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Identification and Characterization of an Eight-cysteine Repeat of the Latent Transforming Growth Factor-β Binding Protein-1 that Mediates Bonding to the Latent Transforming Growth Factor-β1*

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Most cultured cell types secrete small latent transforming growth factor-β (TGF-β) as a disulfide-bonded complex with a member of the latent TGF-β binding protein (LTBP) family. Using the baculovirus expression system, we have mapped the domain of LTBP-1 mediating covalent association with small latent TGF-β1. Coexpression in Sf9 cells of small latent TGF-β1 with deletion mutants of LTBP-1 showed that the third eight-cysteine repeat of LTBP-1 is necessary and sufficient for covalent interaction with small latent TGF-β1. Analysis by mass spectrometry of this eight-cysteine repeat, produced as a recombinant peptide in Sf9 cells, confirmed that it was *N*-glycosylated, as expected from the primary sequence. No other post-translational modifications of this domain were detected. Alkylation of the recombinant peptide with vinyl pyridine failed to reveal any free cysteines, indicating that, in the absence of small latent TGF-β, the eight cysteines of this domain are engaged in intramolecular bonds. These data demonstrate that the third LTBP-1 eight-cysteine repeat recognizes and associates covalently with small latent TGF-β1 through a mechanism that does not require any specific post-translational modification of this domain. They also suggest that this domain adopts different conformations depending on whether it is free or bound to small latent TGF-β.

First identified as a transforming agent on cultured fibroblasts, transforming growth factor-β (TGF-β)[1] is a potent, ubiquitous regulator of cell growth and differentiation and a modulator of the immune system (1–3). TGF-β also stimulates extracellular matrix production both by increasing the rate of synthesis of extracellular matrix components like fibronectin, collagen I, and biglycan, and by down-regulating the overall proteolytic activity. Three isoforms of TGF-β, TGF-β1, TGF-β2, and TGF-β3, have been identified in mammals. These isoforms have similar, but distinct, activities and are differently distributed among tissues. Translation of TGF-β mRNA results in the formation of a 105-kDa dimeric proform, which is proteolytically cleaved in the Golgi apparatus to mature TGF-β (4). Upon secretion, the mature growth factor (m = 25 kDa), remains noncovalently associated with the propeptide (also known as latency-associated peptide or LAP, m = 80 kDa), thus forming an inactive, latent precursor or small latent complex. In order to interact with specific cell surface serine/threonine kinase receptors, TGF-β must dissociate from LAP, a process known as TGF-β activation. Most TGF-β produced by cultured cells and found in tissues is latent, suggesting that the activation process is a key regulatory step in modulating TGF-β activity.

In most cell types and tissues, the small latent complex is disulfide-linked during secretion to a 160–240-kDa glycoprotein called the latent TGF-β binding protein (LTBP). This complex is known as the large latent complex. Three LTBP family members have been described, LTBP-1, LTBP-2, and LTBP-3, which have been cloned respectively from human and rat (5, 6), human and bovine (7, 8), and mouse (9). All three proteins can associate with TGF-β, but differ in their tissue distribution. The first member to be identified, LTBP-1, was shown to be important for assembly and secretion of small latent TGF-β in human erythroleukemic cells (10). After secretion, LTBP-1 can also mediate the association of the latent TGF-β complex to the extracellular matrix (11). Alternative splicing of LTBP-1 pre-mRNA results in two forms of the protein, LTBP-1S and LTBP-1L (12). LTBP-1L includes LTBP-1S plus an N-terminal extension that confers higher affinity for the extracellular matrix. The large latent complex formed by the association of LTBP and small latent TGF-β is released *in vitro* from the extracellular matrix upon treatment with proteases, such as the serine proteases plasmin, mast cell chymase, and leukocyte elastase, through partial cleavage of LTBP-1 (11, 13). Although the release of the large latent complex from the extracellular matrix mediated by cells has not been reported, this step is assumed to play a role in latent TGF-β activation by allowing interaction of the complex with the cell surface (14). Large latent complex activation in cocultures of endothelial and smooth muscle cells or in lipopolysaccharide-activated peritoneal macrophages is inhibited by an excess of LTBP-1 or by anti-LTBP-1 antibodies, suggesting a direct role for LTBP-1 in this process (14, 15).

LTBP primary structure displays multiple tandem calcium binding EGF-like repeats and characteristic eight-cysteine repeats, also called “LTBP-like” repeats. It has been recently shown that LTBP-1 is a component of microfibrils in osteoblast extracellular matrix, independent of its association with latent TGF-β (16). Indeed, LTBPs share extensive structural homol-

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[1] The abbreviations used are: TGF-β, transforming growth factor-β; LAP, latency-associated peptide; LTBP, latent TGF-β binding protein; EGF, epidermal growth factor; PCR, polymerase chain reaction; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; Ab, antibody.

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ogy with the extracellular matrix proteins fibrillin-1 and -2 found in the elastic fibers (17–19). This homology includes multiple EGF-like domains and eight-cysteine repeats, which are unique to LTBP and fibrillins. However, small latent TGF-β was not shown to form a complex with fibrillins, raising the question of the specificity of LTBP interaction with the small latent complex.

To identify LTBP domains involved in the association with latent TGF-β, we have designed deletion mutants of human LTBP-1S and coexpressed them with human latent TGF-β1 in insect Sf9 cells using recombinant baculoviruses, a system suitable for observing the formation of the large latent complex. Our results show that the third eight-cysteine repeat is necessary and sufficient for association to the TGF-β1 precursor. Mass spectrometry analysis of recombinant eight-cysteine repeat-3 shows that, when produced in Sf9 cells, this domain contains no post-translational modification other than an *N*-linked hybrid oligosaccharide.

MATERIALS AND METHODS

Tissue Culture —Sf9 cells were maintained in Hink's TNM-FH supplemented Grace's medium (JRH Biosciences) containing 10% fetal calf serum (Biomed), 0.1% Pluronic (Life Technologies, Inc.), 292 μg/ml glutamine, 100 units/ml penicillin G, and 100 μg/ml streptomycin.

Baculovirus Transfer Vectors—The dual promoter transfer vector pAcUW51 (Pharmingen), which bears the polyhedrin promoter and the p10 promoter, was used to generate recombinant viruses encoding both TGF-β1 and constructs derived from LTBP-1S. To achieve secretion of LTBP-1S mutants deleted at the N terminus, a modified version of pAcUW51, named pAcUGP, was constructed by substituting the *Xba*I-*Bam*HI fragment of pAcUW51, which contains the polyhedrin promoter, with the *Eco*RV-*Bgl*II fragment of pAcGP67-A (Pharmingen), in which the polyhedrin promoter is followed by the baculoviral protein GP67 signal sequence and a polylinker. To produce secreted peptide tagged with a six-histidine motif, a cassette encoding six histidines was retrieved from vector pAcSG-His-NT C (Pharmingen) and subcloned into pAcGP67 downstream of the GP67 signal sequence, which resulted in vector pAcGP67-His.

Coexpression of LTBP-1S Mutants and Small Latent TGF-β1 in Sf9 Cells Using Recombinant Baculovirus—All the following constructs were checked by automated sequencing. The fragment 68-4543 (*Dra*I-*Dra*I) of the human LTBP-1S cDNA (clone BPA13, kindly provided by Dr. Kohei Miyazono) (5), which contains the entire coding sequence, was subcloned into pAcUW51 downstream of the polyhedrin promoter. LTBP-1S mutants were derived from the BPA13 *Dra*I-*Dra*I fragment and placed under the control of the polyhedrin promoter in pAcUW51 or pAcUGP. All subsequent numbering refers to LTBP-1S coding sequence. ΔC1180 and ΔC1014 were obtained by introduction of stop codons after the respective restriction sites *Bst*1107 I (position 3536) and *Dpn*I (position 3037). ΔN273 and ΔN441 were obtained by digestion of BPA13 with *Hpa*I and *Dra*I, and *Sca*I and *Dra*I respectively, and insertion of these fragments into pAcUGP in frame with GP67 signal sequence. Deletion of fragment 3539–3782 in the LTBP-1S coding sequence using flanking restriction sites *Bst*1107I and *Nae*I resulted in mutant ΔCR4. To construct ΔCR3, the region 3264–4194 was amplified by PCR using *Taq* polymerase (Boehringer Mannheim) and BPA13 as a template with primers AATCTCGCGATGAAGCTGGTGGTGAGAAC and GATTCTGTTTCACTCCAGGTC. The *Nru*I site introduced in the forward primer was used to fuse this fragment to the 3′-end of LTBP-1S cDNA digested with *Bsa*BI (position 3031). The same fragment was also retrieved by PCR using GCTCTAGATGAAGCTGGTGGTGAGAAC as a forward primer and subcloned into pAcUGP in frame with GP67 signal sequence to generate ΔN1088. The region 3028–3271, which encodes the eight-cysteine repeat-3, was amplified by PCR with primers AAGGCCTGTAGATGTAGATCAACCCAAAG and GCTCTAGACAGCTTCAGAAGATGATTCTC, which adds a stop codon (TAG) at the 3′-end. Fusion of this PCR product to the GP67 signal sequence in pAcUGP using the *Stu*I site introduced in the forward primer resulted in construct CR3. Also, ligation of this fragment to ΔN441 using *Bsa*BI led to mutants ΔN441CR3. Last, ΔN1009 was obtained by fusing construct CR3 to the 3′-end of LTBP-1S cDNA through the unique *Alw*NI site.

The sequence encoding human TGF-β1 was excised from the vector pRK5-β1E (a gift from Dr. Rik Derynck) (20) with *Eco*RI and *Bgl*II and placed under the control of the p10 promoter in pAcUW51 and pAcUGP in addition to LTBP-1S wild-type or truncated forms. LTBP-1S and TGF-β1 cDNAs were also subcloned individually into pVL1392 (Pharmingen) for separate expression. To generate HisCR3, the PCR fragment encoding the third eight-cysteine repeat (see above) was introduced into pAcGP67-His.

All constructs were used to generate recombinant viruses with linearized AcNPV DNA using the BaculoGold transfection kit (Pharmingen) according to the manufacturer. For protein expression, Sf9 cells were infected with the appropriate virus, medium was changed 4 h post-infection for serum-free 401 medium (JRH Biosciences), and conditioned media were harvested 72 h post-infection for analysis. In the case of HisCR3, coexpression with small latent TGF-β1 was achieved by coinfecting Sf9 cells with the recombinant viruses encoding HisCR3 and small latent TGF-β1.

Western Blot Analysis of LTBP-1S Mutants with Small Latent TGF-β1—LTBP-1S was detected with the polyclonal rabbit sera Ab 39 obtained using LTBP-1 purified from platelets as immunogen (10). A monoclonal antibody directed against recombinant human β1-LAP from Chinese hamster ovary cells (mAb VB3A9) was used to reveal TGF-β1 precursor. Complexes between the small latent TGF-β1 and LTBP-1S mutants in Sf9 cell conditioned media were immunodetected on Western blot using Ab 39 (1/5,000) or mAb VB3A9 (0.6 μg/ml) and the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Corp.). Immunoreactive bands were revealed by processing for chemiluminescence with the Boehringer Mannheim chemiluminescence kit according to the manufacturer's instructions.

The complex of histidine-tagged peptide HisCR3 with small latent TGF-β1 was isolated using TALON chelating resin, as described below for the purification of recombinant peptide HisCR3, and blotted after SDS-PAGE with mAb VB3A9.

Assay for TGF-β Activity—Conditioned media were assayed for TGF-β activity on mink lung epithelial cells stably transfected with the firefly luciferase reporter gene under the control of the plasminogen activator inhibitor-1 promoter, a TGF-β responsive promoter, as described previously (21).

Purification of Recombinant Peptide HisCR3—Sf9 cells (2×10^7 cells/15-cm dish) were infected with the recombinant baculovirus encoding the peptide HisCR3. Medium was changed to 401 serum-free medium 4 h post-infection. At day 3 post-infection, conditioned medium was harvested and dialyzed against 40 volumes of 150 mM NaCl, 20 mM Tris, pH 7.4 (25 °C) for 24 h at 4 °C. Two ml of TALON metal-chelating resin slurry (Clontech) were added to 50 ml of dialyzed conditioned medium and incubated at room temperature for 1 h on a rotating shaker. After three washes with 20 ml of dialysis buffer (one brief, 2 × 10 min), the resin was poured in a column, and the peptide was eluted with 100 mM imidazole in dialysis buffer. Deglycosylation was performed by incubating 3 μg of peptide HisCR3 with 0.5 unit of *N*-glycosidase F (Boehringer Mannheim) in 10 mM phosphate buffer, pH 7.4, for 16 h at 37 °C.

Matrix-assisted Laser Desorption Mass Spectrometry—Protein and peptide samples were prepared for matrix-assisted laser desorption/ionization mass spectrometry using the dried droplet method (22). The matrix used was α-cyano-4-hydroxycinnamic acid (Sigma), which was purified by recrystallization. To produce the dried droplets, a saturated solution of matrix was prepared in 2:1 aqueous 0.1% trifluoroacetic acid:acetonitrile at room temperature. The sample was added to this solution so that the final sample concentration was 1–10 μM. One-half microliter of the solution was placed on the mass spectrometer's probe and allowed to dry. The sample was then analyzed.

RESULTS AND DISCUSSION

Recombinant baculoviruses encoding either human LTBP-1S or human small latent TGF-β1 were generated for expression in insect cells (Sf9). Sf9 cells infected with the virus encoding LTBP-1S secreted a homogenous product with an estimated molecular mass of 195 kDa under nonreducing conditions as revealed by immunoblotting of the conditioned medium (Fig. 1, *lane 1*). Under reducing conditions, the protein migrated at the same position as the 205-kDa marker (not shown) as reported for LTBP-1S from human foreskin fibroblasts (5). Sf9-produced LTBP-1S bound to concanavalin A-Sepharose with high affinity, indicating that it was glycosylated (not shown).

Analysis of the conditioned medium of cells infected with the virus encoding small latent TGF-β1 using an anti-β1-LAP monoclonal antibody revealed two major bands of 80 and 105 kDa under nonreducing conditions (Fig. 1, *lane 3*), correspond-

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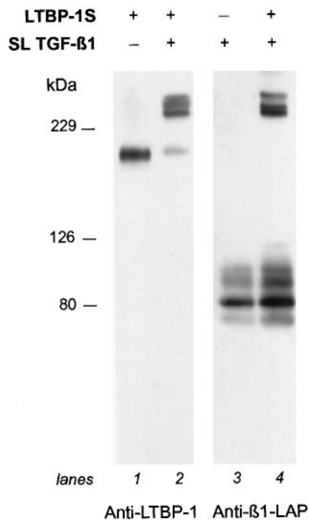

FIG. 1. **Production of small latent TGF-β1, LTBP-1S, and large latent complex by Sf9 cells.** Sf9 cells were infected with recombinant baculovirus encoding small latent TGF-β1 (*SL TGF-β1*), LTBP-1S, or both. At day 3 post-infection, conditioned media were analyzed after SDS-PAGE (7% gel, nonreducing conditions) by immunoblotting with anti-LTBP-1 serum Ab 39 (*lanes 1* and *2*) or with anti-β1-LAP monoclonal antibody VB3A9 (*lanes 3* and *4*).

ing to LAP dimer and unprocessed TGF-β1 precursor. Minor bands, which might correspond to different glycosylation isoforms, were also detected. Two bands of 40 and 53 kDa were seen after reduction of the samples (not shown). These bands correspond to the molecular weights of LAP monomer and uncleaved LAP-TGF-β1 monomer respectively, suggesting that the cleavage of the pro-TGF-β1 into LAP and mature TGF-β1 was incomplete in Sf9 cells. Similar observations were previously described using mammalian cells overexpressing latent TGF-β1 (4). Monomeric pro-TGF-β1 was detectable in Sf9 cell-conditioned medium, probably as a result of high expression level (not shown). *In vitro* activation of latent TGF-β1 by heating of the conditioned medium at 80 °C for 10 min yielded 10–20 ng/ml active TGF-β1, indicating that functional, mature TGF-β1 was produced. Only 1–2% of the mature TGF-β1 was present in the medium as an active form as measured without prior heat activation.

Using a dual promoter transfer vector, we generated a recombinant baculovirus encoding both LTBP-1S and latent TGF-β1 to observe the formation of large latent TGF-β1 complex. When insect cells were infected with this virus, a high molecular mass complex (estimated molecular mass 260–290 kDa) was detected with both anti-LTBP-1 and anti-β1-LAP antibodies by Western blotting under nonreducing conditions, indicating that Sf9 cells assembled the large latent complex (Fig. 1, *lanes 2* and *4*). LTBP-1S and latent TGF-β1 were also detected as free forms. The high molecular mass complex often appeared as two bands, consistent with the inclusion of both processed and unprocessed pro-TGF-β1 in the complex. Secretion of the complex was efficient as more than 90% of the immunoreactive material was recovered in the extracellular medium. In contrast to what was reported previously in human erythroleukemia cells (10), coexpression of latent TGF-β1 with LTBP-1 did not change significantly the rate of latent TGF-β1 secretion (not shown).

The baculovirus expression system was next used to identify the region of LTBP-1S responsible for its disulfide bonding to LAP. Each LAP monomer has three cysteines. Two of these are involved in interchain disulfide bonds in LAP dimer, whereas the third (Cys^{33}) is available for bonding with LTBP-1 (23). LTBP-1S contains 139 cysteines, 10% of the total number of amino acids (5). Each EGF-like repeat has six cysteines, which form three intramolecular disulfide bridges and are unlikely to be engaged in an intermolecular bond with LAP. The remaining cysteines in LTBP-1S are located in the N-terminal region at positions 35, 39, 44, 48, and 298, and in the four eight-cysteine domains.

To determine the region containing the interchain disulfide bonds, deletion mutants of LTBP-1S were constructed (Fig. 2) and coexpressed with latent TGF-β1 in Sf9 cells as described for the wild-type protein. For each mutant, the conditioned medium was immunoblotted with anti-LTBP 1 (Fig. 3*A*, *lanes 1–9*) and anti-β1-LAP (Fig. 3*A*, *lanes 10–21*) antibodies. Deletion of the N-terminal region including the eight-cysteine repeat-1 and -2 did not prevent the formation of a complex with latent TGF-β (mutants Δ*N293*, *lanes 3* and *12*, and Δ*N441*, *lanes 4* and *13*). A mutant LTBP-1S missing the C-terminal sequence including the eight-cysteine repeat-4 (mutant Δ*C1180*, *lanes 5* and *14*) still associated covalently with latent TGF-β1. However, when the C-terminal deletion was extended to include the eight-cysteine repeat-3 (mutant Δ*C1014*, *lanes 6* and *15*), no high molecular weight complex was detected, suggesting that the eight-cysteine repeat-3 was involved in bonding to LAP. To further assess this hypothesis, cDNAs encoding LTBP-1S mutants lacking either the eight-cysteine repeat-3 alone (Δ*CR3*) or the eight-cysteine repeat-4 alone (Δ*CR4*) were constructed. When expressed in Sf9 cells, ΔCR4 formed a complex with latent TGF-β1 (*lanes 8* and *17*), whereas ΔCR3 did not (*lanes 7* and *16*). Conversely, a mutant deleted at the N and the C termini (Δ*N441CR3*), in which all of the eight-cysteine repeats except the eight-cysteine repeat-3 were omitted, associated with latent TGF-β1 (*lanes 9* and *18*). Formation of a complex with latent TGF-β1 was also observed when the C-terminal portion of LTBP-1 was expressed, provided that the sequence included the eight-cysteine repeat-3 (mutants Δ*N1009* and Δ*N1088*, *lanes 19* and *20*). The complex of small latent TGF-β1 with LTBP-1 mutant ΔN1009 was detected with anti-β1-LAP mAb VB3A9, but not with anti-LTBP-1 Ab 39 (not shown). Finally, the eight-cysteine repeat-3 (*CR3*) was expressed together with small latent TGF-β1. Western blot analysis of the conditioned medium with anti-β1-LAP antibodies revealed a complex migrating above latent TGF-β1 (*lane 21*). When the eight-cysteine repeat-3 was fused to a six-histidine tag (*HisCR3*) and coexpressed with small latent TGF-β1, the complex (but not the small latent TGF-β1 alone) was retained on a metal-chelating resin (Fig. 3*B*), confirming that the eight-cysteine repeat-3 associated covalently with latent TGF-β1. Mixing of cells expressing separately the HisCR3 peptide and small latent TGF-β1 did not result in complex formation, indicating that coexpression of the two molecules was necessary for their association (not shown). Bonding of small latent TGF-β1 to the eight-cysteine repeat-3 appeared to be more efficient than to full-length LTBP-1S (Fig. 3*A*); the same phenomenon was also reproducibly observed with constructs ΔN293 and ΔN441, which may be explained by higher expression levels of these mutants as compared to wild-type LTBP-1S. These results show that the eight-cysteine repeat-3 is necessary and sufficient for association of LTBP-1 to latent TGF-β1.

To further characterize the eight-cysteine repeat-3, Sf9 cells were infected with the baculovirus encoding the eight-cysteine repeat-3 fused to a six-histidine motif HisCR3 (see sequence in Fig. 4*A*) and the recombinant peptide was purified by metal-affinity chromatography. The purified peptide, whose predicted molecular mass is 12,237 Da after processing of the signal-sequence, migrated on SDS-PAGE with an estimated molecular mass of 20–21 kDa (Fig. 4*B*). As the eight-cysteine repeat-3 in LTBP-1S contains Asn^{1039} (Asn^{101} in HisCR3, Fig. 4*A*) in the

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proper context for *N*-glycosylation (N/*X*/S or T), we treated the purified peptide with *N*-glycosidase F to determine whether *N*-glycosylation accounted for the distortion in electrophoretic mobility. However, this treatment resulted in a slightly slower migration on SDS-PAGE with an estimated molecular mass of 21–22 kDa (Fig. 4*B*). To assess further the presence of post-translational modifications, the peptide was subjected to mass spectrometry. Analysis of the native peptide (Fig. 5*A*) revealed two major molecular species with molecular masses of 13,266 Da (*peak A*) and 13,345 Da (*peak B*), respectively. Treatment with *N*-glycosidase F decreased the mass of these molecular ions, confirming that the peptide is *N*-glycosylated (Fig. 5*B*). This spectrum showed two peaks, one at 12,230 Da (*peak A1*), which is equal to the predicted molecular mass of the peptide, and one at 12,309 Da (*peak B1*), indicating the presence of a 79-Da post-translational modification resistant to *N*-glycosidase F. The 1,036-Da difference between peaks A and A1 or peaks B and B1 is compatible with the linkage of a truncated high mannose oligosaccharide $Man_3GlcNAc_2$ linked to a fucose residue. To enhance visualization of the glycosylation pattern, the glycosylated peptide was digested with trypsin and analyzed by mass spectrometry (Fig. 5*C*). *Peaks I* (9,011 Da) and *II* (9,198 Da) correspond to the nonglycosylated forms of fragments 65–138 and 65–140, respectively, whereas the same fragments with an additional fucosylated $Man_3GlcNAc_2$ are found in *peaks IV* and *VI*. The nonfucosylated forms of the glycosylated fragments are also detected (*peaks III* and *V*). However, the 79-Da modification was not detected on these peptides. Analysis of the proteolytic fragments obtained with

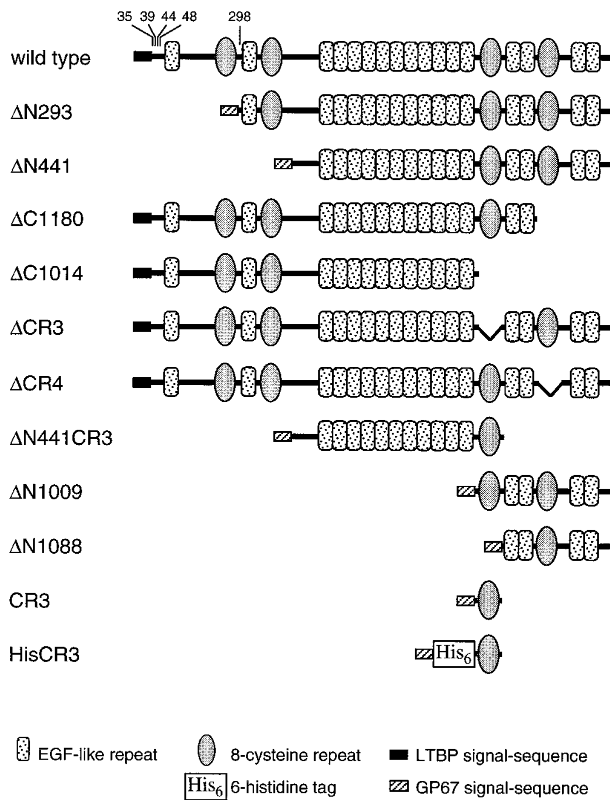

FIG. 2. **Peptides encoded by LTBP-1S constructs.** Cysteines in LTBP-1S are located in EGF-like domains, eight-cysteine repeats, and at positions numbered above the schematic of the wild-type protein. The LTBP-1S domains encoded by these constructs are the following: wild-type, 1–1394; ΔN293, 294-1394; ΔN441, 442-1394; ΔC1180, 1–1179; ΔC1014, 1–1013; ΔCR3, deletion of 1014–1088; ΔCR4, deletion of 1180–1258; ΔN441CR3, 442-1090; ΔN1009, 1010–1394; ΔN1088, 1089–1394; CR3, 1010–1090; HisCR3, 1012–1090.

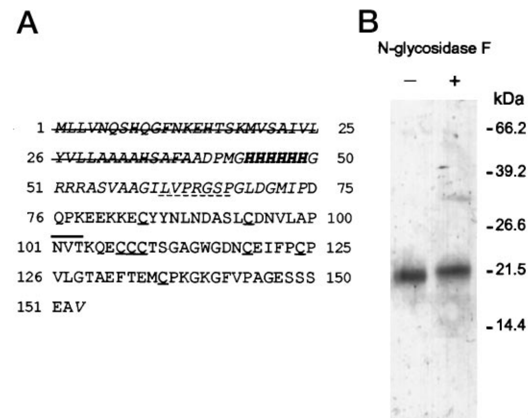

FIG. 4. **Sequence and SDS-PAGE analysis of peptide HisCR3.** *A*, the amino acid sequence of peptide HisCR3 comprises baculoviral protein GP67 signal peptide (*crossed out*), a six-histidine motif (*bold*), a linker with a thrombin cut site (*dashed line*) and LTBP-1S domain 1012–1090 (*plain characters*), which includes a putative *N*-glycosylation site (*overlined*). *B*, HisCR3 peptide purified by immobilized metal affinity chromatography was subjected to SDS-PAGE on a 15% gel (nonreducing conditions) before and after treatment with *N*-glycosidase F. *N*-Glycosidase F appears as a faint band (m = 32.2 kDa).

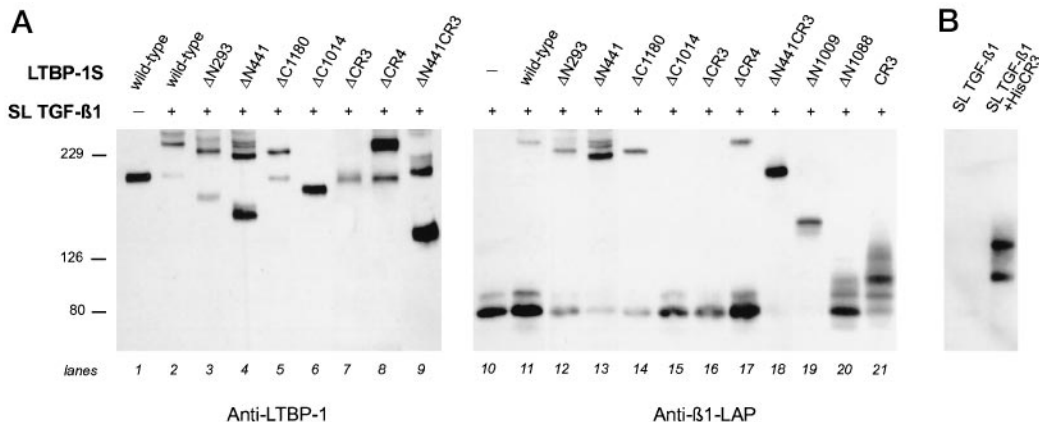

FIG. 3. **Mapping of LTBP-1S domain mediating bonding with small latent complex.** Conditioned media of Sf9 cells infected with recombinant baculovirus bearing both small latent TGF-β1 (*SL TGF-β1*) cDNA and one of LTBP-1S cDNA mutants were subjected to SDS-PAGE (7% gel) under nonreducing conditions and immunoblotted with anti-LTBP-1 serum Ab 39 (*lanes 1–9*) and anti-β1-LAP mAb VB3A9 (*lanes 10–21*). In *B*, Sf9 cells were coinfected with two recombinant baculoviruses, one encoding small latent TGF-β1 and the other histidine-tagged peptide HisCR3; the presence of a complex between the two proteins in the conditioned medium was assessed by precipitation with metal-chelating resin followed by SDS-PAGE and Western blot analysis with mAb VB3A9. The higher immunoreactive band might correspond to the association of the HisCR3 peptide with the small latent complex in a 2:1 ratio, as a result of artifactual disulfide bond exchange on the surface of the chelating resin.

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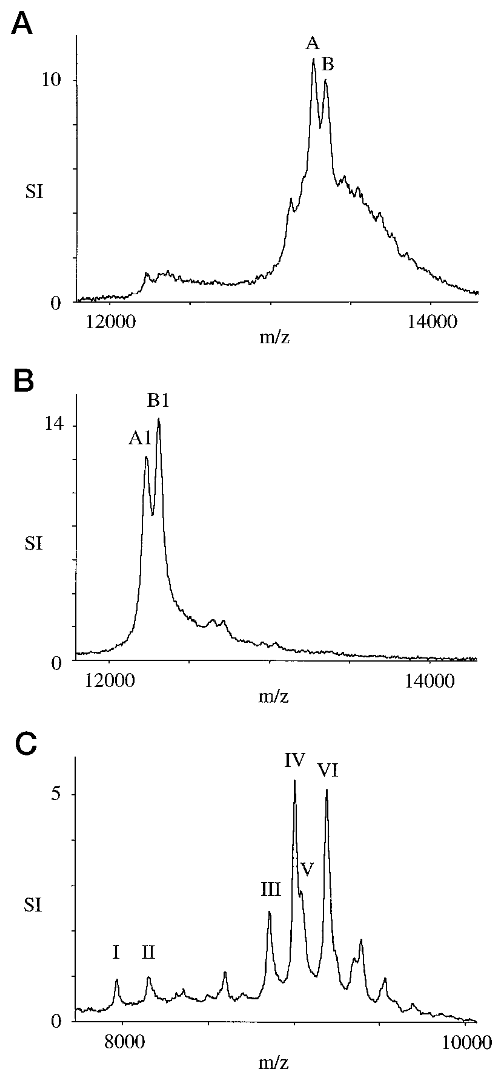

FIG. 5. **Analysis of peptide HisCR3 by mass spectrometry.** Shown are molecular ion regions of mass spectra taken of the following peptide samples: *A*, HisCR3; *B*, HisCR3 after treatment with *N*-glycosidase F; *C*, HisCR3 after partial digestion with trypsin.

trypsin or V8 protease demonstrated that the 79-Da shift in peak B1 was due to an undetermined post-translational modification located in the linker of the histidine tag outside of the eight-cysteine repeat (not shown). Therefore, *N*-glycosylation is the only post-translational modification of the eight-cysteine repeat-3 produced in Sf9 cells.

In order to assess the presence of reduced cysteines in the HisCR3 peptide, alkylation under denaturing conditions was performed using vinyl pyridine and the peptide was analyzed by mass spectrometry. No mass shift was observed, suggesting that the eight cysteines were involved in intramolecular bonds.

The experiments presented here demonstrate that LTBP-1S associates covalently to TGF-β1 through the third eight-cysteine repeat. During the preparation of this manuscript, a study reaching a similar conclusion about the role of the eight-cysteine repeat-3 in the covalent association of LTBP-1S to TGF-β1 in mammalian cells was published by Saharinen *et al.* (24). No other function has been described for this type of repeat, which is found in LTBPs and fibrillin-1 and -2. However, fibrillins do not covalently bind TGF-β, and within LTBP-1, association with TGF-β seems characteristic of the third eight-cysteine repeat. Mass spectrometry analysis of recombinant LTBP-1 eight-cysteine repeat-3 produced in insect cells showed that it is *N*-glycosylated. The *N*-linked glycosidic residue in these cells appears to be mainly a truncated hybrid sugar ($Man_3GlcNAc_2$) with occasional addition of a fucose residue. The $Man_3GlcNAc_2$ pentasaccharide has been described as a common *N*-linked oligosaccharide in lepidopteran cells (25, 26), but is rarely found in proteins synthesized by vertebrate cells. The presence in lepidopteran cells of a fucosyltransferase that can add a fucose residue to the asparagine-linked GlcNAc residue also has been established (27). Among the three LTBPs, the third eight-cysteine repeat is the only one to contain an *N*-glycosylation site. Glycosylation could change the conformation of this domain and favor its association with LAP. However, fibrillin-1 and -2 contain several eight-cysteine domains with putative *N*-glycosylation sites and were not shown to date to associate with small latent TGF-β, suggesting that *N*-glycosylation of the eight-cysteine repeat-3 is not the sole feature for association with small latent TGF-β. Indeed, our results show that LTBP-1S eight-cysteine repeat-3 associates with small latent TGF-β1 even when the *N*-linked glycosidic residue is a trimmed pentasaccharide. Accordingly, as reported by Saharinen *et al.* (24), point-mutagenesis of Asn^{1039} in the eight-cysteine repeat-3 to Ala does not prevent the association process in mammalian cells.

Mass spectrometry characterization of the eight-cysteine repeat-3 shows unambiguously that no other post-translational modification of this domain is needed for bonding to TGF-β1. This conclusion is in contrast with that of Saharinen *et al.* (24), who speculated that other post-translational modifications, such as *O*-glycosylation, might play a role in the association process. This was based on their observation of the anomalous electrophoretic mobility of *N*-glycosidase F treated peptides that contained the eight-cysteine repeat-3, which seemed to indicate that *N*-glycosylation was not the only post-translational modification. From the data presented here, the retarded migration of the eight-cysteine repeat-3 on SDS-PAGE appears to be an intrinsic property of this peptide, which may be related to its conformation. Because insect cells are unable to perform some of the post-translational modifications found in mammals, we cannot rule out the possibility that additional post-translational modifications of this domain occur in mammalian cells. However, such modifications are not necessary for the ability of the eight-cysteine repeat-3 to associate with TGF-β.

The absence of free cysteines in the recombinant HisCR3 peptide suggests that the eight-cysteine repeat-3 adopts an alternative structure for intermolecular bonding with the small latent complex. Remarkably, this domain binds to LAP out of the context of LTBP-1, suggesting the specific recognition of the small latent complex by the eight-cysteine repeat-3. This recognition mechanism may be essential for the formation of the large latent complex. For example, an initial noncovalent interaction of the eight-cysteine repeat-3 with small latent TGF-β could induce a conformational change in the eight-cysteine repeat-3 that would, in turn, favor disulfide bond exchange. Alternatively, this recognition step might occur between partially folded intermediates of LTBP and the TGF-β precursor at an early step during secretion, yielding formation of intermolecular disulfide bonds. The idea of an interaction between LTBP and the TGF-β precursor early in the secretion pathway is supported by the role of LTBP in the assembly and the processing of small latent TGF-β (10).

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Identification and Characterization of an Eight-cysteine Repeat of the Latent Transforming Growth Factor- β Binding Protein-1 that Mediates Bonding to the Latent Transforming Growth Factor- β1

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