Latent Transforming Growth Factor-β Binding Protein Domains Involved in Activation and Transglutaminase-dependent Cross-Linking of Latent Transforming Growth Factor-β

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Abstract. Transforming growth factor-β (TGF-β) is secreted by many cell types as part of a large latent complex composed of three subunits: TGF-β, the TGF-β propeptide, and the latent TGF-β binding protein (LTBP). To interact with its cell surface receptors, TGF-β must be released from the latent complex by disrupting noncovalent interactions between mature TGF-β and its propeptide. Previously, we identified LTBP-1 and transglutaminase, a cross-linking enzyme, as reactants involved in the formation of TGF-β. In this study, we demonstrate that LTBP-1 and large latent complex are substrates for transglutaminase. Furthermore, we show that the covalent association between LTBP-1 and the extracellular matrix is transglutaminase dependent, as little LTBP-1 is recovered from matrix digests prepared from cultures treated with transglutaminase inhibitors. Three polyclonal antisera to glutathione S-transferase fusion proteins containing amino, middle, or carboxyl regions of LTBP-1S were used to identify domains of LTBP-1 involved in cross-linking and formation of TGF-β by transglutaminase. Antibodies to the amino and carboxyl regions of LTBP-1S abrogate TGF-β generation by vascular cell cocultures or macrophages. However, only antibodies to the amino-terminal region of LTBP-1 block transglutaminase-dependent cross-linking of large latent complex or LTBP-1. To further identify transglutaminase-reactive domains within the amino-terminal region of LTBP-1S, mutants of LTBP-1S with deletions of either the amino-terminal 293 (ΔN293) or 441 (ΔN441) amino acids were expressed transiently in CHO cells. Analysis of the LTBP-1S content in matrices of transfected CHO cultures revealed that ΔN293 LTBP-1S was matrix associated via a transglutaminase-dependent reaction, whereas ΔN441 LTBP-1S was not. This suggests that residues 294–441 are critical to the transglutaminase reactivity of LTBP-1S.

Most cell types secrete transforming growth factor-β1 (TGF-β) in a biologically inactive form (42). Mature TGF-β is a homodimer composed of two 12.5-kD polypeptides joined by a disulfide bond at cysteine 77 (14). The monomeric subunits are produced by intercellular cleavage of a higher mol wt precursor at a dibasic site immediately preceding Ala-279 (17, 21). However, after secretion the propeptides remain associated with TGF-β through noncovalent interactions, rendering TGF-β inactive (20). TGF-β with its propeptide, also known as the latency associated peptide (LAP), is referred to as the small latent complex. Both in vitro and in vivo, latent TGF-β is secreted as part of a large latent complex in which a second gene product, the latent TGF-β binding protein (LTBP), is disulfide-linked to LAP (42). The dissociation of TGF-β from LAP is required for TGF-β to bind to its receptors and exert its effects on cell proliferation, extracellular matrix (ECM) deposition, cell migration, and differentiation (34, 38, 58). Latent TGF-β is activated by heat, acid or alkaline treatment, binding to thrombospondin, deglycosylation, proteolysis, or irradiation (5, 9, 34, 35, 56). The most extensively studied process for activating large latent complex is a plasmin-dependent mechanism observed in several tissue culture systems including bovine aortic endothelial (BAE) cells treated with retinoids, cocultures of endothelial cells with smooth muscle cells (MSB), conditioned medium (CM), or extracellular matrix (ECM) and a disulfide bond at cysteine 77 (14). The monomeric subunits are produced by intercellular cleavage of a higher mol wt precursor at a

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1. Abbreviations used in this paper: BAE, bovine aortic endothelial; BSM, bovine smooth muscle; CM, conditioned medium; ECM, extracellular matrix; GST, glutathione S-transferase; LAP, latency associated peptide; LPS, lipopolysaccharide; LTBP-1, latent TGF-β binding protein-1; MDC, monodansylcadaverine; MLEC, mink lung epithelial cells; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; WT, wild type.
molecule or pericytes, and lipopolysaccharide (LPS)–stimulated, thioglycollate-elicited peritoneal macrophages (30, 31, 44, 54). Components of this activation mechanism include the serine protease plasmin, the cross-linking enzyme transglutaminase, LTBP-1, and the mannose 6-phosphate/insulin-like growth factor type II receptor, which appears to bind to mannose 6-phosphate residues in LAP (16, 19, 31, 54, 55).

Interactions of the proteins involved in the activation of large latent complex are not well understood. Plasmin can release TGF-β from large latent complex under cell-free conditions (36). The mannose 6-phosphate/insulin-like growth factor type II receptor binds forms of latent TGF-β, but the role of this interaction is not clear (32). The role of LTBP-1 or tissue transglutaminase in large latent complex activation is not known. As part of an effort to characterize the activation process of large latent complex, we have initiated studies to examine the potential interactions of LTBP-1 and tissue transglutaminase.

LTBP consists of a family of glycoproteins of ~120–210 kD that contain a central core of EGF-like repeats and multiple unique eight-cysteine repeats (22, 28, 43, 46, 64). LTBP-1 and LTBP-2 are structurally similar to the microfibrillar proteins fibrillin-1 and -2 (48, 50, 65). Defects in fibrillins are responsible for the matrix fragility observed in patients with Marfan syndrome and congenital contractual arachnodactyly (29, 47). The best characterized member of the family is LTBP-1, which can exist as either short (LTBP-1S) or long (LTBP-1L) forms (46). A number of cell types secrete LTBP-1 as a higher order complex in which the third eight-cysteine repeat in LTBP-1 is disulfide-linked to the cysteine at position 33 of LAP (22a, 49). It is unknown whether the short and long forms of LTBP-1 are expressed differentially. LTBP facilitates the secretion of small latent complex, participates in the activation of large latent complex, targets large latent complex to the ECM of fetal visceral cells, fibroblasts, epithelial cells, and endothelial cells, and contributes to the formation of fibrillar structures (13, 19, 41, 44, 59–61). LTBP-1S and -1L associate differentially with the matrix, with LTBP-1L having a greater affinity (46). Matrix association of LTBP-1 appears to be covalent, as LTBP in the matrix is deoxycholate insoluble but is released upon proteolysis (Taipale, J., J. Saharinen, K. Hedman, and J. Keski-Oja. 1994. Mol. Biol. Cell. 5 [Suppl.]:311a). However, the mechanism of covalent association between LTBP-1 and the ECM is unknown.

Transglutaminases are a family of structurally and functionally related calcium-dependent enzymes that catalyze the formation of isopeptide bonds between γ-carboxamide groups of glutamine residues and ε-amino groups of lysine residues (24, 33). The family is comprised of five members: plasma Factor XIII, keratinocyte transglutaminase, tissue/endothelial transglutaminase, epidermal transglutaminase, and prostate transglutaminase. Although transglutaminase does not have a signal sequence, the enzyme can be localized to cell surfaces as well as the ECM and reacts to stabilize interactions between extracellular substrates such as plasminogen, fibronectin, nidogen, and vitronectin (3, 6, 37, 51, 63). Pericellular transglutaminase may play a role in stabilizing cell and ECM interactions (24, 37). Transglutaminase is required for the conversion of large latent complex to TGF-β in several systems, including retinoid-treated BAE cells, LPS-stimulated thioglycollate-elicited peritoneal macrophages, and cocultures of BAE and bovine smooth muscle cells (BSM) (30, 31, 44).

Because LTBP-1 is structurally homologous to microfibrillar proteins that are stabilized by transglutaminase-dependent cross-linking (10) and LTBP-2 has been localized to elastin-associated microfibrils (22), we explored the possibility that the covalent association between LTBP-1 and ECM proteins is catalyzed by transglutaminase. Here we show that LTBP-1 and large latent complex are substrates for transglutaminase both as isolated molecules and in cell cultures. We demonstrate that matrix incorporation of LTBP-1 is transglutaminase-dependent and requires the amino-terminal region of LTBP-1 and that matrix association is an intermediate step in the activation mechanism used by cells to generate TGF-β.

Materials and Methods

Materials

Human plasmin (10 U/ml), isopropyl-β-D-thiogalactopyranoside (IPTG), 1-α-n-octyl-β-D-glucopyranoside (α-0ctylglucoside), Pefabloc SC, and protein A–agarose were purchased from Boehringer Mannheim Corp. (Indianapolis, IN). Cystamine, glutathione-agarose, guinea pig liver transglutaminase, monodansylcadieline (MDC), and nonimmune rabbit serum were purchased from Sigma Chemical Co. (St. Louis, MO). Pyrogen-free BSA was purchased from Pierce (Rockford, IL). t-35S]S]ysteine, 35S-labeled express translabeled mix, and 35S-[35S]ouicarbonate (pH 12–14, high concentration) were obtained from Du Pont Company Biotechnology Systems (Wilmington, DE). Recombinant human TGF-β1 was a gift from Berlex Biosciences (South San Francisco, CA). Pan-neutralizing monoclonal mouse anti-TGF-β IgG1 was either purchased from Genzyme (Cambridge, MA) or donated by Celtrix (Santa Clara, CA) (15). Recombinant human LTBP-1S, human large latent complex, and small latent complex were provided by Drs. Hideya Ohashi and Haruhiko Tsumura (KIRIN Brewery Co., Ltd., Pharmaceutical Division, Gunma, Japan). Recombinant Factor XIII (166 kD) was provided by Dr. P.D. Bishop (ZymoGenetics Inc., Seattle, WA) (7). The human fibroblast LTBP-1S cDNA (pSV7d-BP13) was a gift from Drs. K. Miyazono and C. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) (26, 46). Ab 39, a polyclonal rabbit anti-LTBP IgG, was a gift from Dr. K. Miyazono (40). Ab 39 was generated against human LTBP purified from human platelets and recognizes LTBP-1 but not LTBP-2 (28, 43).

Cell Culture

Human fibrosarcoma cells (HT 1080; American Type Culture Collection, Rockville, MD) were grown in DME containing 10% (heat-inactivated) FCS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 2 mM t-glutamine (P/S/Q). Rat osteosarcoma cells (UMR-106; American Type Culture Collection) were cultured in DME containing 10% (heat-inactivated) FCS and P/S/Q. Chinese hamster ovary cells (CHO K1) were cultured in α-MEM containing 10% (heat-inactivated) FCS and P/S/Q.

Primary BAE and BSM cells were isolated and cultured in α-MEM containing 10% (heat-inactivated) FCS and P/S/Q. Human fibroblasts were harvested from Swiss Webster mice, which had been injected i.p. with 4% thioglycollate broth and activated by LPS in vitro as previously described (44).

Expression and Purification of Glutathione S-Transferase–LTBP-1S Fusion Proteins

Three fusion proteins, each consisting of glutathione S-transferase (GST) fused to a region of human fibroblast LTBP-1S, were generated by expressing pGEX1n constructs encoding LTBP-1S fragments in HB101 Escherichia coli followed by purification using glutathione-agarose (57). Fusion proteins GST-450, GST-1065, and GST-849 contain sequences from the amino, middle, and carboxyl regions of human fibroblast LTBP-1S, respectively (Fig. 1) (28, 46).
pGEX-450 was constructed by cloning a 450-bp BamHI fragment of the LTBP-1S cdNA BP13 (nucleotides 419–869) (28) into a small site of pGEX-In. The pGEX-1065 construct was prepared by ligating a 1065-bp BamHI–EcoRI fragment of the LTBP-1S cdNA BP13 (nucleotides 1087–2152) and BamHI–EcoRI-digested pGEX-In. The pGEX-849 construct was generated by cloning an 849-bp StuI–SSpI fragment of the LTBP-1S cdNA BP13 (nucleotides 3779–4628) in the SmaI site of pGEX-In.

Once the insert orientation and sequence were verified for each construct, HB101 E. coli were transformed with the pGEX-In plasmid (control) or a pGEX–LTBP-1S expression construct by electroporation (Cell Porator; Gibco BRL, Gaithersburg, MD). Fusion proteins were purified from IPTG-induced bacteria using glutathione-agarose according to the manufacturer’s instructions (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ). The integrity and apparent mol wts of the fusion proteins were verified by reducing SDS-PAGE followed by Coomassie brilliant blue R staining (26).

Preparation and Characterization of Antibodies to GST–LTBP-1S Fusion Proteins

Polyclonal rabbit antisera raised against purified GST–450, GST–1065, or GST–849 fusion proteins were prepared by Cocalico Biologicals, Inc. (Reamstown, PA). Affinity-purified anti-450, anti-1065, and anti-849 IgG were prepared by incubation of antisera with tosyl-activated agarose (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) at 4°C for 7–10 d. Additions of antibodies or 50 μCi/ml L-[35S]cysteine, which was needed to minimize disulfide exchange within and between proteins.

Immunoprecipitation of Matrix Digests Prepared from Metabolically Labeled Cells

Subconfluent cultures were incubated in DME deficient in cysteine and methionine for 1 h at 37°C. Cultures were treated with 0.06% DMSO (control), 50 μM MDC, 100 μM cystamine, or affinity-purified antibodies during the starvation and subsequent pulse-chase period. After the cells were metabolically labeled for 3 h with 100 μCi/ml of L-[35S]cysteine in cysteine, methionine-deficient DME supplemented with 2% OptiMEM (Gibco Laboratories), and 150 μg/ml of t-methionine, they were transferred to OptiMEM medium for an overnight incubation at 37°C in a 5% CO2 atmosphere. ECMs were prepared from metabolically labeled cells after lysis with 0.5% sodium deoxycholate in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) (59). Matrices were digested with 0.3 U/ml plasmin in 0.1% n-octylglucoside, 3 mM MgCl2, 3 mM CaCl2, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl for 1 h at 37°C. Plasmin was inhibited by 8 mM Pefabloc SC and 1 μg/ml aprotonin. Digests normalized to total TCA-precipitable cpm as described by Harlow and Lane (26) were incubated with either nonimmune or Ab 39 serum, and immune complexes were precipitated using protein A–agarose. Washed immunoprecipitates were separated by reducing SDS-PAGE and visualized by fluorography.

Immunoprecipitation of Matrix Digests Prepared from UMR-106 Matrices

18S-labeled CM were prepared using BAE or UMR-106 confluent cultures that were metabolically labeled for 20 h with 100 μCi/ml of L-[35S]cysteine. Metabolically labeled CM were concentrated fivefold using Centricon-30 ultrafilters (Amicon, Beverly, MA) and immediately added to UMR-106 ECMs prepared using 0.5% sodium deoxycholate in Tris-buffered saline (59). Additions of antibodies or 50 μM MDC were done concurrently. To identify regions of LTBP-1 that contain transglutaminase-reactive sites, cross-linking reactions were performed as described above in the presence of either affinity-purified antibodies generated against GST–LTBP-1S fusion proteins or protein A–purified Ab 39, as well as 0.1 mg/ml t-cystine, which was needed to minimize disulfide exchange within and between proteins.

Immunoprecipitation of Matrix Digests Prepared from Metabolically Labeled Cells

Subconfluent cultures were incubated in DME deficient in cysteine and methionine for 1 h at 37°C. Cultures were treated with 0.06% DMSO (control), 50 μM MDC, 100 μM cystamine, or affinity-purified antibodies during the starvation and subsequent pulse-chase period. After the cells were metabolically labeled for 3 h with 100 μCi/ml of L-[35S]cysteine in cysteine, methionine-deficient DME supplemented with 2% OptiMEM (Gibco Laboratories), and 150 μg/ml of t-methionine, they were transferred to OptiMEM medium for an overnight incubation at 37°C in a 5% CO2 atmosphere. ECMs were prepared from metabolically labeled cells after lysis with 0.5% sodium deoxycholate in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) (59). Matrices were digested with 0.3 U/ml plasmin in 0.1% n-octylglucoside, 3 mM MgCl2, 3 mM CaCl2, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl for 1 h at 37°C. Plasmin was inhibited by 8 mM Pefabloc SC and 1 μg/ml aprotonin. Digests normalized to total TCA-precipitable cpm as described by Harlow and Lane (26) were incubated with either nonimmune or Ab 39 serum, and immune complexes were precipitated using protein A–agarose. Washed immunoprecipitates were separated by reducing SDS-PAGE and visualized by fluorography.
catalyze the cross-linking of large latent complex present in radiolabeled CM and UMR-106 matrix proteins, thrombin-activated Factor XIII (Factor XIIIa) was added (7). Each matrix incorporation reaction was done using 1 ml of concentrated radiolabeled CM, 5 nmol of DTT-pretreated Factor XIIIa, and 4 mM CaCl$_2$ in Tris-buffered saline on 78.5 cm$^2$ of UMR-106 ECM for 1 h at 37°C. The LTBP-1 content of UMR-106 matrices was analyzed by immunoprecipitating plasmin digestes of matrices with Ab 39 followed by SDS-PAGE and fluorography of immunoprecipitates as described in this manuscript.

**Transient Transfection of CHO Cells with LTBP-1S cDNA Constructs**

Three LTBP-1S cDNA constructs were generated using the pcDNA3 (Invitrogen, San Diego, CA) expression vector for transient expression of CHO cells. The three constructs are pcDNA3–wild type (WT), pcDNA–ΔN293, and pcDNA–ΔN441 (see Fig. 5 A). To generate pcDNA3–WT LTBP-1S, the fragment 68–4543 (DraI–DraI) of the human LTBP-1S cDNA BP13 alovirus immediate early gene promoter. pcDNA3–signal sequence as described elsewhere (22a) and subcloned into pcDNA3. Fragments were fused in frame with the baculovirus glycoprotein GP67 1414–4543), and ScaI and DraI (nucleotides 970–4543), respectively; these fragments were fused in frame with the baculovirus glycoprotein GP67 signal sequence as described elsewhere (22a) and subcloned into pcDNA3.

CHO cells were transiently transfected using lipofectamine (Gibco Laboratories) according to the manufacturer’s instructions. Briefly, cells plated in 35-mm dishes received 1–4 μl of plasmid DNA premixed with lipofectamine in Optimem. After 24 h, media were harvested and replaced with fresh Optimem. Transfected cultures were incubated for an additional 24 h, at which time media were collected and matrix digestes were prepared.

**Western Blotting CM and Matrix Digests of Transfected CHO Cultures**

CM and matrix digestes were prepared from transfected CHO cultures for Western blotting analysis using Ab 39. The CM collected after the first and second 24 h of transfection were pooled and concentrated 10-fold. The concentrated CM and matrix digestes were mixed with nonreducing sample buffer for SDS-PAGE. Separated proteins were transferred electrophoretically to Immobilon-P for immunoblotting with Ab 39 serum, and immune complexes were visualized using chemiluminescence reagents as described earlier.

**Preparation of CM from Cultures of BAE and BSM Cells and Stimulated Macrophages**

Serum-free CM were prepared from homo- and heterotypic cultures of BAE and BSM cells and from LPS-stimulated thioglycollate-elicited macrophages as described in previous reports (44, 53, 54). CM collected after 14 h were immediately bioassayed for TGF-β activity (see below). TGF-β–dependent activity was verified by adding neutralizing anti–TGF-β Igs to parallel test samples before assay. Total TGF-β, which consists of cell-activated plus latent TGF-β, was quantitated after heating CM at 85°C for 12 min, a procedure known to convert latent TGF-β to TGF-β, before assay (9).

**Mink Lung Epithelial Cells–Luciferase Assay for TGF-β Activity**

This quantitative bioassay for TGF-β is based on the ability of TGF-β to stimulate plasminogen activator inhibitor-1 (PAI-1) expression (1). Mink lung epithelial cells (MLEC), stably transfected with an expression construct containing a truncated PAI-1 promoter fused to the firefly luciferase reporter gene, were incubated overnight with CM harvested from either stimulated macrophages or cultures of BAE and BSM cells. Recombinant TGF-β1 in α-MEM or DME with 0.1% BSA was used to generate a standard curve of TGF-β activity. Luciferase activity was quantitated using a luciferin substrate buffer in a ML3000 Microtiter Plate Luminometer (Dynatech Laboratories Inc., Chantilly, VA) (1).

**Wound Migration Assay for TGF-β Activity**

To measure TGF-β present in CM, wound migration assays were performed as described by Sato and Rifkin (52). This bioassay is based on the ability of TGF-β to inhibit endothelial cell migration. Confluent monolayers of BAE cells plated in a 35-mm dish were wounded with a razor blade and incubated with serum-free test samples for 18–20 h at 37°C in a 5% CO$_2$ atmosphere. Cells were fixed and stained with 0.5% crystal violet in 20% methanol (44). BAE cells that had migrated from the edge of the wound were counted in successive 125-μm increments at 100× using a light microscope with an ocular grid. The number of cells migrating beyond 125 μm from seven different fields was averaged. Data are presented as a percentage of control, where the control sample consists of homotypic BAE and BSM media mixed 1:1.

**Results**

Transglutaminase Reactivity of LTBP and Large Latent Complex

The apparent covalent interaction of LTBP-1 and ECM (33, 36) as well as the requirement for tissue transglutaminase for activation of large latent complex described for various culture systems suggested (30, 31, 44) that LTBP-1 and large latent complex might be substrates for transglutaminase. Therefore, iodinated recombinant LTBP-1 or large latent complex was incubated with guinea pig liver transglutaminase and analyzed by reducing SDS-PAGE. Samples incubated with transglutaminase revealed high mol wt complexes visible at the top of the 3% stacking polyacrylamide gel as well as intermediate-sized complexes at the interface between the 3% stacking and 7% resolving gels, indicating that both LTBP-1 and the large latent complex were polymerized by transglutaminase (Fig. 2). The efficiency of cross-linking of either LTBP-1 or large latent complex by transglutaminase varied among experiments as the relative amounts of polymerized and unreacted protein differed somewhat between reactions (data not shown).
This may reflect differences in the specific activity of different transglutaminase preparations. The observed polymerization of LTBP-1 and large latent complex implies that LTBP-1 and the large latent complex each contain both acyl donor and acyl acceptor sites necessary for formation of isopeptide bonds. Cross-linking LTBP-1 or large latent complex was dependent on the addition of EDTA or MDC (Fig. 2) (33). MDC acts as a competitive inhibitor of transglutaminase by competing for reactive glutamines (33). As large latent complex consists of small latent complex and LTBP-1, transglutaminase-dependent cross-linking of large latent complex to itself could involve either LTBP-1, small latent complex, or both. Cross-linking reactions using tissue transglutaminase and recombinant small latent complex also yielded high mol wt complexes (data not shown), indicating it also contains acyl donor and acceptor sites. However, this reaction is not responsible for the matrix incorporation of large latent complex as shown by antibody inhibition studies (see below). Iodination of either LTBP-1 or large latent complex did not generate reactive sites absent in nonradiolabeled proteins because transglutaminase-catalyzed incorporation of MDC into nonradiolabeled LTBP-1 or large latent complex was observed using black light to visualize MDC-containing proteins resolved by SDS-PAGE (data not shown) (6).

Transglutaminase-dependent Cross-Linking of LTBP-1 and ECM by Cells

Both free LTBP-1 and large latent complex have been described to associate covalently with ECMs generated by fibroblasts, endothelial cells, and epithelial cells (59–61). The results described above suggested the hypothesis that matrix incorporation of LTBP-1 and large latent complex occurs through transglutaminase-catalyzed cross-linking of LTBP-1 and matrix proteins. To test this hypothesis, we first confirmed that homotypic cultures of HT 1080 and BAE cells as well as heterotypic cultures of BAE and BSM cells generate matrix-associated LTBP-1. ECM-bound LTBP-1 was analyzed by experiments in which matrix digests prepared from metabolically labeled cultures were immunoprecipitated using Ab 39. Immunoprecipitated matrix digests prepared from HT 1080 and BAE cells, as well as from cocultures of BAE and BSM cells, revealed fragments of 120–140 and 180–210 kD, respectively (Fig. 3). These matrix fragments specifically reacted with Ab 39 because they were not observed in control immunoprecipitation experiments with nonimmune serum (data not shown). The difference in mol wt of matrix fragments immunoprecipitated with Ab 39 obtained from HT 1080 and vascular cells is consistent with a report that LTBP-1 recovered from matrix digests prepared from different cell types varies in mol wt (61). The differences in size of the LTBP-1 fragments recovered from the ECM may reflect variations in matrix composition, in differential expression of LTBP-1 isoforms incorporated into the ECM (26), or in endogenous proteolytic activities in the cell culture.

Having confirmed that LTBP-1 was present in the ECM, we next examined whether the incorporation of endogenous LTBP-1 into the ECM by cells was transglutaminase dependent. Matrices were prepared from cultures metabolically labeled in the presence of the transglutaminase competitive inhibitors MDC or cystamine (33), and the LTBP-1 content of the ECM was examined by digesting matrices with plasmin and immunoprecipitating the digests with Ab 39 followed by SDS-PAGE analysis. Significantly less LTBP-1 was recovered from matrices prepared from cultures of HT 1080, BAE, and BSM cells treated with MDC or cystamine than from control cultures (Fig. 4, A and B). Matrix digests from cultures incubated with transglutaminase inhibitors contained 2–10-fold less LTBP-1 than respective control cultures as determined by laser scanning densitometry. Cells treated with MDC incorporated less LTBP-1 into the matrix than did cystamine-treated cells (Fig. 4, second and fourth lanes), probably because the concentration of cystamine used in cultures was below the saturating concentration needed to com-
Assembly and stabilization, matrix incorporation reactions served in culture was a consequence of altered matrix as-
hibitors on the matrix incorporation of LTBP-1 as ob-
erved in cultures treated with MDC. Matrix incorporation reactions of transglutaminase inhibitors did not result from effects on total protein synthesis and secretion, all matrix digests were normalized to total TCA precipitable cpm before immunoprecipitating with Ab 39 serum (11). In addition, the effect of MDC on protein synthesis and secretion was monitored by immu-
noprecipitating fibronectin from metabolically labeled CM harvested from untreated and MDC-treated HT 1080 cells. Fibronectin levels in treated and untreated HT 1080 CM were found to be equivalent (data not shown). Assum-
ing that the transglutaminase inhibitors did not select-
ively affect LTBP-1 expression, the decreased amount of
LTBP-1 observed in matrix digests prepared from cultures treated with inhibitors suggests that the matrix incorpora-
tion of LTBP-1 is transglutaminase dependent.

In similar experiments, twice as much LTBP-1 was re-
covered from BAE matrix digests than from BSM matrix
digests as determined by immunoprecipitating plasmin di-
gests of metabolically labeled ECM using Ab 39 (Fig. 4 B). This may reflect higher levels of tissue transglutaminase expression by BAE cells compared to BSM cells rather than differences in LTBP-1 expression, as BSM cells have been reported to produce more LTBP-1 than BAE cells (19, 31).

To establish whether the effect of transglutaminase in-
hibitors on the matrix incorporation of LTBP-1 as ob-
erved in culture was a consequence of altered matrix as-
sembly and stabilization, matrix incorporation reactions were done using matrices from untreated UMR-106 cells, 35S-labeled BAE CM as an exogenous source of large latent complex, and the plasma transglutaminase Factor XIIIa. Matrices from UMR-106 cells were used because UMR-106 cells produce latent TGF-β only as the small latent complex (12). We confirm that large latent complex was not generated by our UMR-106 cells, as no LTBP-1 was detected in Ab 39 immunoprecipitates of 35S-metabolically labeled CM (Fig. 5A, first lane). Metabolically labeled CM from BAE cells was used as an exogenous source of large latent complex, as we have previously observed (19), and confirmed that they produce LTBP-1 as large latent complex (220 kD; Fig. 5 A, second lane). The LTBP-1 content of UMR-106 matrices incubated with radiolabeled BAE CM in the absence or presence of MDC was analyzed by digesting matrices with plasmin and immunoprecipitating digests with Ab 39 (Fig. 5 B). Visualization of immunoprecipi-
titates by fluorography revealed that matrices reacted with transglutaminase and BAE CM contained cross-
linked LTBP-1 (Fig. 5 B, first lane), whereas digests from a parallel matrix reaction containing MDC did not (Fig. 5 B, third lane). Control matrix incorporation reactions of UMR-106 CM and UMR-106 matrices confirmed that the ECM of UMR-106 cells does not contain endogenous LTBP-1, as no LTBP-1 was recovered from matrix digests. Results from matrix incorporation experiments indicate that the decreased levels of matrix-associated LTBP-1 ob-
erved in cultures treated with MDC were due to the inhi-
bition of cross-linking by transglutaminase rather than ef-
fects on matrix assembly and stability.

Identification of LTBP-1 Domains Involved in
Cross-Linking by Transglutaminase

Results presented in Figs. 2, 4, and 5 demonstrate that
LTBP-1 and large latent complex are substrates for trans-
glutaminase and that matrix incorporation of large latent complex is transglutaminase dependent. To determine whether LTBP-1 is necessary for cross-linking of large la-
tent complex, antibodies to intact LTBP-1 or different
LTBP-1 sequences were included in cross-linking re-
tions of transglutaminase and iodinated large latent com-
plex as described previously. Ab 39 inhibited transglutam-

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To establish whether the amino-terminal region also contained transglutaminase reactive sites involved in the matrix association of LTBP-1, affinity-purified antibodies to the three GST–LTBP-1S fusion proteins were added to cultures of HT 1080 and BAE cells throughout a pulse-chase metabolic labeling period. Because HT 1080 cells are proteolytically more active than BAE cells (18), we added aprotinin, a serine protease inhibitor, to HT 1080 cultures to prevent antibodies from being degraded by endogenous proteases. The amount of LTBP-1 incorporated in the matrix by antibody-treated HT 1080 and BAE cells was assessed by immunoprecipitating matrix digests with Ab 39 followed by SDS-PAGE. The immunoprecipitation experiments revealed that the amount of LTBP-1 recovered from matrices prepared from cultures treated with anti-450 IgG was significantly decreased compared to that recovered from cultures treated with nonimmune, anti-1065, or anti-849 IgG (Fig. 7A). Laser scanning densitometry of fluorographs revealed that digests prepared from HT 1080 and BAE cultures receiving anti-450 IgG contained 5–10-fold and 70-fold less LTBP-1, respectively, than matrices prepared from cultures treated with the other antibodies. Addition of aprotinin did not affect the deposition of LTBP-1 into the matrix, as the cpms recovered from matrices prepared from aprotinin-treated and untreated cultures with Ab 39 were similar (data not shown). The weaker effect of anti-450 IgG on HT 1080 versus BAE cells may reflect degradation of the antibodies by metalloproteases; however, this was not verified. These results support findings reported by others that incorporation of LTBP-1 into the matrix requires its amino-terminal residues (46, 49).

To confirm the observation that addition of anti-450 IgG to cells producing large latent complex interferes with the covalent incorporation of the complex to the ECM, matrix incorporation reactions were done using UMR-106 matrices, 35S-labeled BAE CM, and Factor XIIIa, as described earlier, in the presence of antibodies to GST–LTBP-1S fusion proteins. Western blotting analysis of matrix digests with Ab 39 indicated that addition of anti-450 IgG attenuated matrix deposition of large latent complex, as less LTBP-1 was present in digests from matrix incorporation reactions containing anti-450 IgG compared to those receiving nonimmune, anti-1065, or anti-849 IgG (Fig. 7B). Results from these matrix incorporation reactions confirm
CHO cells incorporate LTBP-1s into ECM, matrix digests were prepared by plasmin treating deoxycholate-extracted matrices followed by immunoblotting using Ab 39. Matrix digests prepared from cells transfected with WT and ΔN293 LTBP-1s cDNA contained LTBP-1s, whereas little to no LTBP-1s was detected in digests from untransfected and ΔN441 LTBP-1s cDNA–transfected cells (Fig. 8 B). To determine whether matrix association of WT and ΔN293 LTBP-1s was transglutaminase dependent, MDC was added to untransfected and transfected CHO cultures. Western blotting analysis of the LTBP-1s content of matrix digests generated from MDC-treated transfected CHO cells revealed negligible levels of LTBP-1s present in digests from WT and ΔN293 LTBP-1s cDNA–transfected cells, indicating that matrix association was transglutaminase dependent (data not shown). Results from the LTBP-1s transfection experiments indicate that, unlike ΔN441 LTBP-1s, ΔN293 LTBP-1s was cross-linked to matrix suggesting that residues 294–441 are critical to the transglutaminase-dependent reactivity of LTBP-1s.

**Effect of Antibodies to GST–LTBP-1s Fusion Proteins on Latent TGF-β Activation**

Activation of latent TGF-β by cocultures of BAE and BSM cells as well as by stimulated macrophages requires transglutaminase and LTBP-1 (19, 31, 44). Transglutaminase-dependent cross-linking of LTBP-1 into the matrix by cocultures is inhibited by anti–LTBP-1 antibodies (Fig. 4 B). Therefore, we examined whether antibodies to GST–LTBP-1s fusion proteins affected activation of latent TGF-β using cocultures of BAE and BSM cells and cultures of stimulated macrophages.

Serum-free heterotypic cultures of BAE and BSM cells were used as a cell system that generates mature TGF-β from endogenous large latent complex (14). Control cultures were serum-free homotypic cultures of BAE and BSM cells. The cocultures of BAE and BSM cells received either affinity-purified antibodies to one of the three GST–LTBP-1s fusion proteins, protein A–purified Ab 39, or nonimmune IgG, as indicated in Fig. 9. After an overnight incubation, CM from these cultures were bioassayed immediately for mature TGF-β using the MLEC-luciferase assay. These experiments revealed that CM from cocultures treated with Ab 39, anti-450, or anti-849 IgG contained less mature TGF-β than CM from untreated cocultures or cocultures treated with nonimmune or anti-1065 IgG (Fig. 9 A). We previously observed that Ab 39 IgG blocks latent TGF-β activation by cocultures of BAE and BSM cells (19). To verify that the measured luciferase activity was TGF-β dependent, neutralizing anti–TGF-β IgG was added to CM from untreated cocultures and found to inhibit 86% of the luciferase response (Fig. 9 A), indicating that most of the measured luciferase activity was TGF-β dependent. Anti-450 and anti-849 IgG blocked TGF-β generation at ID_{50}s of 20 and 1 μg/ml, respectively. CM from cocultures treated with the anti–LTBP-1s IgGs contained levels of latent TGF-β similar to those from untreated cocultures, as determined by the MLEC-luciferase assay (data not shown). Furthermore, addition of the anti–LTBP-1s IgGs to CM from untreated cocultures did not inhibit TGF-β activity (data not shown). Therefore, the ef-
that luciferase production was TGF-β-dependent, 20 μg/ml neutralizing anti–TGF-β IgG was added to CM from untreated cultures. Data presented are from one out of four experiments yielding similar results. (B) The wound migration assay was used to detect TGF-β activity generated by cocultures of BAE and BSM cells. Serum-free CM harvested from untreated cocultures or cocultures treated with the same antibodies as in A were assayed for TGF-β activity using the wound migration assay described in Materials and Methods. Control (100%) is the migration observed in wounded BAE cultures treated with media from homotypic BAE and BSM cells mixed 4:1. These data are presented as the percentage of migration observed in wounded cultures treated with control media. Data are representative of four experiments yielding similar results. (C) The MLEC-luciferase assay was used to detect TGF-β activity generated by stimulated macrophages. Serum-free CM from either untreated or antibody-treated cultures of LPS-stimulated thioglycollate-elicited peritoneal macrophages were harvested and assayed. Antibody treatments were as in A and B. Data presented are from one out of three experiments yielding similar results.

Figure 9. Effect of antibodies to LTBP-1 on latent TGF-β activation by cocultures of BAE and BSM cells, and stimulated macrophages. (A) The MLEC-luciferase assay was used to detect TGF-β activity generated by cocultures of BAE and BSM cells. Serum-free CM harvested from either untreated cultures or cultures treated with 10 μg/ml Ab 39 IgG, 50 μg/ml nonimmune IgG, or 50 μg/ml of affinity-purified anti-450, anti-1065, or anti-849 IgG were assayed using the MLEC-luciferase assay. To verify that luciferase production was TGF-β-dependent, 20 μg/ml neutralizing anti–TGF-β IgG was added to CM from untreated cultures. Data presented are from one out of four experiments yielding similar results. (B) The wound migration assay was used to detect TGF-β activity generated by cocultures of BAE and BSM cells. Serum-free CM harvested from untreated cocultures or cocultures treated with the same antibodies as in A were assayed for TGF-β activity using the wound migration assay described in Materials and Methods. Control (100%) is the migration observed in wounded BAE cultures treated with media from homotypic BAE and BSM cells mixed 4:1. These data are presented as the percentage of migration observed in wounded cultures treated with control media. Data are representative of four experiments yielding similar results. (C) The wound migration assay was used to detect TGF-β activity generated by stimulated macrophages. Serum-free CM from either untreated or antibody-treated cultures of LPS-stimulated thioglycollate-elicited peritoneal macrophages were harvested and assayed. Antibody treatments were as in A and B. Data presented are from one out of three experiments yielding similar results.

The results obtained using the MLEC-luciferase assay to measure mature TGF-β levels present in coculture CM were confirmed using the wound migration assay, in which the inhibitory effect of TGF-β on endothelial cell migration is quantitated. We observed that CM from cocultures of BAE and BSM cells treated with Ab 39, anti-450, or anti-849 IgG did not inhibit the migration of BAE cells, nor did anti–TGF-β IgG–containing CM from untreated cocultures (Fig. 9 B). CM from cocultures treated with non-immune or anti-1065 IgG inhibited the migration of endothelial cells by ~50% (Fig. 9 B). These results are similar to those obtained using the MLEC-luciferase assay, indicating that less TGF-β is present in CM prepared from cocultures treated with Ab 39, anti-450, or anti-849 IgG.

To determine whether the inhibitory effect of anti-450 or anti-849 IgG on latent TGF-β activation was specific to cocultures of BAE and BSM cells, we examined whether these antibodies abrogated TGF-β formation by LPS-stimulated thioglycollate-elicited peritoneal macrophages, another cell system previously demonstrated to convert large latent complex to TGF-β (44). Serum-free CM from stimulated macrophages was prepared and assayed for TGF-β activity using the MLEC-luciferase assay. Levels of mature TGF-β present in CM from cultures treated with Ab 39, anti-450, or anti-849 IgG were similar to those present in anti–TGF-β IgG-containing CM harvested from untreated cultures (Fig. 9 C). Like cocultures, stimulated macrophages were unaffected by anti-1065 IgG with respect to their ability to produce mature TGF-β (Fig. 9 C). Assays of heat-activated CM from untreated or antibody-treated stimulated macrophages revealed that all cultures generated similar levels of total TGF-β (data not shown).

Results obtained using the MLEC-luciferase assay could not be confirmed using the wound migration assay because the wound migration assay was unresponsive to TGF-β present in CM from stimulated macrophages. Addition of either recombinant TGF-β1 or neutralizing anti–TGF-β IgG to CM from stimulated macrophages did not affect the migration of endothelial cells (data not shown). This may be due to the presence of migration stimulatory factors in macrophage CM (2). Nevertheless, anti-450 or anti-849 IgG blocked TGF-β generation by stimulated macrophages as determined by the MLEC-luciferase assay, suggesting that these antibody effects on large latent complex activation were not specific to cocultures of BAE and BSM cells and that the role of LTBP-1 in activation involves interactions with its amino- and carboxyl-terminal regions.

Figure 9. Effect of antibodies to LTBP-1 on latent TGF-β activation by cocultures of BAE and BSM cells, and stimulated macrophages. (A) The MLEC-luciferase assay was used to detect TGF-β activity generated by cocultures of BAE and BSM cells. Serum-free CM harvested from either untreated cultures or cultures and stimulated macrophages. (B) The wound migration assay was used to detect TGF-β activity generated by cocultures of BAE and BSM cells. Serum-free CM harvested from untreated cocultures or cocultures treated with the same antibodies as in A were assayed for TGF-β activity using the wound migration assay described in Materials and Methods. Control (100%) is the migration observed in wounded BAE cultures treated with media from homotypic BAE and BSM cells mixed 4:1. These data are presented as the percentage of migration observed in wounded cultures treated with control media. Data are representative of four experiments yielding similar results. (C) The wound migration assay was used to detect TGF-β activity generated by stimulated macrophages. Serum-free CM from either untreated or antibody-treated cultures of LPS-stimulated thioglycollate-elicited peritoneal macrophages were harvested and assayed. Antibody treatments were as in A and B. Data presented are from one out of three experiments yielding similar results.
Discussion

The reactants involved in the activation of latent TGF-β by cocultures of endothelial and smooth muscle cells, retinoic-treated BAE cells, and stimulated macrophages include plasmin, cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor, tissue transglutaminase, and LTBP-1 (27). Interactions between the reactants involved in the activation of latent TGF-β have not been characterized in cell-free systems, with the exception that plasmin can cleave LAP, destabilizing noncovalent interactions between LAP and TGF-β, and that recombinant small latent complex binds to the cell surface cation-independent mannose 6-phosphate/insulin-like growth factor type II receptor via mannose 6-phosphate residues present on LAP (16, 35, 36). In this article, we have identified and characterized interactions between two reactants, LTBP-1 and tissue transglutaminase, that may be involved in latent TGF-β activation by cocultures of BAE and BSM cells as well as LPS-stimulated thiglycollate-elicited macrophages (31, 44, 54). These interactions include an enzyme–substrate relationship between transglutaminase and LTBP-1 and transglutaminase-dependent anchoring of large latent complex to the ECM. In addition, a second functional domain of LTBP-1 involved in latent TGF-β activation was identified.

Matrix incorporation of LTBP-1 or large latent complex (reported by others and reproduced by us) appears to occur through transglutaminase-dependent cross-linking of LTBP-1 and matrix protein(s) (60). We observed that both large latent complex and LTBP-1 are substrates for transglutaminase, the LTBP-1 of large latent complex contains residues involved in cross-linking large latent complex, and the inhibition of transglutaminase severely attenuates the incorporation of LTBP-1 into the matrix. Transglutaminase-reactive sites of LTBP-1S appear to be located within residues 294 and 441 of the amino terminus, as an LTBP-1S mutant truncated at its amino terminus by 441 amino acids was not cross-linked to the matrix, whereas a 293–amino acid truncated LTBP-1S retained its matrix association.

Transglutaminase is more selective for glutamine residues than acyl acceptor sites (25). A consensus sequence for glutamine residues that serve as amine acceptor sites in transglutaminase-catalyzed isopeptide bond formation has not been identified. However, LTBP-1S shares some structural features present in other transglutaminase substrates. Sequence analysis of reactive glutamine residues reveals that they are exposed in loop structures, as the glutamines tend to be surrounded by positively or negatively charged amino acids (4). In 60% of the substrates, glutamine residues are located at the amino or carboxyl terminus. From sequence alignment analysis of human LTBP-1S and LTBP-2, rat LTBP-1 and murine LTBP-3 (28, 43, 45, 64), glutamine-374 and a positively flanking amino acid within the second cysteine-rich repeat of LTBP-1S are conserved among all LTBP, suggesting that it may be a transglutaminase reactive site. Glutamine-374 is present in ΔN293 LTBP-1S, which was incorporated into ECM but is absent in ΔN441 LTBP-1S, which appeared not to be cross-linked to ECM. Identification of the transglutaminase-reactive glutamine(s) is a current subject of investigation using biochemical and molecular approaches.

The matrix protein(s) to which LTBP-1 is cross-linked has not been identified. However, LTBP-1S has been described to colocalize with fibronectin (Taipale, J., J. Saharinen, K. Hedman, and J. Keski-Oja. 1994. Mol. Biol. Cell. 5[Suppl.]:311a) and with collagen-free fibrillar structures generated by fetal rat calvarial cells (13). LTBP-2 appears to closely associate with elastin-associated microfibrils (22). Thus, fibronectin and proteins of elastic fibers, such as microfibril-associated glycoprotein, are candidate proteins, as they all contain residues susceptible to transglutaminase-catalyzed isopeptide bond formation (10, 37).

In addition to localizing the transglutaminase reactivity of LTBP-1S to amino-terminal residues 294–441, LTBP-1S domains participating in latent TGF-β activation were identified. Both the amino and carboxyl regions of LTBP-1S appear to be involved in protein interactions required for activation as antibodies to these two domains abrogated TGF-β generation by cocultures of BAE and BSM cells as well as LPS-stimulated thiglycollate-elicited macrophages. Previously, we reported that protein A–purified anti-450 IgG (the antibody to the amino terminus of LTBP-1S) did not affect activation of large latent complex by stimulated macrophages (44). This discrepancy results from the fact that in the earlier work total IgG was used, whereas in the experiments reported here affinity-purified IgG was tested. Based upon the concentration of affinity-purified anti-450 IgG required to inhibit activation of large latent complex, the amount of IgG used previously would have been insufficient to observe an effect. Anti-450 IgG also inhibited matrix association of LTBP-1 and large latent complex, suggesting that the role of the amino terminus in latent TGF-β activation may be to mediate the incorporation of large latent complex into the matrix.

We have demonstrated that the carboxyl-terminal region of LTBP-1 is required for large latent complex activation, as antibodies to this region blocked TGF-β generation (44). The carboxyl-terminal sequence of LTBP-1 does not appear to contain sites involved in covalently attaching LTBP-1 to the ECM, as addition of these antibodies to cocultures did not affect the matrix content of LTBP-1. This region does contain a putative protease-sensitive site at residue 1257 located after the third cysteine-rich repeat (28, 49, 60). Cleavage at this site might facilitate activation of soluble forms of large latent complex.

Matrix incorporation of large latent complex may create a concentrated pool of large latent complex. Others have proposed that segregating pools of latent TGF-β may be important in regulating the conversion of large latent complex to mature TGF-β at specific sites (23). Alternatively, we speculate that soluble large latent complex may be resistant to plasmin activation. It has been demonstrated that activation of latent TGF-β in fibroblast CM by plasmin requires nonphysiological levels of plasmin (35). We also find that recombinant large latent complex is not readily activated by plasmin in solution (data not shown). Therefore, cross-linking to the matrix may sequester large latent complex that is not readily activated, and subsequent release from the matrix by proteolysis (60) could generate a modified complex susceptible to plasmin activation.

We propose that the activation of large latent complex, to release, TGF-β occurs by the sequence of reactions il-
residues of LAP involved in localizing latent TGF-β to the cell surface. Proteolytically processed LTBP-1 is proposed to target to the cell surface where cell-associated plasmin cleaves LAP to liberate mature TGF-β from the complex.

This model suggests several testable questions. These include the potential significance of the putative plasmin cleavage sites in LTBP-1S, the availability of the mannose 6-phosphate residues of LAP in the complex, and the location of plasmin-sensitive sites of LAP. It is also unknown whether large latent complexes that differ in their LTBP isoforms are activated by a similar mechanism. Answers to these questions can be approached by altering specific residues in the components of large latent complex and monitoring rates of large latent complex activation.

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References

1. Abe, M., J.G. Harpel, C.N. Metz, I. Nunes, D.J. Loskutoff, and D.B. Rifkin. 1994. An assay for transforming growth factor-β using cells transfected with a plasminogen activator inhibitor-1 promoter luciferase construct. Anal. Biochem. 216:276–284.
2. Adams, D., and T.A. Hamilton. 1984. The cell biology of macrophage activation. Annu. Rev. Immunol. 2:283–318.
3. Aeschlimann, D., and M. Paulsson. 1991. Cross-linking of laminin-nidogen complexes by tissue transglutaminase. J. Biol. Chem. 266:15308–15317.
4. Aeschlimann, D., M. Paulsson, and K. Mann. 1992. Identification of gln726 in nidogen as the amine acceptor in transglutaminase-catalyzed cross-linking of laminin-nidogen complexes. J. Biol. Chem. 267:11316–11321.
5. Barcellos-Hoff, M.H., R. Derynck, M.L. Tsang, and J.A. Weatherbee. 1994. Transforming growth factor-β activation in irradiated murine mammary gland. J. Clin. Invest. 93:892–899.
6. Bendixen, E., B. Wolfgang, and P.C. Harpel. 1993. Transglutaminases catalyze cross-linking of plasminogen to fibroectin and human endothelial cells. J. Biol. Chem. 268:21962–21967.
7. Bishop, P.D., D.C. Teller, R.A. Smith, G.W. Lasser, T. Gilbert, and R.L.
29. Kielty, C.M., T. Rantamaki, A.H. Child, C.A. Shuttleworth, and L. Peltonen. 1995. Cysteine-to-arginine point mutation in a “hybrid” eight-cysteine domain of FBN1: consequences for fibrillin aggregation and microfibril assembly. J. Cell Sci. 108:1317–1323.

30. Kojima, S., and D.B. Rifkin. 1993. Mechanism of retinoid-induced activation of transforming growth factor-β in bovine endothelial cells. J. Cell. Physiol. 155:322–332.

31. Kojima, S., N. Kiyomitsu, and D.B. Rifkin. 1993. Requirement for transglutaminase in the activation of latent transforming growth factor-β in bovine endothelial cells. J. Cell. Biol. 121:439–448.

32. Kovacina, K.S., G. Steele-Perkins, A.F. Purcio, M. Lioubin, K. Miyazawa, C. Heldin, and R. Roth. 1989. Interactions of recombinant and platelet transforming growth factor-β1 precursor with the insulin-like growth factor (IGF)-1 receptor. Biochem. Biophys. Res. Commun. 160:393–403.

33. Lorand, L., and S.M. Conrad. 1984. Transglutaminase. Mol. Cell. Biochem. 58:3–9.

34. Lyons, R.M., and H.L. Moses. 1990. Transforming growth factor-β and regulation of the cell culture proliferation. Eur. J. Biochem. 187:467–473.

35. Lyons, R.M., J. Keski-Oja, and H.L. Moses. 1988. Proteolytic activation of latent transforming growth factor-β from fibroblast-conditioned medium. J. Cell Biol. 106:1659–1665.

36. Lyons, R.M., L.E. Gentry, A.F. Purcio, and H.L. Moses. 1990. Mechanism of activation of latent recombinant transforming growth factor β1 by plasmin. J. Cell Biol. 110:1361–1367.

37. Martinez, J., D.G. Chalupowicz, R.K. Roush, A. Sheith, and C. Barigian. 1994. Transglutaminase-mediated processing of fibronectin by endothelial cell monolayers. Biochemistry. 33:2538–2545.

38. Massague, J. 1990. The transforming growth factor-β family. Annu. Rev. Cell Biol. 6:597–641.

39. McConahey, P.J., and F.J. Dixon. 1980. Radiodiodination of proteins by the use of the chloramine-T method. Methods Enzymol. 70:210–213.

40. Miyazono, K., U. Hellman, C. Wernstedt, and C. Heldin. 1988. High molecular weight complex of transforming growth factor β1. J. Biol. Chem. 263:6407–6415.

41. Miyazono, K., A. Olofsson, P. Colosetti, and C. Heldin. 1991. A role of the latent TGF-β1-binding protein in the assembly and secretion of TGF-β1. EMBO (Eur. Mol. Biol. Organ.) 10:5991–5997.

42. Miyazono, K., H. Ichijo, and C.H. Heldin. 1993. Transforming growth factor-β-beta: latent forms, binding proteins and receptors. Growth Factors. 8:11–22.

43. Morelli, A., A. Olofsson, G. Stenman, P. Sahlin, T. Kanzaki, L. Claesson-Welsh, P. ten Dijke, K. Miyazawa, and C.H. Heldin. 1994. Identification and characterization of LTBP-2, a novel latent transforming growth factor-β-binding protein. J. Biol. Chem. 269:10591–10597.

44. Osofsky, J.M., T. Rantamaki, and D.B. Rifkin. 1995. Characterization of latent TGF-β activation by murine peritoneal macrophages. J. Immunol. 155:1450–1459.

45. Okada, F., K. Yamaguchi, A. Ichihara, and T. Nakamura. 1989. Purification and structural analysis of a latent transforming growth factor-β1 rat platelets. J. Biochem. 106:304–310.

46. Olofsson, A., H. Ichijo, A. Moren, P. ten Dijke, K. Miyazawa, and C.H. Heldin. 1995. Efficient association of an amino-terminally extended form of human latent transforming growth factor-β1 binding protein with the extracellular matrix. J. Biol. Chem. 270:31294–31297.

47. Ramirez, F. 1996. Fibrillin mutations in Marfan syndrome and related phenotypes. Curr. Opin. Genet. Dev. 6:309–315.

48. Rosenthal, J., S. Ribeiro, and R. P. Mecham. 1993. Extracellular matrix type 4: the elastic fiber. FASEB (Fed. Am. Soc. Exp. Biol.) J. 7:1208–1218.

49. Saikawa, T., J. Tanaka, and D.B. Rifkin. 1996. Analysis of latent transforming growth factor-β1 with an eight cysteine repeat of its binding protein LTBP-2. J. Biol. Chem. 271:8091–8097.

50. Schultze-Cherry, S., S. Ribeiro, L. Gentry, and J.E. Murphy-Ullrich. 1994. Vimentin in the sub-stratum of endothelial cells is cross-linked and phosphorylated. Biochem. Biophys. Res. Commun. 174:465–469.

51. Sato, Y., R. Tsuboi, R. Lyons, H. Moses, and D.B. Rifkin. 1990. Characterization of activation of latent TGF-β1 by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J. Cell Biol. 111:757–763.

52. Sakai, L.Y., D.R. Keene, R.W. Glanville, and H.P. Bachinger. 1991. Purification and partial characterization of fibrillin, a cysteine-rich structural component of connective tissue microfibrils. J. Biol. Chem. 266:14763–14770.

53. Sane, D.C., T.L. Moser, and C.S. Greenberg. 1991. Vimentin in the sub-stratum of endothelial cells is cross-linked and phosphorylated. Biochem. Biophys. Res. Commun. 174:465–469.

54. Satoh, T., and D.B. Rifkin. 1988. Autocarboxy-terminal activation of basic fibrillar growth factor: regulation of endothelial cell movement, plasminogen activator synthesis, and DNA synthesis. J. Cell Biol. 107:1199–1205.

55. Satoh, T., and D. Rifkin. 1988. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: activation of latent transforming growth factor-β1-like molecule by plasmin during co-culture. J. Cell Biol. 109:309–315.

56. Seale, R., M. Ono, R. Tsuibo, R. Lyons, H. Moses, and D.B. Rifkin. 1990. Characterization of the activation of latent TGF-β1 by co-cultures of endothelial cells and pericytes or smooth muscle cells: a self-regulating system. J. Cell Biol. 111:757–763.

57. Sato, Y., F. Okada, M. Abe, T. Seguchi, M. Kuwano, S. Sato, A. Furuya, N. Tanaka, and K. Miyazawa. 1988. Characterization of latent transforming growth factor-β1 activity in rat platelets. J. Biol. Chem. 263:14763–14770.

58. Schultz-Cherry, S., S. Ribeiro, L. Gentry, and J.E. Murphy-Ullrich. 1994. Thrombospondin binds and activates the small and large forms of latent transforming growth factor-β1 in a chemically defined system. J. Biol. Chem. 269:26775–26782.
57. Smith, D.B., and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene.* 67:31–40.

58. Sporn, M.B., and A.B. Robert. 1992. Transforming growth factor-β: recent progress and new challenges. *J. Cell Biol.* 119:1017–1021.

59. Taipale, J., K. Koli, and J. Keski-Oja. 1992. Release of transforming growth factor-β1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. *J. Biol. Chem.* 267:25378–25384.

60. Taipale, J., K. Miyazono, C.H. Heldin, and J. Keski-Oja. 1994. Latent transforming growth factor-β1 associates to fibroblast extracellular matrix via latent TGF-β binding protein. *J. Cell Biol.* 124:171–181.

61. Taipale, J., J. Lohi, J. Saarinen, P.T. Kovanen, and J. Keski-Oja. 1995. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-β1 from the extracellular matrix of cultured human epithelial and endothelial cells. *J. Biol. Chem.* 270:4689–4696.

62. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

63. Upchurch, H.F., E. Conway, M.K. Patterson, and M.D. Maxwell. 1991. Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme. *J. Cell. Physiol.* 149:375–382.

64. Yin, W., E. Smiley, J. Germiller, R.P. Mecham, J.B. Florer, R.J. Wenstrup, and J. Bonadio. 1995. Isolation of a novel latent transforming growth factor-β binding protein gene (LTBP-3). *J. Biol. Chem.* 270:10147–10160.

65. Zhang, H., S.D. Apfelroth, E. Hu, E.C. Davis, C. Sanguineti, J. Bonadio, R.P. Mecham, and F. Ramirez. 1994. Structure and expression of fibrillin-2, a novel microfibrillar component preferentially located in elastic matrices. *J. Cell Biol.* 124:855–863.