Latent TGF-β binding protein-3 (LTBP-3) requires binding to TGF-β for secretion

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Abstract Latent transforming growth factor-β (TGF-β) binding protein (LTBP)-1, which is easily secreted, has been shown to enhance the secretion of TGF-β. Here we show that another member of the LTBP family, LTBP-3, is not secreted by several cell types, but secretion occurs after coexpression with TGF-β. The secretion of LTBP-3 requires complexing of LTBP-3 with Cys33 of the TGF-β propeptide.

Key words: Latent transforming growth factor-β binding protein; Transforming growth factor-β

1. Introduction

The latent transforming growth factor-β (TGF-β) binding proteins (LTBPs-1, -2, -3, and -4) comprise a family of extracellular matrix proteins. These proteins share similar modular structures consisting primarily of multiple epidermal growth factor (EGF)-like domains and four cysteine-rich (CR) domains (also called 8-cysteine domains, or TB domains) that are unique to LTBPs and fibrillins. LTBP-1 was originally found as part of a high molecular weight complex containing TGF-β and the TGF-β propeptide [1]. During synthesis, the N-terminal TGF-β propeptide (also called latency-associated peptide, or LAP) is cleaved from the C-terminal mature TGF-β. These two dimeric proteins, LAP and TGF-β, remain associated non-covalently [2,3], and can be secreted from the cell as a small latent complex (SLC). Most cells secrete latent TGF-β as a large latent complex (LLC) consisting of a molecule of LTBP disulfide-bonded to the LAP of the SLC. LTBP-1, -3, and -4 bind to LAP through cysteine residues in the third CR domain of each of these proteins [4]. LTBP-2 does not bind to LAP.

It is believed that LTBP-1 plays an important role in the assembly and secretion of latent TGF-β. TGF-β that is not associated with LTBP-1 is secreted slowly and there is improper formation of disulfide bonds [5], whereas latent TGF-β complexed with LTBP-1 is efficiently secreted and disulfide bond formation is correct. LTBP-3 may also play a role in TGF-β secretion as mice deficient for LTBP-3 have long bone defects resembling those of transgenic mice with impaired TGF-β signaling [6]. These results have been interpreted to indicate that LTBP-3 regulates TGF-β bioavailability, perhaps by affecting secretion [6].

We attempted to study the biological functions of LTBP-3 in cell culture. Our efforts were hindered by the fact that full-length LTBP-3, expressed in mammalian or insect cells, could not be found in the conditioned medium. Interestingly, coexpression of latent TGF-β with LTBP-3 permitted secretion of LTBP-3 as part of the LLC. By engineering various truncated forms of LTBP-3 as well as several chimeric LTBP-1/LTBP-3 proteins, we have identified regions of LTBP-3 that regulate its secretion. In addition, this work provides a potential mechanism for the requirement of LAP/TGF-β in LTBP-3 secretion.

2. Materials and methods

2.1. Cell culture, antibodies and reagents

293T cells were obtained from Dr. D. Ron (NYU School of Medicine, New York, USA). Mouse monoclonal antibody HA.11 against the HA tag was purchased from Babco (Richmond, CA, USA). VB3A9 is a monoclonal antibody directed against the LAP portion of human TGF-β1 [7]. Anti-L3C is a rabbit polyclonal antiserum directed against the C-terminal portion of mouse LTBP-3. To generate this antibody, the cDNA fragment encoding the C-terminal portion of LTBP-3 was cloned into an insect cell expression vector pAcGP67-His [7] in frame with a 6×His tag downstream of the Gp67 signal sequence and expressed in insect cells. The recombinant protein failed to be secreted and formed inclusion bodies inside the cells. The inclusion bodies were dissolved in 8 M urea and purified using TALON (Clontech) under denaturing conditions. The imidazole-eluted protein was renatured by dialysis against buffers with decreasing concentrations of urea. Purified protein was injected into two rabbits to produce the anti-L3C antisera. Pwo DNA polymerase and molecular biology enzymes were purchased from Roche Molecular Biochemicals. LipofectAMINE and LipofectAMINE PLUS reagents were purchased from Gibco BRL.

2.2. cDNA cloning and constructs

Mouse LTBP-3 specific primers were designed based on the published sequence [8] and used in reverse transcription-PCR to amplify a fragment (nucleotides 461–893) of mouse LTBP-3 cDNA from RNA extracted from 2T3 cells. This fragment was used as a probe to screen a mouse heart cDNA library (Clontech). Several clones were isolated and cloned as *Eco*RI–*Eco*RI fragments into pBluescript SK vector. Sequencing confirmed that these clones contain mouse LTBP-3 cDNA.

Human TGF-β1 cDNA was from Dr. Rik Derynck (UCSF, San Francisco, CA, USA). Human Cys33→Ser TGF-β1 cDNA (TGF-β1C33S) was a kind gift from Dr. Jorma Keski-Oja (University of Helsinki, Helsinki, Finland). The eukaryotic expression vector pSignal (construct pJS34 containing nucleotides 2667–3423 of mouse LTBP-3

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Abbreviations: TGF-β, transforming growth factor-β; LTBP, latent TGF-β binding protein; LAP, latency-associated peptide; EGF, epidermal growth factor

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cDNA) was a kind gift from Dr. Juha Saharinen (University of Helsinki, Helsinki, Finland). It has a pcDNA3 backbone, a partially optimized Kozak sequence, a mouse IgG heavy chain signal sequence, a synthetic HA epitope and a STOP codon [9]. Fragments of the mouse LTBP-3 or human LTBP-1 cDNA were amplified by PCR with oligonucleotides containing appropriate restriction sites. PCR fragments were cloned into the pSignal vector in frame with the signal sequence at the N-terminus and the HA epitope at the C-terminus. All constructs were verified by automated sequencing. Construct numbers refer to the numbers listed in Fig. 3. Constructs L3CR34 (#6; nucleotides 2686–3372), L3CR3 (#7; 2676–2916), L3CR4 (#8; 3201–3411), L1CR34 (#11; 3102–3903) were generated by PCR and cloned as *Bam*HI-*Xho*I fragments into pSignal. L3 (full-length; 1–3714) was cloned as an *Eco*RI–*Eco*RI fragment from a pBluescript SK into the *Eco*RI site of pcDNA3. L3NCR4 (#1; 86–3423) was generated by replacing the *Bam*HI–*Cel*II fragment of JS34 (2667–3423) with the *Fse*I–*Cel*II fragment of the mouse LTBP-3 cDNA (*Bam*HI and *Fse*I sites were blunted by using Klenow enzyme and T4 DNA polymerase respectively after digestion). L3NCR2 (#2; 86–1458) was generated by cloning the *Fse*I–*Xho*I fragment (*Fse*I site blunted after digestion) into the *Bam*HI (blunted), *Xho*I sites of pSignal. L3LEGC (#3; 1661–3714) and L3CR3C (#5; 2686–3714) were generated by PCR with *Eco*RI and *Xho*I sites at the ends filled in, and cloned into the blunted *Bam*HI and *Xho*I sites of pSignal. L3LEGCR4 (#4; 1455–3423) was generated by replacing the *Bam*HI (blunted)–*Cel*II fragment of the construct L3CR34 with the *Xho*I (blunted)–*Cel*II fragment of the mouse LTBP-3 cDNA. L3CR3E2C (#9) was generated by cloning the *Xho*I–*Xho*I PCR fragment of L3E2C (3394–3714) into the *Xho*I site of the L3CR3 construct. L3CR4E2C (#10) was generated by cloning the *Xho*I–*Xho*I PCR fragment of L3E2C (3394–3714) into the *Xho*I site of the L3CR4 construct. L1CR34.L3E2C (#15) was generated by cloning the *Xho*I–*Xho*I PCR fragment of L3E2C (3394–3714) into the *Xho*I site of the L1CR34 construct. L1LEG.L3CR34 (#12) was generated by cloning the *Bam*HI–*Bam*HI PCR fragment of L1LEG (1326–3092) into the *Bam*HI site of the L3CR34 construct. L3CR34.L1E2C (#13) was generated by cloning the *Xho*I–*Xho*I PCR fragment of L1E2C (3904–4269) into the *Xho*I site of the L3CR34 construct. L3LEG.L1CR34 (#14) was generated by cloning the *Bam*HI–*Bam*HI PCR fragment of L3LEG (1488–2682) into the *Bam*HI site of the L1CR34 construct.

2.3. Transfection and Western blot analysis

Cells were plated in 35 mm diameter tissue culture dishes at a density of 6×10^5 cells per dish the day before transfection and were transfected with 1 μg of the indicated plasmids. Transfections were carried out using a LipofectAMINE PLUS transfection kit (Invitrogen) in Opti-MEM I (Gibco BRL) following the manufacturer's instructions.

The cells were washed with Opti-MEM I medium 3 h after transfection, and the conditioned medium was collected 48 h after transfection. The remaining cells were washed once with phosphate-buffered saline, collected with a rubber policeman, pelletted, and lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 2 mM EDTA, 0.5% NP40, 0.5% Triton X-100, 0.1% SDS). Proteins in the conditioned media or whole cell extract from the transfected cells were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with TBS-T (Tris-buffered saline with 0.1% Triton X-100) with 5% non-fat dry milk, incubated with primary antibody (HA.11, VB3A9 or anti-L3C) for 1 h at room temperature, washed three times with TBS-T, and incubated with an appropriate secondary antibody conjugated with horseradish peroxidase (Amersham Life Science). After washing, immunoreactive bands were revealed by processing with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) following the manufacturer's instructions.

3. Results

3.1. Transfection of full-length LTBP-3 in mammalian cells caused its accumulation inside the cells

The mouse LTBP-3 protein sequence deduced from the cDNA sequence contains a signal sequence at its N-terminus [8], and the protein has been reported to be secreted [8,10].

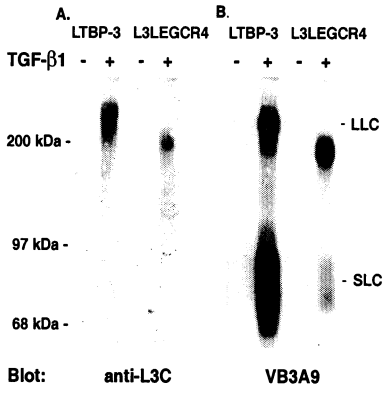

Fig. 1. Coexpression with TGF-β1 helps LTBP-3 secretion. cDNAs encoding full-length LTBP-3 or a truncated form, LEGCR4, were transfected into 293T cells alone or together with TGF-β1 cDNA as indicated. Conditioned media collected 48 h after transfection were separated by SDS–PAGE under non-reducing conditions, transferred to nitrocellulose membrane and blotted either with a specific antibody for LTBP-3 (A) or with antibody against TGF-β1: LAP (B). The positions of the SLC and the LLC are indicated.

However, after we transfected the full-length LTBP-3 cDNA into 293T cells, we could not detect the protein in the conditioned media using a polyclonal antibody against the C-terminal region of LTBP-3 (Fig. 1). Using immunohistochemical methods, we detected the protein as aggregates in the cytosol (see below). The lack of secretion was not unique to 293T cells, as expression in other mammalian cells, such as CHOK7, 2T3, COS7 or insect cells, such as SF9 and High 5, gave similar results. Furthermore, the use of different expression vectors and/or different secretion signals did not result in the secretion of LTBP-3 (data not shown). These results suggested that the intracellular accumulation of LTBP-3 is of functional significance.

3.2. Coexpression of TGF-β1 allows the secretion of LTBP-3

LTBP-3 has been shown to bind with the TGF-β1 SLC [4,8]. Therefore, we hypothesized that LTBP-3 secretion might require association with latent TGF-β. To test this, we co-transfected 293T cells with mouse LTBP-3 and human TGF-β1 cDNAs and monitored the conditioned medium for LTBP-3. After SDS–PAGE under non-reducing conditions, followed by Western blotting, no protein immunoreactive with anti-L3C was observed in the conditioned medium in cells transfected only with LTBP-3 cDNA (Fig. 1A). However, a band at a position corresponding to 220 kDa, which was immunoreactive with the LTBP-3 antisera (anti-L3C), was observed when cells were transfected with TGF-β and LTBP-3 cDNAs (Fig. 1A). A monoclonal antibody against the LAP portion of TGF-β1 (VB3A9) revealed a band corresponding to the SLC in the conditioned media (Fig. 1B). A higher molecular mass band migrating at an apparent molecular mass of more than 200 kDa corresponding to the TGF-β LLC containing LTBP-3 was present. A truncated form of LTBP-3, L3LEGCR4, which encodes the region starting from the long EGF-like repeat stretch to the end of CR domain 4 (Fig. 3A), was also not secreted when expressed alone (Fig. 1A), but was when coexpressed with TGF-β1 (Fig. 1B). These data indicate that TGF-β1 assists LTBP-3 secretion by forming a complex with LTBP-3. Coexpression of TGF-β2 or -β3 also facilitates LTBP-3 secretion (data not shown).

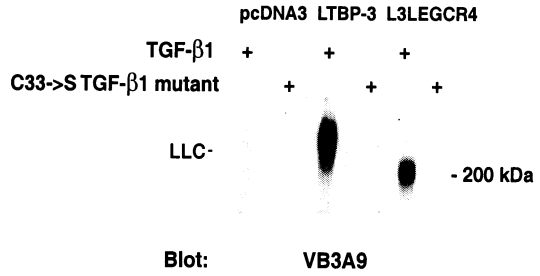

Fig. 2. Coexpression with TGF-β1 Cys33→Ser does not help LTBP-3 secretion. The empty vector (pcNDA3), vector with a cDNA encoding full-length LTBP-3 or a vector with cDNA encoding L3LEGCR4 were transfected into 293T cells alone, together with wild-type TGF-β1 cDNA or with a mutant Cys33→Ser TGF-β1 cDNA as indicated. Conditioned media were collected 48 h after transfection, separated by SDS–PAGE under non-reducing conditions, transferred to nitrocellulose membrane and blotted with antibody against TGF-β1 LAP.

3.3. TGF-β-facilitated secretion of LTBP-3 is dependent on the formation of a disulfide bond between Cys33 of the LAP and cysteines in LTBP-3

It has been shown that LTBP-1 associates with TGF-β1 through the Cys33 of LAP bonding with cysteine(s) in the CR3 domain [7,9]. We asked whether the TGF-β1-assisted secretion of LTBP-3 is dependent on the formation of disulfide bonds between LTBP-3 and Cys33 of the TGF-β1 LAP. Cells were cotransfected with a TGF-β1 cDNA containing a Cys33→Ser mutation (TGF-β1C33S) and an LTBP-3 cDNA. The mutant TGF-β1 SLC was secreted, but it did not promote the secretion of LTBP-3, as no LLC was detected in the conditioned medium by immunoblotting (Fig. 2). Cotransfection of cells with the truncated LTBP-3 cDNA L3LEGCR4 and TGF-β1C33S yielded a similar result (Fig. 2). These results indicate that TGF-β-enhanced secretion of LTBP-3 is dependent upon the formation of disulfide bonds between Cys33 of the LAP and cysteine(s) in LTBP-3.

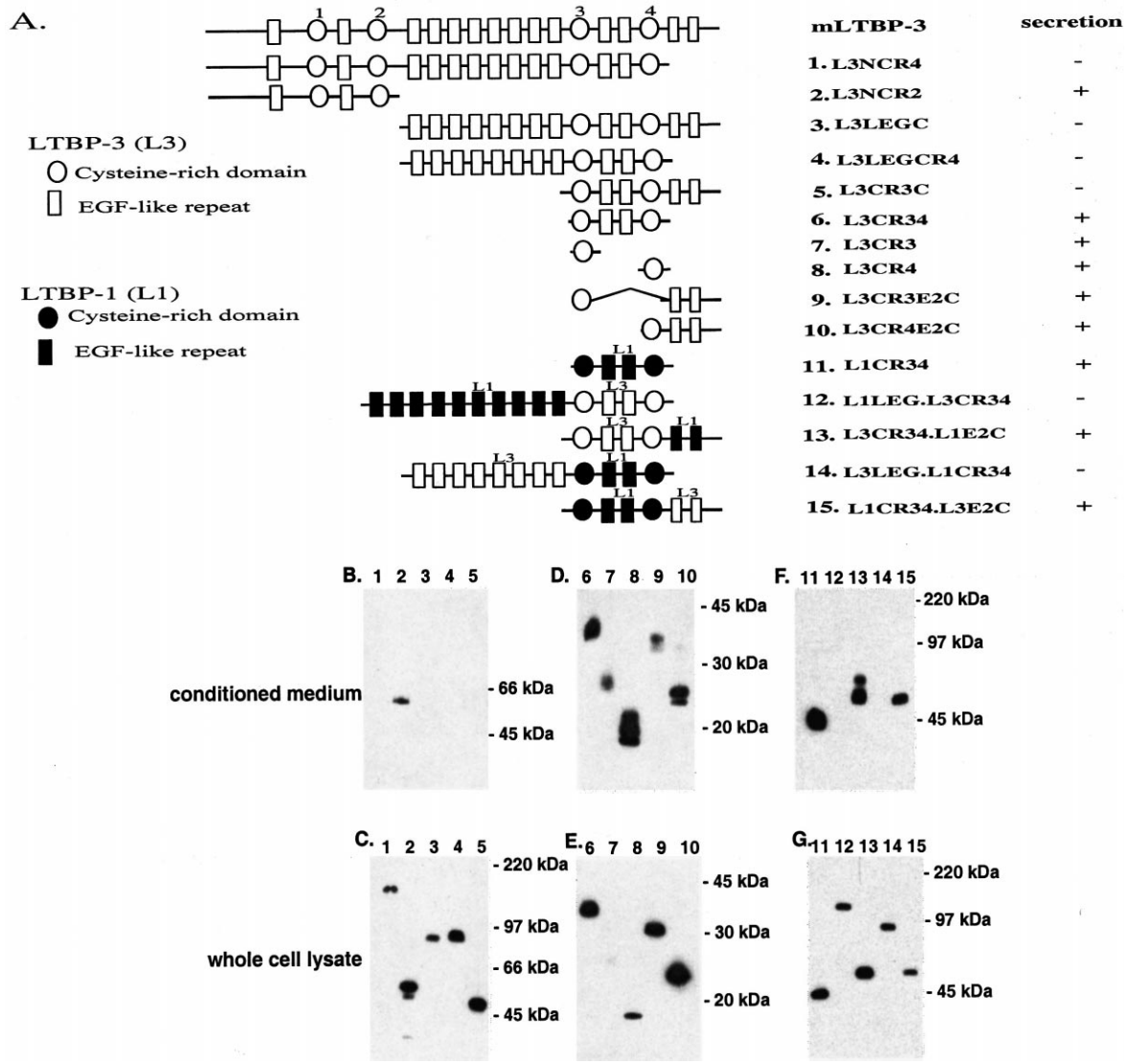

Fig. 3. Schematic description of the LTBP-3 and LTBP-3/LTBP-1 constructs and influence of different regions of LTBP-3 and LTBP-1 on the secretion of LTBP-3. A: Description of constructs. B–G: Constructs encoding truncated LTBP-3 or chimeras of LTBP-3/LTBP-1 were transfected into 293T cells. Conditioned media and whole cell extract were collected 48 h after transfection, separated on SDS–PAGE under reducing conditions and revealed by Western blotting using HA antibody. B, D, F: Conditioned medium; C, E, G: cell lysate.

3.4. Secretion of truncated forms of LTBP-3 with LTBP-3/LTBP-1

To identify the region(s) of LTBP-3 that regulate its secretion, we designed a number of expression cassettes that encoded either truncated LTBP-3 forms or chimeric forms of LTBP-3 plus LTBP-1 (Fig. 3).

With constructs 1–5, representing large regions of LTBP-3, only the N-terminal portion of LTBP-3 (L3NCR2, construct 2) was secreted (Fig. 3B). The failure to detect these proteins in the conditioned medium was not due to poor expression as immunoreactive protein was found in the cell extracts of cells transfected with all constructs (Fig. 3C). Interestingly, the proteins that were not secreted (encoded by constructs 1, 3, 4, 5) all have in common the LAP binding domain of LTBP-3.

To further our analysis, smaller fragments of the C-terminal region of LTBP-3 (constructs 6–10) were expressed and the conditioned medium monitored for secreted protein (Fig. 3A,D). The CR3 or CR4 region either alone (L3CR3, construct 7; L3CR4, construct 8) or together with the two intervening EGF-like domains (L3CR34, construct 6) are all secreted (Fig. 3D). If the EGF-like repeats at the most C-terminus are added to L3CR34 (L3CR3C, construct 5), this protein is not secreted (Fig. 3B). These results suggest that the two terminal EGF-like domains control secretion. Interestingly, when these two domains are combined with either CR3 (L3CR3E2C, construct 9) or with CR4 (L3CR4E2C, construct 10) secretion is normal. These results indicate that the two EGF-like repeats at the C-terminus hinder the secretion of LTBP-3 but only when both CR3 and CR4 are present. The long EGF-like repeat stretch in combination with L3CR34 (L3LEGCR4, construct 4) (Fig. 3B) also blocks secretion, suggesting that this N-terminal sequence is interactive with CR34. Thus, both flanking domains of L3CR34 may participate in regulating LTBP-3 secretion.

Many truncated forms of LTBP-1 have been described, and all have been reported to be secreted [7,9,11]. Therefore, we constructed chimeras combining different regions of LTBP-3 and LTBP-1 to test whether the regulatory domains of LTBP-3 acted in a dominant fashion, i.e. blocked the secretion of LTBP-1 domains (Fig. 3F). When the last two EGF-like repeats of LTBP-1 are added to L3CR34 the resulting chimeric construct L3CR34.L1E2C (construct 13) is secreted (Fig. 3F). Similarly, when the last two EGF-like domains of LTBP-3 are added to the carboxy-terminus of L1CR34 (construct 11, the LTBP-1 equivalent of construct 6) the resulting chimeric protein (L1CR34.L3E2C, construct 15) is also efficiently secreted. These results are in contrast to the inability of the corresponding protein derived entirely from LTBP-3, L3CR3C (construct 5), to be secreted. Therefore, the failure of L3CR3C to be secreted is the result of specific interactions between L3CR34 and EGF-like domains 13, 14 of LTBP-3 as neither domain acts in a dominant fashion. When the long stretch of EGF-like repeats of LTBP-1 is linked to L3CR34 (construct 6), the resulting protein, L1LEG.L3CR34 (construct 12), is not secreted. Similarly, when the long stretch of EGF-like repeats of LTBP-3 is added to L1CR34 (construct 11), the resulting chimera, L3LEG.L1CR34 (construct 14), is also not secreted (Fig. 3F). However, similar constructs derived only from the LTBP-1 sequence are efficiently secreted. Therefore, a dominant regulatory role is implied for both the stretch of EGF-like repeats and, in this context, the L3CR34 of LTBP-3. Finally, the protein products of all constructs (constructs 1–15) are secreted when coexpressed with latent TGF-β (data not shown). Thus, we believe that the covalent interaction between LAP and LTBP-3 prevents the regulatory domains of LTBP-3 from interacting and thereby allows secretion.

4. Discussion

Like LTBP-1, LTBP-3 has multiple EGF-like and CR domains. The full-length and many truncated forms of LTBP-1 have all been reported to be well secreted. Interestingly, LTBP-3 forms intracellular aggregates under the same conditions that permit LTBP-1 to be secreted. It is intriguing that these two proteins, which are so similar in their overall domain structure, behave so differently with respect to secretion.

Our results indicate that LTBP-3 cannot be secreted from the cell in a free form and that both the long stretch of EGF-like repeats and the two EGF-like repeats at the C-terminus hinder secretion. Miyazono et al. demonstrated that latent TGF-β1 was secreted very slowly without LTBP-1 and contained incorrect disulfide bonding [5]. When coexpressed, LTBP-1 assisted the assembly and secretion of the TGF-β1 LLC. Here, we show that when coexpressed with TGF-β1, LTBP-3 is secreted as the LLC. It is interesting that through this association, TGF-β1 coordinates the folding and secretion of LTBP-3. When Cys33, the residue critical for TGF-β LAP binding to LTBP, is mutated, the association of LTBP and LAP is abolished, and the mutant TGF-β1 does not assist LTBP-3 secretion. This demonstrates that this disulfide bonding between TGF-β1 and LTBP-3 is critical for this 'chaperone function' of TGF-β1. These results imply that both TGF-β and LTBP may function as chaperones for each other depending upon the LTBP isoforms involved. It is possible that this requirement for pairing of TGF-β and LTBP-3 serves as a mechanism to regulate the extracellular availability of these proteins.

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