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Latent transforming growth factor-β: Structural features and mechanisms of activation

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Latent transforming growth factor-β: Structural features and mechanisms of activation. Transforming growth factors-β are cytokines with a wide range of biological effects. They play a pathologic role in inflammatory and fibrosing diseases such as nephrosclerosis. TGF-βs are secreted in a latent form due to noncovalent association with latency associated peptide (LAP), which is a homodimer formed from the propeptide region of TGF-β. LAP is disulfide linked to another protein, latent TGF-β binding protein (LTBP). LTBP has features in common with extracellular matrix proteins, and targets latent TGF-β to the matrix. Activation of latent TGF-β can be accomplished *in vitro* by denaturing treatments, plasmin digestion, ionizing radiation and interaction with thrombospondin. The mechanisms by which latent TGF-β is activated physiologically are not well understood. Results to date suggest an important role for proteases, particularly plasmin, although other mechanisms probably exist. A general model of activation is proposed in which latent TGF-β is released from the extracellular matrix by proteases, localized to cell surfaces, and activated by cell-associated plasmin.

Transforming growth factors-β (isoforms 1, 2 and 3) are mammalian cytokines involved in development, proliferation, angiogenesis, regulation of inflammation, extracellular matrix production, integrin expression, protease activity and apoptosis [1, 2]. Evidence from animal and clinical studies indicates they play important etiologic roles in various inflammatory and fibrotic diseases, including nephrosclerosis [3]. These cytokines belong to the TGF-β superfamily, which in mammals includes Mullerian inhibitory substance, activins, inhibins and bone morphogenic proteins, and is characterized by a distinctive "cysteine knot" structure [4, 5].

The TGF-βs are notable among cytokines because of the number of ways in which their bioavailability is regulated by additional proteins. Newly synthesized TGF-β is released in a latent form (that is, a form in which it cannot interact with TGF-β receptors) due to noncovalent association with the TGF-β propeptide homodimer, also termed latency associated peptide (LAP). TGF-β must be liberated from this complex before it can exert its actions, a process termed activation. Latent TGF-β can be targeted to the extracellular matrix (ECM) by a second molecule, latent TGF-β binding protein (LTBP). One LTBP monomer is disulfide linked to LAP. Finally, free TGF-β can interact with and be inactivated by a number of soluble or matrix molecules, including alpha$_2$-macroglobulin, decorin, betaglycan, heparin, and fucoidan [6–9] These interactions are thought to prevent TGF-β from diffusing from loci of activation and thereby exerting effects in inappropriate sites. TGF-β in plasma is found almost exclusively bound to alpha$_2$-macrogloblin and presumably represents previously activated TGF-β that will be cleared by the liver [6]. The processes of activation, localization and subsequent inactivation by extracellular molecules are reminiscent of the control of protease systems (such as the plasminogen/plasminogen activator and coagulation systems) in which proteases are activated when required, concentrated at the cell surface, and inactivated by a variety of soluble inhibitors. As discussed below, there is evidence that TGF-β activation is also localized to cell surfaces.

Conditions under which cells secrete TGF-β in a completely free form have not been described, and the concentration of free TGF-β in equilibrium with the LAP-bound form is probably physiologically negligible (the apparent K_d of this interaction being ~1 nM) [10]. Thus, activation of latent TGF-β must be a major regulatory step controlling TGF-β effects. Although a variety of activation mechanisms have been established in cell culture and *in vitro* systems, almost nothing is known about how TGF-β is activated *in vivo*. Indeed, the precise mechanism of activation in certain cell culture systems remains unknown despite intense investigation. Why is this a refractory problem? First, activation of TGF-β can be difficult to detect. Typically, conditioned medium from the cells being tested is employed in bioassays or immunological assays of active TGF-β. However, if TGF-β is efficiently activated at cell surfaces and rapidly cleared from the medium by specific receptors, little TGF-β will remain free for detection. The detection of active TGF-β is even more difficult *in vivo*, although recently two techniques for recognizing active TGF-β in tissue sections have been reported [11, 12]. Second, it is likely that there are multiple mechanisms of activation that may operate simultaneously. Third, a given mechanism may involve multiple steps (such as release of latent TGF-β from matrix, modification of the latent complex, and association of latent TGF-β with the cell surface), any of which might be accomplished in more than one fashion. Finally, in a cell culture system, the activation of TGF-β can lead to a down-regulation of the activating pathway, as when active TGF-β results in increased levels of plasminogen activator inhibitor-1 (PAI-1), leading to down-regulation of a plasmin-mediated activation pathway [13].

This paper reviews the structure of latent TGF-β, mechanisms

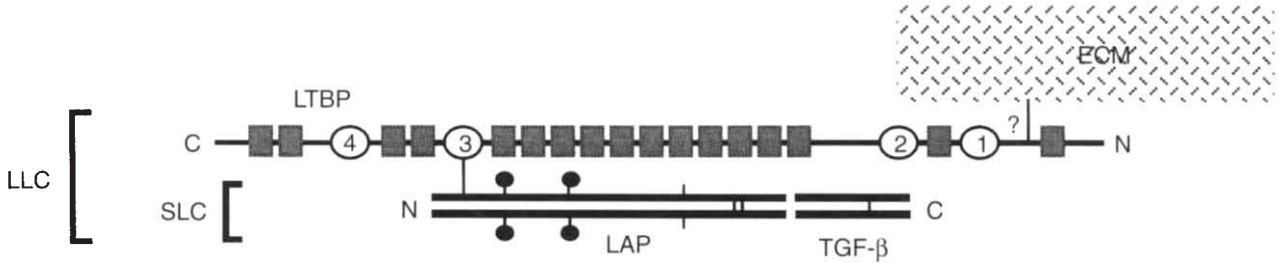

Fig. 1. *Schematic representation of latent TGF-β.* The putative tissue transglutaminase-mediated covalent bonding between the N-terminus of LTBP and the ECM is indicated by a question mark (?). Disulfide bonds between LAP monomers, between TGF-β monomers and between LAP and LTBP are indicted by thin lines. Carbohydrate residues on LTBP are not shown. Abbreviations are in the text. Symbols are: (|) carbohydrate; (ϕ) mannose-6-phosphate; (○) 8-cysteine domain; (■) EGF-like domain.

of activation identified by our lab and others, and questions that remain to be addressed.

Structure of latent TGF-β

The free TGF-β cytokines are 25-kDa homodimeric proteins. (Small amounts of TGF-β heterodimers can be detected, such as in platelets.) TGF-β associated with LAP is termed the small latent complex (SLC). If LAP is disulfide linked to latent TGF-β binding protein (LTBP), the resultant complex is termed the large latent complex (LLC). These associations are depicted in Figure 1.

LAP has several important structural features. Like TGF-β, LAP is a homodimer, which is formed within the secretory pathway after the removal of a signal sequence and cleavage from the mature TGF-β peptide sequence at a processing site containing basic amino acids. In addition to rendering TGF-β latent, LAP enhances the proper folding and secretion of the cytokine [14]. Other members of the TGF-β superfamily have similarly sized propeptide sequences. These sequences have little homology to the TGF-β propeptides, and although they also play a role in the proper folding and secretion of the active factors [14], no other functions have been ascribed to them. There are three cysteines per LAP-1 monomer. The LAP-1 monomers are thought to be joined by intrachain disulfide bonds involving the two cysteines located near the C-termini (at amino acids 223 and 225). A third cysteine near the N-terminus (amino acid 33) is disulfide-bonded to LTBP [15]. The other two isoforms of LAP contain three cysteines in equivalent locations along with two additional cysteines of unknown function. Each monomer of LAP (isoform 1) contains three N-linked carbohydrate residues, two of which contain mannose-6-phosphate (M6P) [16]. LAP can associate with the insulin-like growth factor II/mannose-6-phosphate receptor (IGF-II/M6PR) [17] via interactions with these M6P residues, suggesting a mechanism by which latent TGF-β can be localized to cell surfaces. The IGF-II/M6PR is required for activation of latent TGF-β in cocultures of endothelial and smooth muscle cells [18]. Because M6P residues target newly synthesized proteins to the lysosomes, it is important to understand how latent TGF-β escapes this fate. One possibility being examined in our laboratory is that LTBP in the LLC masks the M6P residues; alternatively, TGF-β itself or a specific conformation of LAP might mask the M6P residues within the secretory pathway. Isoforms 1 and 3 of LAP each contain one arginine-glycine-aspartate (RGD) sequence per monomer, which might also function to localize latent cytokine to the cell surface by binding RGD-recognizing integrins. Recently, Grainger and colleagues have reported indirect evidence that the RGD sequence in platelet-derived SLC may be recognized by platelet integrins [19]. However, no other reports that these RGD sequences are capable of binding integrins have appeared.

The three dimensional structure of LAP has not been reported, so information about its interaction with TGF-β is indirect. Recombinant LAP expressed by itself is secreted from mammalian cells and is able to bind and inactivate recombinant TGF-β [14, 20]. We (J.G. Harpel and D.B. Rifkin, unpublished observations) and others [21] have found that recombinant LAP produced by insect cells is also active, both *in vitro* and *in vivo*. Mutational analysis indicates that regions near the N-termini are important for interacting with TGF-β [22], and circular dichroism measurements indicate that recombinant LAP undergoes significant conformational change on binding to TGF-β [23].

As discussed below, plasmin can activate latent TGF-β (LLC) by acting on LAP [24, 25]. The exact proteolytic site(s) for this activation have not been determined. Studies by Lyons et al using immunoblots with antibodies specific for different regions of LAP suggest that the site is near the N-terminus [25]. However, a different site appears to be preferentially cleaved when plasmin acts on free LAP (J.S. Munger, unpublished observation).

LTBP (now known as LTBP-1, see below) is a 125 to 190 kDa glycoprotein [26]. The range of observed sizes is thought to be due to differences in proteolytic processing. In free form, it assumes a linear shape as revealed by rotary shadowing (P-E. Gleizes, D.R. Keene and D.B. Rifkin, unpublished observations). It has a distinctive structure closely related to that of the fibrillins. This structure consists of multiple epidermal growth factor (EGF)-like domains, and four domains that contain up to eight cysteines in a conserved arrangement. The latter domains, termed eight cysteine domains (although the first such domain in LTBP contains only 7 cysteines), are unique to the LTBPs and fibrillins. Miyazono et al have reported that LTBP enhances the secretion of latent TGF-β via formation of the LLC [27]. Taipale et al [28] have proposed the existence of a protease-sensitive "hinge" region. Cleavage at this site may be involved in protease-mediated release of LLC from the matrix, with which LTBP-1 can associate (see below).

LTBP-2 and -3 are recently described molecules [29, 30] with similar structures that can also form LLCs with TGF-β 1 when overexpressed in mammalian cells. The relative biological roles of the three isoforms, including any role they may play as matrix proteins unassociated with TGF-β, remain unclear. Bonewald and colleagues have suggested that LTBP-1 has a distinct role as an ECM protein [31], and conversely have shown that certain cells can secrete predominantly the SLC form of TGF-β [32]. Thus,

TGF-β bioavailability may be governed in part by whether the LLC is formed and, if so, which LTBP isoform is involved. Although in most cell systems in which the latent complex has been defined TGF-β is found as a LLC containing LTBP-1, in most *in vivo* settings and cell culture systems the proteins with which TGF-β is associated remain undefined.

LLC produced by cells in tissue culture is secreted into the medium and is also incorporated into the matrix. Recently a form of LTBP-1 with an extended amino terminus due to alternate mRNA splicing, termed LTBP-1L, has been described [33]. LTBP-1L associates more readily with the matrix than does the previously described form (LTBP-1S). LTBP can be released from matrix by protease digestion but not by denaturing treatments [28, 34] (and I. Nunes, unpublished observations), which suggests that LTBP is covalently linked to the matrix. Work by our laboratory suggests that this cross-linking may be mediated by the enzyme tissue transglutaminase (tTGase). As discussed below, inhibitors of tTGase block TGF-β activation by cocultures of endothelial and smooth muscle cells [35]. Also, Nunes et al (unpublished observations) have found that transglutaminase inhibitors and antibodies to the N-terminus of LTBP-1 block incorporation of LTBP-1 into the matrix formed by HT1080 fibrosarcoma cells. Together, these observations suggest that covalent association of LLC to the ECM is an important context for activation.

Recently, Saharinen et al and Gleizes et al [15, 36] have shown that the third 8 cysteine domain is necessary and sufficient for the disulfide bonding of LAP and LTBP-1. The number of bonds between domain 3 and LAP is not yet known. This is the first reported function for these domains. These observations suggest that the eight cysteine domains may be involved in protein-protein interactions.

Like several other matrix proteins, human LTBP-1 contains an RGD sequence. However, there are no reports that this sequence serves as an integrin ligand, and two facts argue that it is nonfunctional. First, fibrillin-1 contains RGD motifs that can mediate cell binding [37], but these are located in eight cysteine domains, whereas the sole RGD sequence in hLTBP-1 is located in an EGF-like domain. Second, the RGD in hLTBP-1 is not present in the rat homologue of LTBP-1.

How does the intact large latent complex function in activation? The structural features described above suggest the following general concepts. First, LTBP acts to target latent TGF-β to sites in the matrix where it remains until activated. (In some cases, latent TGF-β may be released as SLC to avoid matrix sequestration). Latent TGF-β can be released from the matrix by limited proteolysis of LTBP by proteases such as thrombin, chymase, elastase and plasmin, as might occur during matrix remodeling or inflammation. Structural features of LAP and/or LTBP might allow this soluble form of latent TGF-β to associate with the cell surface where TGF-β would be released from LAP. The final mechanisms for TGF-β release from LAP are not known, and, as discussed below, might involve proteolytic and/or nonproteolytic events.

Activation of latent TGF-β

How does activation occur? Studies addressing this question employ either *in vitro*, cell culture, or *in vivo* systems. In general, *in vitro* experiments reveal only direct activators of latent TGF-β. In contrast, cell and *in vivo* systems might identify both preliminary steps in activation (such as release of matrix-bound TGF-β) and activation *per se*. One cannot necessarily distinguish between indirect and direct effects on activation. For example, the observation that inhibitors of plasmin block activation in a cell culture system does not distinguish between the action of plasmin to release latent TGF-β from matrix reservoirs and the direct cleavage of LAP resulting in activation. Table 1 lists known conditions for activating TGF-β. These conditions are briefly discussed below.

Table 1. Mechanisms of latent TGF-β activation

	In vitro	Cell culture	*In vivo*
Nonproteolytic	heat detergents pH extremes chaotropes radiation thrombospondin-1 deglycosylation sialic acid, M6P	coculture[a] matrix vesicles	radiation?
Proteolytic	plasmin cathepsin D	coculture (plasmin)[a] other proteases	plasmin?

[a] Activation by cocultures of endothelial cells with pericytes or smooth muscle cells; activation involves proteolytic and nonproteolytic events.

Denaturing conditions

TGF-β is more stable than LAP under a number of denaturing conditions [38]. Thus, latent TGF-β can be activated by denaturing by heat, chaotropes, detergents, and extremes of pH. These conditions are useful in assays of total TGF-β; heating and acidification are commonly used for this purpose. It is unlikely that such mechanisms operate *in vivo*, with two possible exceptions. First, Lyons, Keski-Oja and Moses [24] reported that some activation occurs *in vitro* in the pH 4 to 5 range (albeit less than at pH 2), which suggests that activation of TGF-β might occur in physiologically acidified zones, such as along the ruffled border of the osteoclast. Indeed, activation by cultured osteoclasts occurs [39, 40]. Second, TGF-β might be activated in areas of thermal burns.

Radiation

Barcellos-Hoff and Dix [41] have found that reactive oxygen species, generated by either ionizing radiation or metal-catalyzed ascorbate oxidation, can activate latent TGF-β. This suggests that there is a redox-sensitive element in LAP, which is yet to be defined but might be one or more of the cysteines. These *in vitro* results are consistent with immunologic evidence of rapid (within 1 hr) activation of TGF-β in irradiated breast tissue [12]. Whether this system operates *in vivo* only under the extreme conditions of ionizing radiation or also under physiological variations in tissue redox potential needs to be determined.

Thrombospondin

Thrombospondin-1 (TSP-1) is a trimer of disulfide-linked 180-kDa subunits found at high concentration in platelet alpha-granules and also produced by a number of other cell types. It is an adhesive protein with a number of domains available for binding to cell surface or matrix proteins. Murphy-Ullrich and

colleagues have reported that TSP-1 isolated from human platelets is tightly associated with TGF-β that can be released at high pH, and that latent TGF-β is nonproteolytically activated by TSP-1 *in vitro* [42]. TSP-1 also activates TGF-β in cell culture assays when added to endothelial cells. This activation appears to be related to binding of TSP-1 to latent TGF-β at the so-called type I (properdin-like) domains of TSP-1 [43]. There may also be an important interaction with LAP as antibodies against a region near the N-terminus of the LAP monomers block activation. These results have been extended by Souchelnitskiy, Chambaz and Feige [44], who have shown that both TSP-1 and -2 can activate LLC, and that neither can activate TGF-β bound to alpha$_2$-macroglobulin.

These observations suggest that TSP may play an important role as an activator or stabilizer of TGF-β. However, other observations suggest that the situation is more complex. For example, cultures of sparse, rapidly growing cells produce more TSP-1 than confluent, growth-arrested cells; however, because TGF-β inhibits the proliferation of these cell types one might expect the opposite relationship between proliferation and TSP-1 levels [45]. Also, although both TSP-1 and TGF-β can affect angiogenesis, some reported effects of TSP-1 on angiogenesis are independent of TGF-β [45].

Deglycosylation

Miyazono and Heldin reported that latent TGF-β (LLC) can be activated by enzymatic removal of carbohydrate moieties on LAP [46]. This suggests that carbohydrate structures are important in the interaction of LAP and TGF-β and, in fact, competing concentrations of sialic acid and M6P are also able to activate latent TGF-β [46]. It is not known if this represents a physiological activation mechanism. However, cells such as activated macrophages, in which sialidase activity is induced, might use such a mechanism.

Proteases

Lyons et al showed that two proteases, plasmin and the lysosomal serine protease cathepsin D, can activate LLC *in vitro* [24]. Some LLC remained unactivated in these experiments, suggesting that there are two or more populations of LLC with differing sensitivities to plasmin. Immunoblotting with site-specific antibodies suggested that the important proteolytic event occurs near the N-terminus [25]. Taipale et al have shown that several proteases (plasmin, thrombin, neutrophil elastase and mast cell chymase) can readily release LTBP (as latent TGF-β) from matrix produced by cells in tissue culture [47]. This occurs by cleavage at a specific protease-sensitive site in LTBP, an event that separates a truncated form of LLC from the N-terminus of LTBP that remains bound to the matrix.

Thus, plasmin might promote TGF-β activation in two ways: by liberating a form of latent TGF-β from storage sites in the matrix, and by directly activating this latent TGF-β. What are the relative rates of these different actions by plasmin? Experiments in our laboratory, using recombinant LLC in solution exposed to plasmin, indicate that the truncation of LTBP occurs much more rapidly than the degradation of LAP (J.S. Munger, unpublished observations). Further questions need to be answered to understand the role of plasmin in activation; for example, can LAP's sensitivity to plasmin be modulated, perhaps by conformational changes induced by binding to cell surface proteins?

Cocultures of endothelial cells and smooth muscle cells

Antonelli-Orlidge and colleagues reported in 1989 that cocultures of capillary endothelial cells and pericytes activate latent TGF-β produced by the cells; monocultures of the same cells produced only latent TGF-β, and close cell contact was required [48]. Our laboratory obtained similar results, using an assay system in which serum-free conditioned medium from mono- or cocultures of endothelial cells and smooth muscle cells was assayed for its ability to inhibit endothelial cell migration in a TGF-β-dependent manner [49]. This system has now been studied extensively.

Several results indicate that plasmin is required for activation in this coculture system. Both plasmin inhibitors and depletion of plasminogen from the culture medium block activation [13]. (Note that because the assays are done in serum-free conditions, the source of plasminogen used in activation is cell- or matrix-associated plasminogen derived from previous culture in serum-containing medium.) Lipoprotein (a), which contains regions homologous to plasminogen and can displace plasminogen from cellular binding sites, also blocks activation [50]. Our results are consistent with both a direct activating effect of plasmin on latent TGF-β, and indirect effects (such as release of latent TGF-β from matrix stores). The major effect of plasmin is not likely to be simply the liberation of latent TGF-β from matrix, because the conditioned medium of the cultures already contains concentrations of soluble latent TGF-β in excess of 1 ng/ml. However, the truncated, released form of LLC may be more readily activatable than the LLC. Because of increased PAI-1 levels due to TGF-β, this activation system is self-regulating. TGF-β is formed within the first 12 hours of the coculture. Within the same time frame, PAI-1 levels increase 20-fold, and no further TGF-β activation occurs after this period unless antibodies to PAI-1 are added.

There are three other requirements for activation in this system. First, LTBP-1 is necessary since antibodies to LTBP-1, and exogenously added excess recombinant LTBP-1, block activation [51]. The underlying mechanism is not understood. Second, interference with ligand binding to the IGF-II/M6PR by addition of M6P or antibodies to the receptor blocks activation [18]. Again, the mechanism of this effect is not known. However, because LAP contains M6P residues and has been reported to bind to the IGF-II/M6PR, we have postulated that latent TGF-β is localized to the cell surface by binding the IGF-II/M6PR where it can be efficiently activated by cell-associated plasmin. Third, the cross-linking enzyme tissue transglutaminase is involved since both nonspecific and specific inhibitors of this enzyme block activation [35]. Thus, association of LLC to the matrix may be an important preliminary step in activation.

It is not yet known why coculture is required. Although each cell type produces all of the reactants described above, the levels of specific molecules vary, and the combination of two cell types may be important for this reason. Other activation systems with similar characteristics have been reported. For example, coculturing carbon tetrachloride-exposed hepatic fat-storing cells with sinusoidal endothelial cells results in TGF-β activation associated with IGF-II/M6PR expression [52]. Also, retinoid-treated endothelial cells and endotoxin-treated peritoneal macrophages can activate TGF-β via a similar plasmin-based mechanism in monoculture [53, 54].

Table 2. Activation mechanisms in cell culture systems

Cell type	Stimulus	Mechanism	Reference
Endothelial cells	retinoic acid	plasmin	[53]
	bFGF	plasmin	[67]
Osteoblast	glucorticoid	proteases	[60]
Osteoclast	(none)	proteases	[39]
Chondrocyte	Vitamin D_3	vesicles	[61]
Gastric carcinoma	(none)	protease	[75]
Macrophage	endotoxin	plasmin	[54]
	bleomycin	plasmin	[57]

Other cell culture systems

There are over one hundred reports of cell culture systems in which TGF-β is activated. In most of these reports, the mechanism by which TGF-β is activated was not investigated. Most frequently reported have been various cancer cell lines [55, 56], monocytes and macrophages [54, 57], lymphocytes [58, 59], bone-derived cells [39, 40, 60, 61], mesangial and renal tubular epithelial cells [62–65], fibroblasts [66], and vascular (endothelial [53, 67] and smooth muscle [68]) cells. In almost all of these systems the cells do not activate TGF-β unless they are stimulated in some way. For example, mesangial or proximal tubule cells in culture have been reported to activate endogenous TGF-β following exposure to angiotensin II [62], high glucose concentration [63], thromboxane A2 [64] and low density lipoprotein [69]. Stimuli that result in activation in more than one system include retinoic acid [53, 70], sex hormones [55, 71, 72] and vitamin D_3 [61, 73, 74]. In a small number of reports, the mechanism of activation has been investigated, as shown in Table 2.

In vivo activation

Animal studies already indicate that inhibitors of active TGF-β (such as blocking antibodies or decorin) can ameliorate the course of diseases such as nephrosclerosis [76, 77]. An understanding of TGF-β activation *in vivo* might lead to alternate therapies that block pathologic activation. Grainger and colleagues [11] and Barcellos-Hoff et al [12] have reported promising results on the use of histochemical techniques to assess TGF-β activation *in vivo*.

Grainger et al examined levels of active TGF-β in the vascular walls of mice genetically engineered to overexpress lipoprotein (a), which is associated with coronary atherosclerotic disease in humans. They used a soluble type II TGF-β receptor to localize active TGF-β, and attempted to avoid the activation of latent TGF-β in the tissues by closely regulating the fixation process. There was less active TGF-β in the vessel walls of mice overexpressing the lipoprotein. This decrease is consistent with a plasmin-mediated activation of TGF-β in the vessel wall, since lipoprotein (a) displaces plasminogen from cell binding sites and can block TGF-β activation in plasmin-dependent cell activation systems [50].

Barcellos-Hoff et al used specific anti-TGF-β antibodies to examine tissue sections of irradiated mammary tissue. They performed careful control studies using tumor masses formed by injected cells that had been stably transfected to express either latent or constitutively active TGF-β in order to identify antibodies and fixation conditions in which active TGF-β is detected [78]. Their results indicate a rapid activation of TGF-β after irradiation, and a later sustained increase in activity. They hypothesize that, because ionizing radiation activates TGF-β rapidly *in vitro*, the initial surge of activation is a direct effect of the radiation. They suggest that the later sustained increase is due to increased tissue plasminogen activator (and hence plasmin).

Conclusion and future directions

We propose a general model of TGF-β activation. Latent TGF-β is targeted to the extracellular matrix by LTBP. A truncated form of latent TGF-β is released from the matrix by the action of one or more proteases on LTBP. Structural features of LAP and/or LTBP allow the latent complex to associate with the cell surface. Finally, latent TGF-β is activated by cell-associated plasmin.

Much remains to be discovered about TGF-β activation. Any of the steps proposed above could be modulated in ways yet to be discovered. Other important mechanisms of physiological activation probably exist. What new studies might further the understanding of TGF-β activation?

(1.) Determining the three dimensional structure of LAP (and the SLC). This may suggest regions of LAP available for protein-protein interactions or proteolysis, and information about possible conformational changes that might promote activation.

(2.) Developing in vivo systems for testing activation systems. The systems described above represent good starting points and could be extended by interfering with potential activating mechanisms.

(3.) Comparative studies of the three isoforms. Experiments to date have mainly involved TGF-β1. The other two isoforms, which are more dissimilar in the propeptide (LAP) region than in the cytokine region, may have quite different modes of activation.

(4.) Determining the mechanism of association of LTBP to the matrix. Indirect evidence suggests that tissue transglutaminase cross-links LTBP to the matrix. Alternatively, the eight cysteine domains, which are involved in other protein-protein interactions, may be involved in bonding to the matrix. Studies are needed to determine the mechanism of LTBP association to the matrix and to identify the matrix proteins with which it interacts.

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