflammation, TGF- β activation by integrin β_8 plays a role in wound closure in vitro (Neurohr et al. 2006) and may act as an angiogenic control switch regulated by perivascular astrocytes (Cambier et al. 2005). Specific expression of integrin β_8 by nonmyelinating Schwann cells in bone marrow may assist in maintaining dormancy in hematopoietic stem cells by generating a highly localized niche of active TGF- β near blood vessels (Yamazaki et al. 2011).

Unlike TGF- β 1 and 3, TGF- β 2 lacks an RGD motif and was not activated in experiments testing latent TGF- β activation by specific integrins (Annes et al. 2002). TGF- β 2 contains other conserved motifs in the RGD region that are not found in TGF- β 1 and - β 3 (Robertson and Rifkin 2013), which suggests the possibility that TGF- β 2 is the target of specific activators that bind TGF- β 2 LAP in a similar fashion to integrin binding to TGF- β 1 and TGF- β 3 LAP RGD.

Proteases

A number of proteases of various classes can activate latent TGF- β in vitro, including cysteine proteases like calpain (Abe et al. 1998), aspartyl proteases like cathepsin D (Lyons et al. 1988), and numerous serine and metalloproteases (Maeda et al. 2001; Jenkins 2008). However, the relevance of these assorted proteases for latent TGF- β activation in vivo is unclear, as mice deficient in individual proteases fail to display TGF- β , null-like phenotypes. Although this may seem to suggest a minor biological role for protease-mediated activation, it may also reflect the general ability of multiple proteases to activate latent TGF- β , creating significant redundancy in vivo.

Many of the MMPs, including MMP2, MMP9, MMP3, MMP13, and MMP14 (MT1-MMP) (Jenkins 2008), activate latent TGF- β in vitro. MMP2 and MMP9 activate latent TGF- β when localized by CD44 to the surface of tumor cells during angiogenesis (Yu and Stamenkovic 2000), and MMP3 from chondrocyte-derived matrix vesicles activates latent TGF- β 1 (Maeda et al. 2001). Interestingly, although MMP2 is present in these chondrocyte vesicles, it did not activate TGF- β in this context.

Mice deficient in many of these proteases have been generated, but none clearly show TGF- β -related phenotypes. Inactivation of *MMP3* yields mice with no visible abnormalities (Mudgett et al. 1998; Johnson et al. 2011), and loss of *MMP13* yields only mild skeletal defects in mice (Stickens et al. 2004). Mice lacking expression of both *MMP2* and *MMP9* are viable (Garg et al. 2009), as are mice lacking *MMP9* and *MMP13* expression (Stickens et al. 2004), although these mice are runted because of defects in their growth plates. *MT1-MMP* (*MMP14*)-deficient mice suffer from osteopenia, dwarfism, and fibrosis, but this is likely a result of deficiencies in collagen turnover and not because of insufficient TGF- β activation (Holmbeck et al. 1999). *MMP2*- and *MT1-MMP*-deficient mice display respiratory failure, abnormal blood vessel development, immature muscle fibers, and die immediately after birth (Oh et al. 2004). However, none of these

phenotypes are reminiscent of defective TGF- β signaling.

The BMP-1/tolloid MMPs also play a role in latent TGF- β activation. However, rather than directly degrading LAP, they catalyze the cleavage of LTBP-1 from the ECM, releasing latent TGF- β bound to LTBP-1, which requires activation by other factors, such as MMPs (Ge and Greenspan 2006). Mice with null mutations in BMP-1 and the closely related tolloid1 (*Tll1*) protease display excessive accumulation of LTBP-1 in embryonic tissues and reduced phospho-Smad2/3 staining, suggesting defective activation of latent TGF- β (Ge and Greenspan 2006). Mice deficient in BMP-1 or *Tll1* expression die before birth and these proteins process a variety of other matrix and signaling molecules; therefore, it is difficult to attain a clear assessment of their importance for latent TGF- β activation in vivo (Pappano et al. 2003).

Various serine proteases, including kallikreins and plasmin, activate TGF- β (Lyons et al. 1990). Plasmin mediates TGF- β activation in cocultures of endothelial cells and pericytes (Antonelli-Orlidge et al. 1989; Sato and Rifkin 1989), in cultures of endothelial cells treated with retinoids (Kojima and Rifkin 1993), and on the surface of activated macrophages (Nunes et al. 1995; Yehualaeshet et al. 1999; Jenkins 2008). Activation in endothelial-pericyte cocultures was inhibited by antibodies to LTBP-1 (Flaumenhaft et al. 1993) and inhibitors of the mannose-6-phosphate receptor (Dennis and Rifkin 1991). Activation by macrophages was dependent on plasmin and latent TGF- β bound at the cell surface by thrombospondin1 (TSP1) and CD36 (Yehualaeshet et al. 1999; Jenkins 2008). Despite the importance of plasmin in several TGF- β -activating cell systems, mice with targeted deletion of the plasminogen gene show no clear developmental abnormalities and are viable (Bugge et al. 1995), which rules out a nonredundant role for this protease in latent TGF- β activation.

Kallikreins are a large family of serine proteases, several of which activate latent TGF- β . Kallikreins appear to play a role in TGF- β -mediated immunosuppression in seminal plasma (Emami and Diamandis 2010) and lipopolysaccharide impairment of liver regeneration

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(Akita et al. 2002). Plasma kallikrein (PLK) cleaves TGF- β 1 LAP between Arg58 and Leu59. Antibodies have been raised against the resulting LAP peptides and used to show that the specific PLK-LAP cleavage products are significantly more abundant in the liver tissue of patients with hepatic fibrosis (Hara et al. 2014). Prostate-specific antigen (PSA), a member of the kallikrein family, activates TGF- β 2 but not TGF- β 1 (Dallas et al. 2005b). The large number of kallikrein proteases again provides significant potential redundancy and, at present, only limited data on kallikrein-null mice are available (Bergaya et al. 2001).

Although many proteases activate latent TGF- β in a variety of contexts, most of these studies have been conducted in vitro, and obtaining clear in vivo data on the role of proteases in latent TGF- β activation is difficult for the reasons discussed above. One way to gain future insight into the significance of protease-mediated latent TGF- β activation in vivo might be to identify specific cleavage sites in LAP or LTBP responsible for activation, and generate mice with mutations replacing these sites with protease-insensitive sequences.

Deglycosylation

Deglycosidases, including endoglycosidase F (Miyazono and Heldin 1989) and influenza neuramidase, activate latent TGF- β (Schultz-Cherry and Hinshaw 1996; Carlson et al. 2010). The LAPs of human TGF- β 1 and - β 2 possess three potential *N*-glycosylation sites, whereas TGF- β 3 LAP has only two sites. It is not clear which sites are important for activation by deglycosidases or what the activation mechanism may be. One possibility is that removing sugar groups exposes protease-sensitive sites, as activation by neuramidase is at least partially protease dependent (Carlson et al. 2010). The in vivo role of deglycosylation-driven latent TGF- β activation also remains undefined.

Other Protein Factors

As well as the molecules discussed above, a diverse range of other proteins have been implicated as latent TGF- β activators.

TSP-1 has been the subject of particularly intense study with respect to latent TGF- β activation. Recombinant TSP-1 activates latent TGF- β when added to cell cultures or purified latent TGF- β in vitro (Murphy-Ullrich and Poczatek 2000). TSP-1 interacts with both LAP and active TGF- β in copurification and pull-down assays (Murphy-Ullrich et al. 1992; Yang et al. 1997; Ribeiro et al. 1999). A KRFL peptide that mimics the RFL sequence between the first and second TSP-1 domains activates latent TGF- β (Schultz-Cherry et al. 1994, 1995) and inhibits TSP-1 binding. The ability of an LSKL peptide to inhibit this activation further suggested that interactions between the RFL motif of TSP-1 and the LSKL motif in the amino terminus of LAP were responsible for TGF- β release (Ribeiro et al. 1999). Consistent with this hypothesis is the observation that TSP-2 does not activate latent TGF- β , as the RFL motif is absent from TSP-2 (Schultz-Cherry et al. 1995).

Although these are interesting results, some groups have failed to observe latent TGF- β activation in response to TSP-1 (Grainger and Frow 2000), and surface plasmon resonance studies failed to show TSP-1 binding to either LAP, TGF- β , or SLC (Bailly et al. 1997). Moreover, platelet α -granules activate latent TGF- β and are a rich source of TSP-1, but α -granules derived from TSP-1-null platelets activate latent TGF- β just as effectively as those from wild-type platelets (Abdelouahed et al. 2000).

TSP-1-null mice suffer from inflammation of the lung and pancreas as well as various other defects (Lawler et al. 1998). The sites of inflammation overlap, in part, with those observed in the multiorgan inflammation phenotype of TGF- β 1 knockout mice (Kulkarni et al. 1993; Crawford et al. 1998). Reduced inflammation was observed in *Tsp1*^{-/-} animals treated with the latent TGF- β -activating KRFL peptide, whereas treating wild-type mice with the LSKL peptide that inhibits activation resulted in increased inflammation (Crawford et al. 1998). However, *Tsp1*^{-/-} mice fail to mimic all of the features of TGF- β 1-null mice, such as cardiac inflammation. *Tsp1*^{-/-} animals also display phenotypes, like kyphosis, not seen in TGF- β 1-null animals. Moreover, although

TSP-1 and the KRFLK peptide-activated latent TGF- β 2 in vitro (Ribeiro et al. 1999), TSP-1-null animals do not display any of the serious defects seen in TGF- β 2 knockout mice. This leaves the debate over the exact role of TSP-1 in vivo unresolved, but rules out TSP-1 as a central activator of all three TGF- β isoforms.

F-spondin (or spondin-1) increases TGF- β activity in cartilage explants (Attur et al. 2009). An antibody targeted to the F-spondin thrombospondin type 1 repeats blocks this activation, suggesting parallels with activation by TSP-1. However, more information is needed on whether F-spondin activates latent TGF- β by binding directly to LAP or if some other mechanism is at work in these cultures.

Neuropilin (Nrp) is a cell-surface protein reported to act as an activator of latent TGF- β on the surface of T cells (Glinka and Prud'homme 2008; Glinka et al. 2011) and in some tumor lines (Glinka et al. 2011). The mechanism by which Nrp activates latent TGF- β remains undefined, but in solid-phase assays Nrp binds mature TGF- β 1, LAP, and both type I and II TGF- β receptors (Glinka et al. 2011). Nrp may initiate intracellular signaling events and could act as a co-receptor for TGF- β (Glinka and Prud'homme 2008; Glinka et al. 2011; Grandclement et al. 2011).

Pregnancy specific glycoprotein 1 (PSG1) is another protein with no known enzymatic activity recently shown to activate both latent TGF- β 1 and 2. PSG1 also inhibits dextran sodium sulfate-induced colitis in a TGF- β -dependent manner (Blois et al. 2013). However, both the molecular mechanism and in vivo significance of this activator remain unknown. Tenascin-X has also been reported to promote epithelial to mesenchymal transition by activating TGF- β through interactions between its fibrinogen-like domain and the SLC. These interactions may deform LAP and also rely on cell adhesion via integrin $\alpha_{11}\beta_1$ to promote activation (Alcaraz et al. 2014).

Activation by Physicochemical Factors

Latent TGF- β can be activated by exposure to specific physical or chemical conditions, in-

cluding detergents, ionizing, and ultraviolet (UV) radiation (Barcellos-Hoff et al. 1994; Barcellos-Hoff 1996; Ehrhart et al. 1997; Wang and Kochevar 2005; Anscher et al. 2006; Biswas et al. 2007), reactive oxygen species (ROS) (Barcellos-Hoff and Dix 1996), heat (Brown et al. 1990), physical shear (Ahamed et al. 2008), and extremes of pH (Lyons et al. 1988).

Not all of these conditions are encountered in vivo, but some may be biologically relevant. For example, ROS activate latent TGF- β both in cell cultures and in cell-free systems (Barcellos-Hoff and Dix 1996), and ROS are proposed to mediate latent TGF- β activation by asbestos, which can lead to mesothelioma in exposed lungs (Pociask et al. 2004). ROS produced by HIV-infected regulatory T cells are also proposed to activate latent TGF- β and lead to immunosuppression (Amarnath et al. 2007). ROS may mediate latent TGF- β activation caused by ionizing (Barcellos-Hoff 1996) and UV-B radiation (Wang and Kochevar 2005). One study suggested that ROS activation was specific to TGF- β 1 and relied on the presence of Met253 (Jobling et al. 2006), but this residue is not well conserved (Robertson and Rifkin 2013) and the exact mechanism by which ROS activate latent TGF- β is unclear. One possibility is that ROS catalyze direct breaks in the backbone of LAP to release the active growth factor (Barcellos-Hoff and Dix 1996), but it is not known if LAP is particularly sensitive to ROS-driven scission.

Latent TGF- β activation by low pH could play a physiological role in some circumstances. Osteoclasts reduce extracellular pH to ~ 4.5 during bone resorption (Teitelbaum 2000), and these cells activate latent TGF- β (Oreffo et al. 1989). Tumor cells also significantly lower the pH of their microenvironment (Jullien et al. 1989). However, whether the drop in pH is both necessary and sufficient for latent TGF- β activation in this later context has not been clearly shown. Lactic acid also promotes TGF- β activity at physiological concentrations and may be a player in idiopathic pulmonary fibrosis (Kottmann et al. 2012). But, although the ability of lactate to up-regulate α -smooth muscle actin was dependent on TGF- β signaling and the lowered pH of the medium, studies of lactic

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acid in other contexts have shown that lactate up-regulates genes that may affect TGF- β bio-availability (Seliger et al. 2013). The effects of physiological levels of lactic acid on latent TGF- β in a cell free system have not yet been reported.

Physical shear or stirring can activate a significant percentage of the latent TGF- β present in platelet releasate (Ahamed et al. 2008). This reaction is dependent on LTBP binding to LAP, as activation is not seen with the releasate from C33S TGF- β 1 mice (Ahamed et al. 2008). The full activation mechanism is not known, but it is inhibited by the thiol isomerase-binding peptide mastoparan and appears to involve disulfide exchange (Brophy et al. 2013).

Although there is compelling evidence for many physicochemical factors playing important roles in TGF- β biology, the current lack of detailed activation mechanisms and specific residues to target for knockin studies prevent us from testing their true significance in vivo.

ACTIVATION OF TGF- β —AN EVOLUTIONARY PERSPECTIVE

The range of latent TGF- β activation mechanisms may seem daunting, especially when trying to consider what the trigger for TGF- β activity may be in different tissue contexts. In vivo studies knocking out specific activators or key activation sequences cast some light on their relative significance. However, these experiments are difficult and time consuming and so there remain many potential activators whose significance has not yet been tested.

An alternative approach to qualitatively gauge the significance of various latent TGF- β activators is to examine the evolutionary time frames in which they emerge and to analyze the conservation of residues critical to specific activation mechanisms.

This evolutionary analysis has been performed for a variety of established latent TGF- β activators and has provided a number of insights (Fig. 4) (Robertson et al. 2011; Robertson and Rifkin 2013). One interesting observation is that bona fide TGF- β seems to have coevolved with LTBP and that the key sequence features for the attachment of LTBP and LAP are present

in the most evolutionary distant TGF- β and LTBP sequences, including those in sea urchins, lancelets, and acorn worms (Robertson et al. 2011; Robertson and Rifkin 2013). Additionally, integrin-binding RGD motifs are also present in these evolutionary distant TGF- β sequences and are highly conserved through to humans (except in TGF- β 2, where they are consistently absent).

These facts suggest that all elements required for traction-induced latent TGF- β activation by integrins were present soon after “true” TGF- β diverged from the rest of the TGF- β family. If integrins were initially the main activators of latent TGF- β , this would be consistent with their continued in vivo significance, as observed in mouse models (Yang et al. 2007; Aluwihare et al. 2009). On the other hand, the evolutionary data do not support an early evolving function for TSP-1, plasmin, or many other specific proteases, which in many cases do not seem to emerge until well after the LTBP–TGF- β complex first appeared (Robertson and Rifkin 2013).

If we make the assumption that the earliest evolving latent TGF- β activators have remained important throughout evolution, traction-mediated activation by integrins may continue to be an important mechanism for releasing TGF- β in many tissue contexts. Therefore, we propose that future studies examining the role of TGF- β in development and homeostasis should give careful consideration the dynamic relationship between cells and their biomechanical environment.

POSTACTIVATION BIOAVAILABILITY OF TGF- β AND OTHER TGF- β FAMILY MEMBERS

One generalization that distinguishes bona fide TGF- β from many other members of the family, such as the BMPs and GDFs, is that TGF- β bio-availability is primarily regulated by binding and release from LAP, which locks up TGF- β before it leaves the cell. However, the biological regulators characterized for BMPs and GDFs generally bind to the active forms of these growth factors after they have been secreted.

Regulation of the Bioavailability of TGF- β Evolutionary history of TGF- β and its regulatory proteins

Figure 4. Evolution of transforming growth factor β (TGF- β) and its extracellular regulators. Phylogenetic tree highlighting the evolutionary relationships between a selection of organisms with sequenced genomes. Green arrows show where specific proteins appear to have evolved based on their presence or absence from available genome and other sequence libraries. Red crosses indicate where specific genes appear to have been lost. The variable quality of some shotgun genome sequences means that the absence of a gene from a specific species is not always definitive, as some genes may be missed or fragmented in the available sequence data. This introduces some uncertainty to the evolutionary story, but ambiguity is kept to a minimum by searching through the many different genomes available. GARP, Glycoprotein A repetitions predominant protein; TSP1, thrombospondin 1; LTBP, TGF- β -binding protein; MMP, matrix metalloproteinase; *H. sapiens*, *Homo sapiens*; *G. gallus*, *Gallus gallus*; *A. carolinensis*, *Anolis carolinensis*; *X. tropicalis*, *Xenopus tropicalis*; *L. chalumnae*, *Latimeria chalumnae*; *D. rerio*, *Danio rerio*; *P. marinus*, *Perkinsus marinus*; *C. intestinalis*, *Ciona intestinalis*; *B. floridae*, *Branchiostoma floridae*; *S. purpuratus*, *Strongylocentrotus purpuratus*; *S. kowalevskii*, *Saccoglossus kowalevskii*; *N. vectensis*, *Nematostella vectensis*; *T. Adhaerens*, *Trichoplax Adhaerens*; *A. Queenslandica*, *Amphimedon Queenslandica*.

The release of certain BMPs and GDFs by specific cells can generate morphogen gradients, and their antagonists help to localize and confine the activity of these growth factors once in

the extracellular space. These regulation mechanisms have been discussed in more detail elsewhere (Hinck et al. 2016; Kim et al. 2016), but they are mentioned here in passing to highlight

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key conceptual differences between signaling by bona fide TGF- β and signaling by other members of the family.

The simplified concepts of growth-factor signaling by localized activation or morphogen gradients are shown in Figure 5. In a morphogen gradient, the active growth factor is released by a specific cellular source and its concentration reduces as it diffuses away from that source. Further control may also be introduced by antagonists that prevent the diffusing growth factors from binding their receptors and add further 3D structure to the growth-factor gradient. The active concentration of a growth factor in any given region of the morphogen gradient can provide individual cells with an indication of their relative position within a developing organism, which may help define their niches within a tissue. In the localized activation model, on the other hand, the growth factor is predistributed throughout the ECM in its latent state. Its local concentration is determined by the rate of activation rather than its rate of release by cells and its diffusion from a central source. One might speculate that this mode of activation may allow bona fide TGF- β to act as a more direct readout of local extracellular events rather than a long distance signal of developmental status.

Although we currently consider factors that regulate TGF- β latency and activation to be the key controllers of its signaling, there are also a variety of proteins that bind and regulate active TGF- β after its release from LLC. For example, the proteoglycan decorin binds active TGF- β (Hildebrand et al. 1994; Schonherr et al. 1998) and appears to inhibit TGF- β signaling in many contexts (Isaka et al. 1996; Teicher et al. 1997), although early studies of decorin suggested that it could enhance TGF- β bioactivity in the bone matrix (Takeuchi et al. 1994). Decorin can inhibit TGF- β signaling through calcium-dependent phosphorylation of Smad2 at Ser240 (Abdel-Wahab et al. 2002), and may interface with TGF- β signaling on a more complex level than just binding the active growth factor. In the bone marrow stroma, both decorin and biglycan appear to work together to inhibit TGF- β signaling and maintain sufficient osteoblast

proliferation (Bi et al. 2005). Other molecules that may interact with active TGF- β include collagen IV in the basement membrane (Paralakar et al. 1991), human IgG (Stach and Rowley 1993; Bouchard et al. 1995), and the cell-surface proteins β glycan (also known as TGF- β type III receptor) and endoglin, which may play a role as TGF- β co-receptors.

It is important to consider that TGF- β is a “sticky” molecule that associates nonspecifically with hydrophobic surfaces. TGF- β is active at very low concentrations and copurifies with a number of His-tagged proteins when they are expressed and purified from HEK293 cells (Kaur and Reinhardt 2012), as well as with gelatin-purified fibronectins (Fava and McClure 1987). These properties further complicate interpretation of in vitro experiments, and underline the importance of in vivo studies to confirm proposed biochemical mechanisms.

To ensure that the TGF- β signal does not persist after activation, mature TGF- β is rapidly cleared from the extracellular space. α_2 -Macroglobulin binds TGF- β and may mediate its endocytosis (LaMarre et al. 1990, 1991). However, α_2 -macroglobulin knockout mice have no clear defects to suggest excessive accumulation of active TGF- β in tissues (Umans et al. 1995), and so the full mechanisms by which active TGF- β is cleared from the ECM remain a mystery.

Although the biological data on how active TGF- β is regulated after its release from LAP remain patchy, antagonists of other members of the TGF- β family have been well described and shown to play significant roles in vivo. Myostatin (GDF-8) and activin, for example, are more closely related to TGF- β than many other TGF- β family members (Burt and Law 1994; Herpin et al. 2004), and although not much is known about the significance of their pro-peptides in their regulation, follistatin and follistatin-related proteins play a significant role in controlling these two growth factors by binding and blocking their interaction with receptors (Walton et al. 2011). The interplay of myostatin and follistatin has been particularly well examined in the context of muscle growth, where loss of myostatin causes widespread muscle hypertrophy and hyperplasia (McPherron

A Morphogenic gradient

Growth factor diffuses away from source

Cells expressing antagonist help control and shape gradient

B Latent cytokine activation

Figure 5. Conceptual mechanisms for the extracellular regulation of transforming growth factor β (TGF- β) and bone morphogenetic protein (BMP)/growth and differentiation factor (GDF) compared. Growth factors may convey information to cells in a number of ways. Shown here are some hypothetical schemes for ways in which active growth factors may be distributed in the extracellular matrix (ECM). Cells are colored purple to indicate their responsiveness to growth-factor signaling, or light blue if unresponsive. (A) Here, cells are provided with a measure of their position relative to the source of the growth factor, as its concentration will decrease when diffusing away from the site of release. Antagonists further control this gradient, which becomes even more relevant when considering the three dimensions of a developing tissue. Growth factors are light green and antagonists light brown. (B) Here, the growth factor does not come from a localized source but is distributed through the matrix in a latent state. The local concentration of growth factor is determined by the availability of activators to release it from this state. Also, depending on how quickly the growth factor is cleared from the extracellular space, it may not have time to diffuse far, which could limit gradient formation. Growth factors are red, whereas the ECM and proteins that anchor them are dark green. These different concepts could be important when considering growth-factor signaling from a systems biology perspective.

et al. 1997). Transgenic overexpression of follistatin in skeletal muscle causes similar muscle overgrowth (Lee and McPherron 2001), but follistatin also stimulates muscle hypertrophy through myostatin-independent mechanisms (Winbanks et al. 2012). Follistatin-null mice display multiple developmental defects, reflecting its important role in regulating bioavailability of both activin and myostatin (Matzuk et al. 1995).

A diverse array of other proteins is hypothesized to regulate the bioavailability of TGF- β family members by binding and sequestration. Unlike TGF- β , many BMPs are biologically active when they are secreted from cells, and form morphogenic gradients (Gazzerro and Canalis

2006). A number of BMP antagonists share little clear sequence homology, but several contain a “cystine-knot” structural motif, which is a highly versatile fold that often forms dimers and is found in a diverse selection of ECM proteins. For example, cystine-knot motifs also comprise the growth factor domains of the TGF- β family (Vitt et al. 2001), suggesting a common, if extremely distant, evolutionary ancestor for both the TGF- β family and many of its antagonists. Noggin, chordin, and DAN family proteins all interact with multiple members of the BMP family and are crucial in the biology of these signaling proteins.

There are clearly a large number of both TGF- β family members and their antagonists.

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The full significance of this diversity is far from well understood, and the ability of multiple TGF- β family members to bind multiple antagonists and receptors introduces significant potential for redundancy, multifunctionality, and the formation of complex protein gradients in a developing organism. This complexity may, in turn, play an important role in directing the elaborate patterns adopted by our organs and tissues during development.

CONCLUDING REMARKS

The bioavailability of TGF- β and TGF- β family members is regulated in complex and diverse ways. It is rarely appropriate to consider these growth factors as simple autocrine or paracrine signals sent from one cell to another. Instead, they must be considered in the broader structural context of the ECM, both in terms of how they may be sequestered, stored, and concentrated although interactions with insoluble matrix components, and also how these growth factors will be regulated by gradients and localization of specific inhibitors and activators.

This field poses many complex challenges for future study, which will require extensive and coordinated efforts to decipher the key mechanisms at work and their significance in health and disease. However, the considerable therapeutic potential of modulating TGF- β family signaling in an extracellular context makes this research a particularly pressing endeavor.

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