

Latent Transforming Growth Factor- β 1 Associates to Fibroblast Extracellular Matrix via Latent TGF- β Binding Protein

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Abstract. The role of latent transforming growth factor- β (TGF- β) binding protein (LTBP) in the association of TGF- β 1 to the extracellular matrix of cultured fibroblasts and HT-1080 fibrosarcoma cells was studied by immunochemical methods. The matrices were isolated from the cells, and the levels of LTBP and TGF- β 1 were estimated by immunoblotting and immunoprecipitation. LTBP, TGF- β 1, and its propeptide (latency-associated peptide, LAP) were found to associate to the extracellular matrix. Immunoblotting analysis indicated that treatment of the cells with plasmin resulted in a concomitant time and dose dependent release of both LTBP and TGF- β 1 from the extracellular matrix to the supernatant. Comparison of molecular weights suggested that plasmin treatment resulted in the cleavage of LTBP from the high molecular weight fibroblast

form to a form resembling the low molecular weight LTBP found in platelets. Pulse-chase and immunoprecipitation analysis indicated that both the free form of LTBP and LTBP complexed to latent TGF- β were efficiently incorporated in the extracellular matrix, from where both complexes were slowly released to the culture medium. Addition of plasmin to the chase solution resulted, however, in a rapid release of LTBP from the matrix. Fibroblast derived LTBP was found to associate to the matrix of HT-1080 cells in a plasmin sensitive manner as shown by immunoprecipitation analysis. These results suggest that the latent form of TGF- β 1 associates with the extracellular matrix via LTBP, and that the release of latent TGF- β 1 from the matrix is a consequence of proteolytic cleavage(s) of LTBP.

GROWTH factors of the TGF- β family have diverse effects on cell growth, morphology, and differentiation. TGF- β 's also have an important role in controlling the proteolytic balance of cells and the production and structure of the extracellular matrix (for review see Lyons and Moses, 1990; Massagué, 1990; Laiho and Keski-Oja, 1992; Miyazono et al., 1993). Three isoforms of TGF- β have been found in mammals, TGF- β 's 1, 2, and 3 (Derynck et al., 1985, 1988; de Martin et al., 1987; ten Dijke et al., 1988). After synthesis, two chains of pro-TGF- β associate to form a disulfide bonded dimer. Homodimeric forms are most common, but the heterodimers TGF- β 1.2 and TGF- β 2.3 have also been isolated (Cheifetz et al., 1987; Ogawa et al., 1992). TGF- β 's are secreted by cultured cells as latent complexes. During secretion the carboxy-terminal chains constituting mature TGF- β are cleaved from their amino-terminal propeptides. The disulfide linked propeptide dimer (TGF- β 1 latency associated protein, LAP¹) renders the

secreted TGF- β latent by remaining noncovalently associated to dimeric TGF- β .

A fraction of latent TGF- β contains additional high molecular weight proteins, which are associated with LAP (Miyazono et al., 1988; Wakefield et al., 1988; Olofsson et al., 1992). Best characterized of these is latent TGF- β binding protein (LTBP), which is bound to LAP by a disulfide bond (Miyazono et al., 1988). The amino acid sequence of LTBP is highly repetitive, containing 16 EGF-like repeats and three novel "LTBP-like" repeats (Kanzaki et al., 1990; Tsuji et al., 1990). LTBP-like repeat structures are also present in fibrillin (Maslen et al., 1991), a proteinase sensitive component of the elastic fibers, suggesting that LTBP is a component of the extracellular matrix. Fibroblasts produce high molecular mass (190 kD) form of LTBP, while the form of LTBP contained in the platelet α -granules (Assoian and Sporn, 1986; Fava et al., 1990) is smaller, 125–160 kD, due to proteolytic processing (Kanzaki et al., 1990; Tsuji et al., 1990). The expression of LTBP is often co-regulated with TGF- β 1 (Miyazono et al., 1991; Taipale, J., S. Matikainen, M. Hurme, and J. Keski-Oja, manuscript submitted for publication).

TGF- β has a major role in the regulation of extracellular matrix synthesis and degradation. TGF- β stimulates the synthesis of multiple extracellular matrix components, including collagens, fibronectin, vitronectin, tenascin, and pro-

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1. *Abbreviations used in this paper:* LAP, latency-associated protein; LTBP, latent TGF- β binding protein.

teoglycans (Ignatz and Massagué, 1986; Koli and Keski-Oja, 1991; Pearson et al., 1988; Bassols and Massagué, 1988). It also suppresses matrix degradation by downregulating the expression of proteinases, such as plasminogen activators (Laiho et al., 1986), collagenase (Edwards et al., 1987), and stromelysin (Kerr et al., 1990), and by inducing proteinase inhibitors, such as plasminogen activator inhibitor-1 (PAI-1; Laiho et al., 1986, 1987) and tissue inhibitor of metalloproteinase-1 (TIMP-1; Edwards et al., 1987). Interestingly, TGF- β itself is a component of the extracellular matrix, and can be released by proteinases (Taipale et al., 1992). Furthermore, the presence of extracellular matrix has been found to downregulate the expression of the TGF- β gene (Streuli et al., 1993). TGF- β may thus act as a feedback regulator of extracellular matrix formation.

Latent forms of TGF- β can be activated by extremes of pH, heat treatment, certain glycosidases and by the protease plasmin (Lawrence et al., 1985; Brown et al., 1990; Miyazono et al., 1988; Lyons et al., 1988). Latent TGF- β is also activated in co-cultures of endothelial cells and smooth muscle cells by plasmin-mediated proteolysis (Antonelli-Olridge et al., 1989; Sato and Rifkin, 1989). Studies using these co-culture models also suggest that transglutaminase, an enzyme involved in the cross-linking of extracellular matrix proteins, and latent TGF- β binding protein, are required for the activation of latent TGF- β (Kojima et al., 1993; Flaumenhaft et al., 1993).

We have found earlier that TGF- β 1 is associated with the extracellular matrix of human fibroblasts and fibrosarcoma cells, and that it can be released from the extracellular matrix in a latent form by plasmin and thrombin (Taipale et al., 1992). The release of latent TGF- β 1 from the matrix of endothelial cells by plasmin (Falcone et al., 1993) and by thrombin (Benezra et al., 1993) have also been observed. The structure of the matrix form as well as the molecular basis of latency of the TGF- β 1 released by proteinases have not been understood. This study was carried out to analyze the association of LTBP with the extracellular matrix and its role in the proteolytic release of latent TGF- β 1.

Materials and Methods

Reagents

Plasmin (specific activity 21.3 CU/mg protein) was purchased from Kabi (Mölnådal, Sweden). [35 S]cysteine (specific activity 1300 Ci/mmol) was from Amersham (Buckinghamshire, England). Bolton-Hunter labeled 125 I-TGF- β 1 (specific activity 158 μ Ci/ μ g) used as a standard was from Du Pont (NEN Research Products, Wilmington, DE). Human LTBP was purified from outdated platelets according to Miyazono et al. (1988). Recombinant small latent TGF- β 1 was purified according to Miyazono et al. (1991) from Chinese hamster ovary cells transfected with TGF- β 1 cDNA (gift from Dr. Hideya Ohashi, Kirin Brewery Co., Ltd., Gumma, Japan). All other reagents were from commercial sources and of the highest purity available.

Cell Culture

Human fibrosarcoma HT-1080 cells (CCL-121; American Type Culture Collection, Rockville, MD) and human embryonic lung fibroblasts (CCL-137; ATCC) were grown in Eagle's modification of minimal essential medium (MEM) containing 10% FCS, 100 IU/ml penicillin and 50 μ g/ml streptomycin. All experiments were carried out under serum-free conditions. Before commencing experiments, the cells were washed twice with serum free medium and incubated in serum free medium for 1-6 h to remove traces of serum proteins. Essentially similar results were obtained

using fibroblasts and HT-1080 cells in all experiments. HT-1080 cells were used in immunoprecipitation experiments due to lower background levels.

Antibodies

Affinity-purified peptide antibody No. 627 to TGF- β 1 amino acids 78-109 was used in immunoblotting analyses of TGF- β 1 (Taipale et al., 1992). This antibody is functional in immunoblotting, but does not immunoprecipitate TGF- β 1 or neutralize TGF- β 1 activity in biological assays. Antibody LT-2 was raised using purified recombinant small latent TGF- β 1 as antigen (Miyazono et al., 1991). This antibody recognizes TGF- β 1 propeptide (β 1-LAP) in immunoprecipitation. IgG fraction was purified from LT-2 serum by protein A-Sepharose (Pharmacia, Uppsala, Sweden) affinity chromatography according to manufacturer's instructions. Antibody Ab-39 (Miyazono et al., 1988, 1991) was raised against purified human platelet LTBP (free form, devoid of LAP and TGF- β). This antibody is functional in immunoblotting and immunoprecipitation of LTBP under nonreducing conditions, but does not react with reduced LTBP. All antibodies used were polyclonal rabbit antibodies.

Extracellular Matrix Preparations

Extracellular matrices were prepared according to Hedman et al. (1979). Briefly, cell cultures were washed once with PBS (0.14 M NaCl in 10 mM sodium phosphate buffer, pH 7.4) and then treated three times with 0.5% sodium deoxycholate in 10 mM Tris-HCl buffer, pH 8.0, at 0°C for 10 min. The plates were then washed again with PBS and allowed to dry overnight at room temperature. The residue was collected to nonreducing SDS-PAGE sample buffer (2% SDS) by rubber policeman and treated at 95°C for 5 min. These preparations are thus sodium deoxycholate insoluble, SDS soluble cell extracts, referred as "matrix" in the text. In some preparations, LTBP cross-linked to the matrix was unmasked and made soluble by digesting the sodium deoxycholate insoluble residues by plasmin (0.3 CU/ml) in matrix digestion buffer (PBS containing 1 mM Ca $^{2+}$, 1 mM Mg $^{2+}$, and 0.1% *n*-octyl-d- β -glucopyranoside) at 37°C for 1 h. These preparations are referred to as "matrix digest" in the text, and they contain also proteins derived from the highly cross-linked SDS-insoluble extracellular matrix.

Immunoprecipitation analyses of the matrix were carried out after plasmin digestion. The supernatants of plasmin digested matrices were incubated with the antibodies (see below) after inhibition of plasmin by phenylmethylsulfonyl fluoride (PMSF, 1 mM), leupeptin (100 μ g/ml), and aprotinin (200 IU/ml). For immunoblotting of the digested matrices, SDS was added (1% final concentration) without removing the plasmin solution, and the plates were scraped with a rubber policeman. The plasmin digested matrix samples (matrix plus supernatant) were then transferred to microcentrifuge tubes and treated at 95°C for 5 min before SDS-PAGE.

SDS-insoluble extracellular matrix proteins were prepared from the conventional matrices by treatment in 1% SDS at 95°C for 5 min, followed by centrifugation at 100,000 *g* for 1 h. The pellets were then washed once with PBS to remove excess of SDS. To release associated LTBP, the pellets were treated with plasmin (0.3 CU/ml) in matrix digestion buffer at 37°C for 1 h.

Metabolic Labeling Studies

Extracellular Matrix. For metabolic labeling of the extracellular matrix, HT-1080 cells were seeded to new plates and cultured for 3 d in the presence of [35 S]cysteine (50 μ Ci/ml) in MEM containing 5% FCS and 10% of the usual amount of cysteine (MEM contains 200 μ M cysteine). The cells were washed three times and incubated for 24 h in serum-free MEM (no radioactive Cys). The cells were then washed three times to remove soluble radioactivity. This method produced extracellular matrixes of low specific radioactivity, but ensured that all cysteine containing proteins of the extracellular matrix were labeled, irrespective of turnover rate.

Conditioned Medium. For production of [35 S]cysteine-labeled conditioned medium, confluent cultures of human fibroblasts were incubated under serum free conditions for 16 h. The cells were subsequently labeled with [35 S]cysteine (200 μ Ci/ml) in MEM containing 1% of the usual amount of methionine and cysteine for 16 h. Nonmetabolic labeling of proteins by disulfide exchange with [35 S]cysteine was minimized by dilution of the medium to MEM containing cysteine (1:1) directly after collection.

Pulse-Chase. For pulse-chase studies, HT-1080 cells were changed to serum free conditions, starved for 30 min in methionine- and cysteine-free MEM, followed by metabolic labeling with [35 S]cysteine in cysteine-free MEM containing 1% of the normal amount of methionine as indicated in each experiment. The medium was then collected and the cells were lysed

with 20 mM Tris-HCl buffer, containing 0.5% sodium deoxycholate, pH 8.0, at 0°C. Extracellular matrices were prepared as described above.

Immunoprecipitation Analyses

For immunoprecipitation, cell lysate, culture medium, and matrix digestion samples were preabsorbed by incubation with preimmune serum (1:500 dilution) or control hyperimmune rabbit IgG (5 µg/ml) at 0°C for 10 h followed by the addition of protein A-Sepharose beads. After mild shaking at 4°C for 2 h, the beads were collected by centrifugation at 13,000 rpm for 10 min in a microcentrifuge. The supernatants were transferred to fresh tubes and the preabsorption procedure was repeated. Subsequently, the samples were incubated with the specific antiserum (Ab-39, 1:500 dilution) or purified IgG (LT-2, 2 µg/ml) in fresh tubes for 2 h, followed by incubation with protein A-Sepharose in an end-over rotary shaker for 1 h. The beads were collected by slow centrifugation (2,000 rpm in a microcentrifuge for 5 min). The beads were then washed twice with PBS, twice with detergent buffer (1% sodium deoxycholate, 1% TX-100, 150 mM NaCl in 50 mM Tris-HCl buffer, pH 7.0), once with high salt buffer (500 mM NaCl, 0.2% TX-100 in 20 mM Tris-HCl buffer, pH 7.0) followed by three washes with PBS. During the last wash the beads were transferred to fresh tubes to avoid contamination by plastic-binding material. Bound material was then eluted from the beads by treating at 95°C for 5 min in gel sample buffer containing 2% SDS. Where indicated, the antibodies were incubated with respective antigens prior to immunoprecipitation (1 µg of antigen incubated with 30 µl of serum or 30 µg IgG at room temperature for 15 min).

PAGE and Immunoblotting

Gradient (4–15%) SDS-PAGE analysis was carried out using the Laemmli (1970) buffer system. The gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) and the filters were treated at 80°C under vacuum for 1 h to fix the transferred proteins.

For sodium deoxycholate-PAGE, samples were cooled to 0°C and an equal volume of sample loading buffer (200 mM Tris-HCl, pH 8.0, containing 10% glycerol and 1% sodium deoxycholate) was added at 0°C. The samples were then electrophoresed on pre-cast 4–15% polyacrylamide minigels not containing SDS (Bio Rad Laboratories, Hercules, CA) on a minigel apparatus (Mini protean II; Bio Rad Laboratories). Electrophoresis buffer was 50 mM Tris/192 mM glycine (pH ~8.3) containing 0.1% sodium deoxycholate. The electrophoresis was carried out at 0°C (20 mA constant current) until the phenol red marker dye exited the gel. Proteins from sodium deoxycholate-PAGE gels were electrophoretically transferred to Immobilon P (Millipore Corp., Richmond, CA) membranes according to manufacturer's instructions (Millipore Corp., and Bio Rad Laboratories). Transfer was carried out in a 30 mM Tris/200 mM glycine buffer (pH ~8.3) containing 0.01% SDS and 10% methanol. Extensive characterization of the electrophoretic properties of this system was not done. Since the migratory rate of proteins greatly decreases as the pore size of acrylamide approaches their radius, gradient deoxycholate-PAGE is likely to separate proteins dominantly according to size. Some proteins with positive or weakly negative charge may migrate anomalously, since sodium deoxycholate may not confer an overall negative charge to all proteins.

Immunodetection of LTBP and TGF-β1 from both sodium deoxycholate and SDS-PAGE filters was performed essentially as described (Taipale et al., 1992). The filters were blocked by PBS containing 1% TX-100 and 5% nonfat milk powder. The filters were then reacted with antibodies, washed, and bound antibodies were detected using biotin-streptavidin amplification and enhanced chemiluminescence detection (ECL; Amersham). Incubations and washes were carried out in 50 mM Tris-HCl buffer, pH 8.5, containing 0.5 M NaCl, 0.1% BSA, and 0.1% Tween 20.

Results

LTBP Is Associated with the Extracellular Matrix of Fibroblasts and Fibrosarcoma Cells

To determine whether LTBP is associated with the extracellular matrix, we labeled HT-1080 fibrosarcoma cells with [³⁵S]cysteine for three days (see Materials and Methods). The cells were then washed, changed to serum free medium, followed by treatment with plasmin (1 CU/ml) at 37°C for 1 h. The plasmin concentration used releases over 90% of

matrix-associated TGF-β1 in 30 min (Taipale et al., 1992). The medium was collected and plasmin was inhibited by proteinase inhibitors. LTBP was immunoprecipitated from the medium with specific antibodies (see Materials and Methods). Analysis of the precipitated material by 4–20% SDS-PAGE under reducing conditions followed by autoradiography revealed that LTBP immunoreactivity is rapidly released from the cell layer by plasmin (Fig. 1). Two major and a minor polypeptides (140–180 kD) were observed in the autoradiogram.

Subsequently, we prepared extracellular matrices from the respective cells by sodium deoxycholate extraction (see Materials and Methods). (Note that since sodium deoxycholate insoluble fraction of cells contains both substratum attached proteins and proteins derived from the extracellular matrix, we cannot differentiate between these two. The term "extracellular matrix" is therefore used synonymously with "sodium deoxycholate insoluble fraction" throughout the text.) Immunoprecipitation analysis of these preparations after solubilization with 8 M urea, 100 mM NaOH or 1% SDS and dilution to non-denaturing conditions revealed no detectable LTBP immunoreactivity (data not shown). The observed release of LTBP immunoreactivity (Fig. 1) could not be explained by release from the cell surface, since sufficient amounts of LTBP were not found in cell lysates containing cell surface bound proteins (data not shown).

To resolve the question whether LTBP is bound to the extracellular matrix, we used immunoblotting analysis to determine the approximate amount of LTBP released by plasmin. Confluent cultures of human lung fibroblasts, which build heavy meshworks of extracellular matrix, were changed to serum free medium and treated with plasmin. Extracellular matrices were prepared from the same cells by sodium deoxycholate extraction. Compared with the amount of LTBP released by plasmin (see below), only very low levels

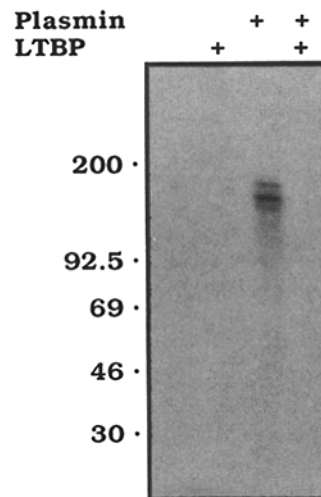


Figure 1. LTBP is associated with the cell layer. Immunoprecipitation analysis of LTBP released from the cell layer by plasmin. Extracellular matrices of HT-1080 cells were labeled by culturing the cells in the presence of [³⁵S]cysteine for 3 d (see Materials and Methods). The cells were subsequently treated with plasmin (1 CU/ml) at 37°C for 1 h as indicated (+). The medium was collected and plasmin activity was neutralized by 1 mM PMSF and 200 IU/ml aprotinin. LTBP was immunoprecipitated from the medium with an LTBP specific antibody (Ab-39) with

(+) or without competing unlabeled platelet LTBP. The precipitates were analyzed on 4–20% gradient SDS-PAGE under reducing conditions, followed by fluorography. Plasmin treatment is indicated on the top of the figure (+). The mobilities of molecular mass markers (kD) are indicated on the left. Note that TGF-β1 and LAP are not visible in this fluorogram due to weak labeling of the respective proteins (low content of cysteine).

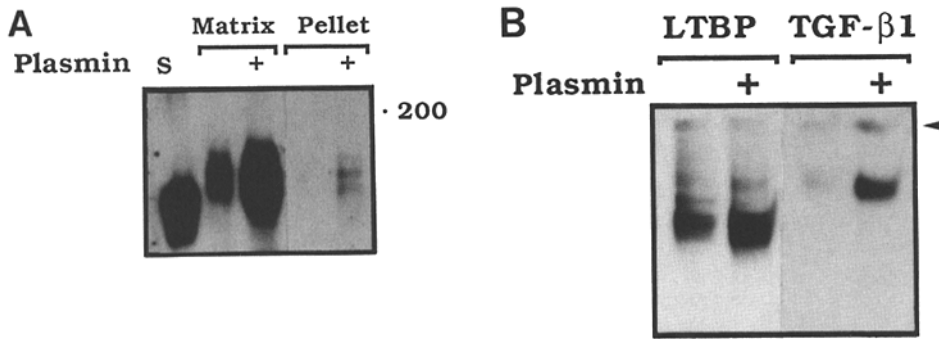


Figure 2. Association of LTBP with the extracellular matrix and its release by plasmin. (A) Plasmin releases covalently bound LTBP from fibroblast extracellular matrix. Extracellular matrices were prepared from human lung fibroblasts by sodium deoxycholate extraction (see Materials and Methods). The extracellular matrices were collected in 1% SDS with or without prior partial digestion with plasmin (0.3 CU/ml, 37°C for 1 h) and solubilized by heating to 95°C. SDS-insoluble

material was pelleted from two untreated matrices by ultracentrifugation (see Materials and Methods). The pellets were treated with plasmin (0.3 CU/ml) or control buffer. SDS-containing sample buffer was added and the samples heated again to 95°C. The samples were then analyzed by 5–15% gradient SDS-PAGE under nonreducing conditions, followed by immunoblotting analysis, using anti-LTBP antiserum (Ab-39). All lanes are from a single exposure of one filter and represent material derived from 10 cm² of extracellular matrix. Standard (S) contains 10 ng of purified platelet LTBP. Plasmin treatment is indicated on the top of the figure (+). Note that plasmin treatment increased the amount of LTBP immunoreactivity in the matrix preparations 10–100-fold, and revealed the presence of LTBP also in the SDS-insoluble pellet fraction. (B) Analysis of the complex structure of plasmin released latent TGF-β1 by deoxycholate-PAGE. Confluent cultures of human fibroblasts were changed to serum free medium, and the conditioned medium prepared in the presence or absence of plasmin (0.01 CU/ml), was collected after 3 d. The molecular nature of secreted TGF-β1 complexes was analyzed from the concentrated conditioned medium (500 μl/lane) by sodium deoxycholate-PAGE followed by immunoblotting with antibodies specific for TGF-β1 and LTBP as indicated in the figure (see Materials and Methods for details). Both chemiluminescence signals are from the same filter. TGF-β1 was detected first (5-min exposure) followed by washing and detection of LTBP (3-s exposure). Arrow represents the bottoms of the sample wells (stacking gel was not used).

were detectable in the untreated matrix preparations by immunoblotting (data not shown), indicating that LTBP is either masked or cross-linked to very high molecular weight form that does not enter the gel.

Previous cross-linking experiments suggested that matrix-bound TGF-β1 is cross-linked to very high molecular weight complexes (see Taipale et al., 1992). Highly cross-linked complexes formed by disulfide linkages and covalent association of proteins catalyzed by lysyl oxidase and transglutaminase are known to be present in the ECM (see Keski-Oja et al., 1976; Hedman et al., 1979; Reiser et al., 1992). These cross-linked complexes appear as particulate material that is not dissociated by heat treatment in the presence of SDS. To determine whether LTBP is a component of the fibroblast SDS-insoluble extracellular matrix, we treated isolated extracellular matrices with plasmin (0.3 CU/ml) at 37°C for 1 h to partially digest the extracellular matrix prior to solubilization of the matrix in SDS containing sample buffer (see Materials and Methods). Immunoblotting analysis indicated that ~10–100-fold higher levels of LTBP immunoreactivity were present in the matrix digest compared to a conventional matrix preparation (Fig. 2 A, Matrix). In subsequent experiments, we were able to immunoprecipitate LTBP and LAP from the supernatant fluids of isolated extracellular matrix preparations treated with plasmin.

To confirm that LTBP is present in the SDS-insoluble matrix fraction, we prepared extracellular matrices from confluent fibroblasts by sodium deoxycholate extraction, and solubilized them to 1% SDS by treatment at 95°C for 5 min. The matrix preparations were then centrifuged to pellet SDS-insoluble material, and the pellets washed with PBS to remove excess SDS. The pellets were subsequently treated with plasmin or control buffer, and immunoreactive LTBP was assayed by immunoblotting (see Materials and Methods). A small amount of LTBP immunoreactivity was recovered from the pellet by the plasmin treatment (Fig. 2 A, Pel-

let), indicating that at least some LTBP is present in the cross-linked extracellular matrix (ECM). Since heat treatment in SDS denatures proteins and renders them very sensitive to proteinases, quantitative recovery by this method is not practical. These results suggest that LTBP is associated covalently with the extracellular matrix, either by disulfide bond(s), or reducible or nonreducible cross-links formed by matrix cross-linking enzymes. Since the anti-LTBP antibody (Ab-39) does not react with LTBP under reducing conditions, we were unable to determine the exact molecular nature of the bond between LTBP and the extracellular matrix.

To analyze the complex structure of latent TGF-β1 released by plasmin, we used novel analysis method consisting of sodium deoxycholate-PAGE and immunoblotting (see Materials and Methods). Fibroblast cultures were washed with serum-free medium and incubated with or without 0.01 CU/ml plasmin for 3 d under serum-free conditions. The conditioned medium was subsequently concentrated 30-fold by centrifugal ultrafiltration (Centriprep 3; Amicon Corp., Beverly, MA). Immunoblotting analysis of the medium using sodium deoxycholate-PAGE with antibodies specific for LTBP and TGF-β1 indicated that TGF-β1 is bound to a species co-migrating with a minor component of LTBP (~200 kD), ~100 kD larger than the major LTBP species (Fig. 2 B). These results suggest that fibroblasts secrete an excess of LTBP, and that only a fraction of LTBP is associated to the small latent TGF-β1 (LAP·TGF-β1) complex. These results also suggest that soluble proteins migrate in gradient sodium deoxycholate-PAGE similarly to gradient SDS-PAGE. Since TGF-β1 did not dissociate from β1-LAP during electrophoresis, sodium deoxycholate-PAGE can be used to study the molecular complexes of TGF-β1. The molecular weights obtained are, however, highly approximate (actually an upper limit) due to nondenaturing nature of this gel system. The active form of TGF-β1, a highly hydrophobic and positively charged protein, obtained by heat treatment of the

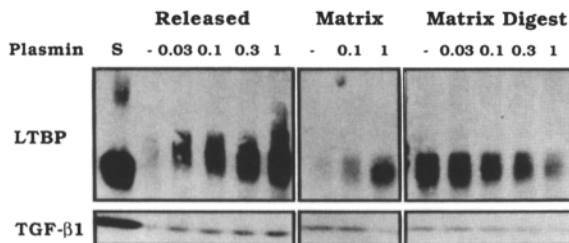


Figure 3. Corelease of LTBP and TGF- β 1 from the extracellular matrix by plasmin. Confluent cultures of HT-1080 cells were treated for 30 min with increasing concentrations of plasmin under serum-free conditions. LTBP and TGF- β 1 were assayed from the conditioned medium (*Released*), extracellular matrix preparations (*Matrix*, SDS-soluble, sodium deoxycholate insoluble cell extract), and isolated extracellular matrices digested with plasmin prior to addition of SDS (*Matrix Digest*, see Materials and Methods for details) by immunoblotting (nonreducing 4–20% gradient SDS-PAGE). Immunoblotting was first carried out using anti-LTBP antibodies and subsequently anti-TGF- β 1 antibodies. All signals are from the same filter (separate exposures for LTBP and TGF- β 1). Specific bands are shown. *Released* and *Matrix Digest* samples contain proteins derived from 1 cm² of cell culture, while 2 cm² of matrix was loaded to lanes *Matrix* for clarity. The concentrations of plasmin in the figure refer to the original concentration of plasmin (CU/ml) used to release LTBP from the cells prior to matrix preparation by sodium deoxycholate extraction. In *Matrix Digest* the second treatment with plasmin was 0.3 CU/ml at 37°C for 30 min. Standard (S) lane contains approximately equimolar amounts of purified human platelet LTBP (8 ng) and TGF- β 1 (1 ng).

conditioned medium, did not migrate at 25 kD in deoxycholate-PAGE, but aggregated on the top of the gel (data not shown). The reason for this is unclear at present.

LTBP and TGF- β 1 Are Co-released from the Extracellular Matrix of HT-1080 Cells by Plasmin

To analyze the interrelationships between LTBP and TGF- β 1, confluent cultures of HT-1080 cells were treated with increasing concentrations of plasmin for 30 min under serum-free conditions. The medium was collected and plasmin was inhibited by the addition of PMSF and aprotinin. Alterations in the amounts of released LTBP and TGF- β 1 were analyzed by immunoblotting (see Materials and Methods). It was found that the amounts of LTBP and TGF- β 1 released to the supernatant increased in a dose-dependent manner and concomitantly as a function of plasmin concentration (Fig. 3, *Released*). The molar amounts of LTBP and TGF- β 1 suggested that there is an excess of LTBP in the matrix. The size of LTBP (170–120 kD) decreased stepwise suggesting that several proteolytic cleavages may occur (Fig. 3, *Released*). The final fragment of digestion was in the same range as the platelet LTBP used as standard (Fig. 3, lane S; 120–140 kD). The molecular weight of TGF- β 1 did not decrease during the experiment, as reported earlier (Lyons et al., 1990; Taipale et al., 1992).

To analyze the relative amounts of TGF- β 1 and LTBP in the matrix, extracellular matrices were prepared from the respective cells by extraction with sodium deoxycholate and analyzed by immunoblotting. The amount of TGF- β 1 was found to decrease in these matrix preparations with increasing plasmin concentrations as shown earlier (Taipale et al., 1992). Only low amounts of immunoreactive LTBP were

found in the matrices of untreated cells, but the level of 120–140 kD LTBP increased with increasing plasmin dose (Fig. 3, *Matrix*). Immunoreactive LTBP thus probably appeared in the matrix preparations due to cleavage(s) from the high molecular weight cross-linked insoluble form that does not enter the resolving gel. The reason why this minor fraction of LTBP is cleaved but remains associated to the matrix is unclear at present. The result does not imply that TGF- β 1 and LTBP would be released from the matrix independently, since TGF- β 1 is released from the latent complex by SDS used in the preparation of the SDS-PAGE sample (Miyazono et al., 1988).

To determine whether LTBP released to the supernatant by plasmin is derived from the SDS-insoluble extracellular matrix, we treated HT-1080 cells with the same doses of plasmin as above and isolated the extracellular matrices. Here, instead of directly collecting the matrices, we treated these isolated matrices again with a constant concentration of plasmin (0.3 CU/ml) to partially digest the extracellular matrix (see *Matrix Digest* in Materials and Methods) and unmask covalently bound LTBP before addition of SDS containing buffer (see above). Analysis of these preparations by immunoblotting indicated that the initial plasmin treatment resulted in concomitant, dose dependent loss of LTBP and TGF- β 1 from these matrix preparations (Fig. 3, *Matrix Digest*). Comparison of the amounts of LTBP released from the cell layer to the supernatant (Fig. 3, *Released*) and the amount of total LTBP present in the extracellular matrix (Fig. 3, *Matrix Digest*), indicate that a major fraction of plasmin released LTBP is derived from the extracellular matrix. Slightly lower amounts of LTBP were however found in the solubilized matrix (*Matrix Digest*) than in the supernatant (*Released*). This is probably due to losses occurring during the preparation of the matrices and during plasmin digestion. In earlier experiments (Taipale et al., 1992) the recovery of TGF- β 1 from the matrix was ~70–80%. We cannot, however, rule out the possibility that plasmin could release some LTBP from the cell surface as well. Comparison between the levels of LTBP and TGF- β 1 also suggest that there is an excess of LTBP in the matrix (see below).

Secreted Large Latent TGF- β Complex Associates with the Extracellular Matrix Rapidly After Secretion

To study the kinetics of latent TGF- β association with the extracellular matrix, we performed pulse-chase experiments. Subconfluent, actively growing cultures of HT-1080 cells were incubated in serum free MEM for 1 h to remove serum proteins. The cells were subsequently labeled with [³⁵S]cysteine (500 μ Ci/ml) for 10 min (see Materials and Methods), washed twice, and chased with MEM (>500-fold excess of unlabeled cysteine) as indicated (Fig. 4). The medium was then collected and the cells were lysed with 0.5% sodium deoxycholate containing buffer. Extracellular matrices were prepared from the same cells, and treated with plasmin to release LTBP covalently bound to the matrix. Cell lysates, extracellular matrix digests (see Materials and Methods) and the medium were analyzed by immunoprecipitation with antibodies specific for the small latent TGF- β 1 complex (LT-2) and LTBP (Ab-39). The specificity of the LAP (LT-2) bands was judged by precipitation with LT-2 in the presence of competing unlabeled antigen.

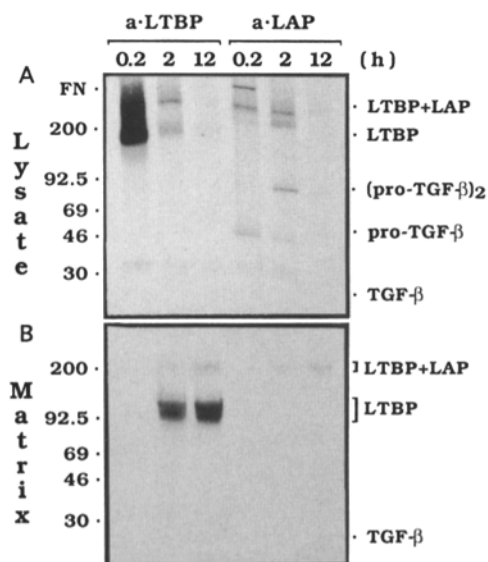


Figure 4. Deposition of LTBP and latent TGF- β to the extracellular matrix of HT-1080 cells. Cultures of HT-1080 cells (70% confluent) were changed to serum-free medium and incubated in cysteine- and methionine-free MEM for 30 min to deplete intracellular pools of cysteine. Cells were subsequently labeled with 500 μ Ci/ml [35 S]cysteine in cysteine-free MEM for 10 min. The cells were then washed rapidly two times and chased with MEM (>500-fold excess of unlabeled cysteine). Medium was then collected and the cells were lysed with sodium deoxycholate containing buffer (see Materials and Methods) at the time points shown on the figure. Extracellular matrices were prepared from the same cells and treated with plasmin (0.3 CU/ml) at 37°C for 1 h to release latent TGF- β complexes covalently bound to the matrix. The medium, cell lysates and extracellular matrix digests were incubated with antibodies specific for LTBP (Ab-39) and LAP (LT-2) followed by precipitation of immune complexes by protein A-Sepharose beads. The precipitates were analyzed by 4–15% gradient SDS-PAGE under nonreducing conditions followed by fluorography. The specificity of precipitation was assessed by precipitation with anti-LAP antibodies in the presence of competing antigen. The positions of the specific complexes and a single background band probably representing fibronectin (FN) are also indicated. The mobilities of the molecular mass markers (kD) are shown on the left. (A) Cell lysates; (B) material released from the pericellular matrix by plasmin. Immunoprecipitation analysis of the medium was negative.

Analysis of the material immunoprecipitated by anti-LTBP antibodies in 4–15% gradient SDS-PAGE under nonreducing conditions, followed by fluorography, indicated that after 12 min of chase, all detectable radioactivity was present in the cell lysate (Fig. 4 A, *a-LTBP* lanes). A predominant band of 180 kD and a less intense band of 250 kD were observed (Fig. 4 A). The 180-kD band represents free LTBP, while the 250-kD band is evidently a complex between LTBP and LAP, since it was also present in the immunoprecipitate obtained by anti-LAP antibodies (Fig. 4 A, *a-LAP* lanes). Under reducing conditions, the 250-kD band was reduced to 180-kD (gel not shown), which is in accordance with the finding that LTBP and LAP are bridged by a disulfide bond (Miyazono et al., 1988, 1991). A component of \sim 45 kD, corresponding in size to the nascent, uncleaved chain of pro-TGF- β , was also observed in the anti-LAP immunoprecipitates after 12 min of chase (Fig. 4 A, lanes *a-LAP*). After

2 h of chase, this band is converted to a disulfide bonded dimer of 90 kD (Fig. 4 A; see Miyazono et al., 1991). In addition to the 250-kD band representing LAP-LTBP complex, two specific bands of 195 and 200 kD were also detected in the anti-LAP immunoprecipitates. These components were not present in the material precipitated with anti-LTBP antibodies, suggesting that they represent complexes between LAP and a high molecular weight protein that is distinct from LTBP (see Olofsson et al., 1992). Two nonspecific bands, a low molecular mass species migrating slightly above the 30-kD marker, and a high molecular mass band, probably representing fibronectin (denoted FN), was also present in some of the immunoprecipitates (Fig. 4 A).

After 2 h of chase, the majority of LTBP immunoreactivity was lost from the cell lysate, while approximately equal levels of LAP immunoreactivity were present in the samples collected at 12 min and 2 h. This is consistent with earlier results showing that the free form of LTBP and LTBP complexed to latent TGF- β are secreted rapidly, while latent TGF- β not containing LTBP is retained intracellularly (Miyazono et al., 1991, 1992). Since the cell lysates contain also cell surface bound material, these results also suggest that significant amounts of LTBP do not remain associated with the cell layer due to association with cell surface alone.

After 2 h of chase, the major fraction of LTBP immunoreactivity was found in association with the extracellular matrix, while negligible levels of immunoreactivity were present in the medium (data not shown). Two groups of LTBP related components were precipitated from the extracellular matrix released material, representing the free form of LTBP (100–130 kD) and LTBP complexed to LAP (200 kD; Fig. 4 B). The 200-kD component was also detectable in the anti-LAP immunoprecipitates (Fig. 4 B). A very weak band of 25 kD corresponding TGF- β , as judged by comigration with 125 I-TGF- β 1, was also detectable in the fluorograms of both the anti-LTBP and anti-LAP immunoprecipitates.

These results indicate that in actively growing, subconfluent cells, secreted free form of LTBP and LTBP complexed to latent TGF- β rapidly associate with the extracellular matrix. The difference between the migration of free LTBP here and above (Fig. 1) is due to the fact that the free form of LTBP migrates as a component of slightly lower molecular weight under nonreducing than under reducing conditions (Miyazono et al., 1991).

Free and TGF- β 1-bound Forms of LTBP Are Released from the Extracellular Matrix by Plasmin

To study the effects of plasmin on the association of LTBP with the extracellular matrix, we pulse labeled subconfluent HT-1080 cultures with [35 S]cysteine (200 μ Ci/ml) for 1 h. The cultures were subsequently chased with MEM containing a relatively low concentration of plasmin (0.03 CU/ml). Medium and extracellular matrix digests were analyzed by immunoprecipitation with the LTBP specific antibody followed by SDS-PAGE under nonreducing conditions and fluorography. Inspection of the fluorograms indicated that in control cells LTBP was associated with the extracellular matrix, reaching peak levels after 7 h of chase (Fig. 5 A). A component comigrating with 125 I-TGF- β 1 was also seen in the matrix after 7 and 26 h of chase. Substantial amounts of

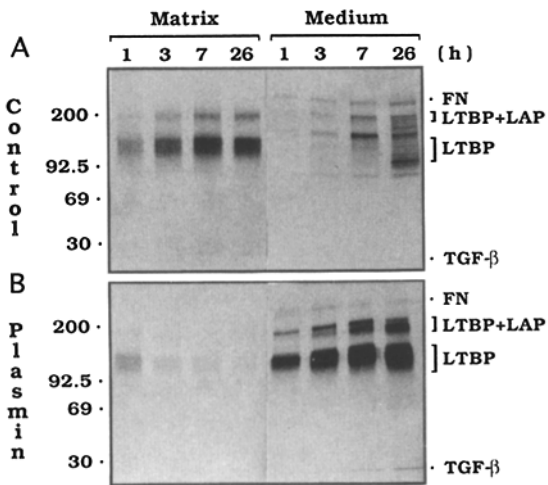


Figure 5. Plasmin releases latent TGF- β complexes from the extracellular matrix of HT-1080 cells. Cultures of HT-1080 cells were labeled with 200 μ Ci/ml [35 S]cysteine in cysteine-free MEM for 1 h. The cells were washed two times and chased with MEM with or without plasmin (0.03 CU/ml). Medium was collected after different chase periods and plasmin activity was neutralized by proteinase inhibitors. Extracellular matrices were prepared from the respective cells and treated with plasmin to release LTBP covalently bound to the matrix (see Materials and Methods). LTBP was immunoprecipitated from the cell supernatant and extracellular matrix digests, and the precipitates were analyzed by 4–15% gradient SDS-PAGE under nonreducing conditions followed by fluorography. Specificity of precipitation was assessed by precipitation with unrelated control serum. The migration of molecular mass markers (kD) is shown on the left. A plasmin sensitive background band probably representing fibronectin is denoted by FN. Positions of the specific complexes and 125 I-TGF- β 1 used as standard are indicated.

LTBP immunoreactivity were not observed in the medium until after 26 h (Fig. 5 A). At this time point several LTBP immunoreactive components between 100 and 200 kD were observed, suggesting that LTBP is cleaved by endogenous proteinases.

In contrast, when plasmin (0.03 CU/ml) was included in the chase solution, two groups of components of 180–200 kD and 110–130 kD were precipitated from the medium even after 1 h of chase. The intensities of these components reached a peak after 7 h chase (Fig. 5 B). The 180–200 kD components represent LTBP complexed to β 1-LAP (see above), while the lower group of components represents the free form of LTBP. The free form of LTBP and LTBP complexed to LAP·TGF- β were present in roughly 10:1 ratio. The relative intensities of the bands may, however, not reflect the actual levels of these complexes, since binding of LTBP to LAP is likely to mask some immunoreactive epitopes and hence decrease the binding of the antibodies. After 7 h of chase, a 25-kD band representing TGF- β 1 (as judged by specificity of precipitation and comigration with 125 I-TGF- β 1) was also detected (Fig. 5 B).

Association of Exogenous LTBP with the Matrix

To determine whether the release of latent TGF- β 1 occurs by cleavage of LTBP or the LTBP binding component in the extracellular matrix, we studied the association of exogenous

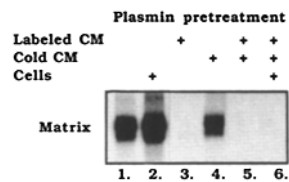


Figure 6. Association of exogenous LTBP to the extracellular matrix of growing HT-1080 fibrosarcoma cells. Human lung fibroblasts were grown to confluency and changed to serum-free medium for the collection of soluble fibroblast

derived proteins. Cell-conditioned medium was produced by incubating the cells in MEM containing 1% of the normal amount of methionine and cysteine with or without [35 S]cysteine (see Materials and Methods). The conditioned medium was collected by centrifugation and treated with plasmin (+). Plasmin was inhibited by the addition of proteinase inhibitors. Radiolabeled and unlabeled plasmin treated and untreated conditioned media were mixed to generate samples representing all possible plasmin treatment variations containing equal amounts of radioactivity. To prepare recipient cells, actively growing, subconfluent cultures of HT-1080 cells were changed to serum free medium and treated with plasmin (+) or medium alone, followed by inhibition of protein synthesis by cycloheximide. Samples of fibroblast conditioned medium, prepared as above, were diluted to MEM containing cysteine (1:1) and incubated with the HT-1080 cultures for 1 h. Subsequently, extracellular matrices were prepared from the HT-1080 cells (see Materials and Methods) and analyzed by immunoprecipitation with antibodies specific for LTBP (Ab-39). The immunoprecipitates were analyzed by 5–15% SDS-PAGE under nonreducing conditions followed by fluorography. All samples thus contain HT-1080 cultures and a similar amount of metabolically labeled and unlabeled conditioned medium. Treatments of the HT-1080 cells (Cells) and fibroblast conditioned media (Labeled CM and Cold CM) by plasmin, prior to mixing and incubation, are indicated by (+). Note that the association of radioactive LTBP, derived from the fibroblast conditioned medium, to the ECM of HT-1080 cells is totally abrogated in all incubations where labeled conditioned media had been pretreated with plasmin (lanes 3, 5, and 6).

radioactive LTBP with the extracellular matrix of HT-1080 cells. Although low to negligible levels of immunoreactive LTBP were detected by immunoprecipitation from the culture supernatants of subconfluent, actively growing cells (above), confluent, resting cultures of fibroblasts were found to secrete relatively large fraction (50–80%) of produced LTBP immunoreactivity in 16 h (data not shown). The conditioned medium of [35 S]cysteine labeled fibroblasts was thus used as a source of soluble radioactive LTBP (see Materials and Methods). To obtain unlabeled conditioned medium control cells were treated identically but radioactive cysteine was omitted. The cell conditioned medium was collected, clarified by centrifugation and treated with plasmin (0.1 CU/ml) at 37°C for 1 h as indicated. Plasmin was inhibited by the addition of proteinase inhibitors, and the incubation was continued for 30 min. Radioactive and unlabeled conditioned media, treated or not with plasmin, were mixed to yield samples containing equal amounts of radioactivity (see Fig. 6). The samples were then added to the medium of subconfluent cultures of HT-1080 whose protein synthesis had been inhibited by pretreatment with cycloheximide (5 μ g/ml, 5 min), and incubated at 37°C for 1 h. Before addition of labeled conditioned media, some cultures were treated with plasmin (0.1 CU/ml) at 37°C for 1 h to partially digest the extracellular matrix as indicated on the figure. Subsequently, extracellular matrices were prepared from the HT-1080 cells and immunoprecipitation analysis was carried

out with anti-LTBP antibodies (see Materials and Methods for details).

In control cells incubated with untreated radioactive conditioned medium mixed with untreated unlabeled conditioned medium, significant amounts of LTBP were found to associate with the matrices of HT-1080 cells (Fig. 6, lane 1). While plasmin treatment of the recipient cells slightly enhanced LTBP association (Fig. 6, lane 2), treatment of labeled conditioned medium by plasmin totally abrogated binding of radioactive LTBP to the ECM (Fig. 6, lanes 3, 5, and 6). When radioactive untreated conditioned medium was mixed with nonradioactive plasmin treated conditioned medium, the association of radioactive LTBP to the ECM was inhibited only partially (Fig. 6, lane 4), indicating that plasmin preparation or proteolytic products of the conditioned medium are not responsible for the inhibition of LTBP binding to the extracellular matrix. The partial inhibition of binding could be due to degradation of other soluble extracellular matrix components required for proper matrix assembly. Note that the banding pattern of LTBP in all lanes is identical because the matrices were solubilized by limited plasmin digestion before immunoprecipitation.

Discussion

The present results indicate that latent TGF- β 1 (LAP·TGF- β 1) is bound to the extracellular matrix of cultured fibroblasts and fibrosarcoma cells via LTBP. The association between LTBP and the extracellular matrix is mainly covalent, and the major fraction of LTBP cannot be released from the matrix even by heat treatment in SDS. Immunoreactive LTBP can be released from the matrix, however, by plasmin-mediated proteolysis. On the other hand, TGF- β 1 is released from the extracellular matrix preparations by SDS, indicating that TGF- β 1 is noncovalently associated to β 1-LAP, which in turn binds to LTBP by a disulfide-bond, as described earlier for the soluble, platelet form of latent TGF- β 1 (Miyazono et al., 1988; Wakefield et al., 1988).

In accordance with previous findings (Miyazono et al., 1991, 1992), our pulse-chase experiments revealed that the synthesized LTBP is secreted relatively rapidly from HT-1080 cells, while the secretion of LAP is slower. After secretion, the major fraction of LTBP associates with the extracellular matrix, and only traces of LTBP immunoreactive material are detectable in the culture medium. Both the free form of LTBP, and LTBP complexed to LAP·TGF- β 1 were found associated with the ECM. Exogenous LTBP from the conditioned medium of fibroblasts also associated with the matrix of HT-1080 cells, and the association was inhibited by plasmin pretreatment of the conditioned medium. Treatment of the cell layers with plasmin, in contrast, slightly enhanced the binding of exogenous LTBP to the extracellular matrix. These results indicate that secreted large latent TGF- β 1 associates with the extracellular matrix via LTBP, and β 1-LAP or TGF- β 1 are not necessarily required for the interaction. The release of latent TGF- β 1 is likely to be due to a cleavage of LTBP between the domains involved in binding β 1-LAP and the extracellular matrix (schematically illustrated in Fig. 7). The molecular character of these domains and the proteins to which LTBP is bound in the extracellular matrix is not understood at present. The results do not, however, exclude the possibility that also the small latent TGF- β 1

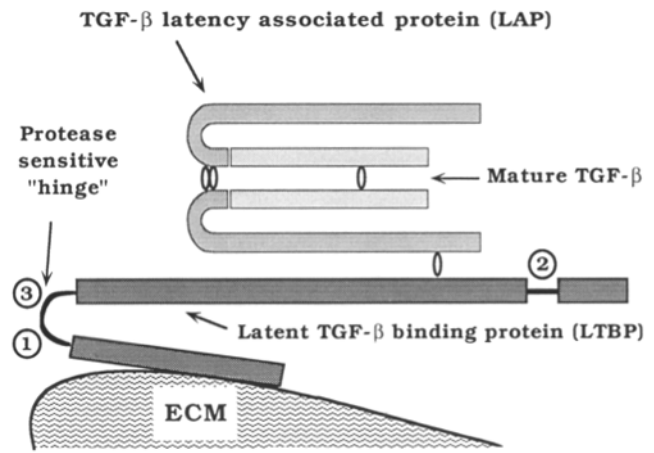


Figure 7. Proposed structure of matrix-associated latent TGF- β 1. A schematic representation of the proposed structure of extracellular matrix-associated latent TGF- β 1. TGF- β 1 dimer is bridged by a single disulfide bond (cys 77-77 of the mature sequence in TGF- β 2) (Daopin et al., 1992; Schlunegger et al., 1992). β 1-LAP dimer is held together via cysteine residues 223 and 225, and the dimer is associated to TGF- β 1 by noncovalent interactions, rendering the complex latent. LTBP is associated to the extracellular matrix covalently, and to the small latent TGF- β 1 complex by disulfide bond(s). Unknown cysteine residue in LTBP binds probably to cys-33 in β 1-LAP, since this cysteine is not involved in LAP-dimer formation, and forms an anomalous disulfide bond with active TGF- β 1 in recombinant latent TGF- β 1 (see Brunner et al., 1989). Proposed proteinase sensitive hinge region, rich in proline and basic residues (amino acids 413–506), separates LTBP domains responsible for binding to LAP and to the extracellular matrix. Plasmin cleavage of this hinge region results in the formation of soluble large latent TGF- β 1 complex, containing an \sim 135-kD fragment of LTBP, while an \sim 50-kD fragment possibly remains associated to the extracellular matrix. An additional cleavage occurs later, resulting in an \sim 10-kD decrease in molecular weight of the large fragment (see Fig. 5 B, Medium, LAP+LTBP). Proposed cleavage sites at human LTBP are a tribasic (RRRP; ①) site starting at amino acid 415 and dibasic site (RR; ②) at 1257, respectively (Kanzaki et al., 1990; Tsuji et al., 1990). In cells that store latent TGF- β , LTBP could be cleaved at these sites by processing endoproteinases that are exclusively present at the regulated secretory pathway, like PC2 and PC1/3 (see Barr, 1991; Nakayama et al., 1992; Steiner et al., 1992). An additional site that resembles a monobasic site cleaved by PC1/3 is also present in the human, but not rat, LTBP sequence (starting at amino acid 461, REHGARS; ③) (see Nakayama et al., 1992). Small ovals represent disulfide bridges.

complex (LAP·TGF- β 1) could interact with the extracellular matrix.

Active TGF- β 1 binds to multiple extracellular matrix components, including type IV collagen, fibronectin, and the proteoglycans betaglycan, endoglin and decorin (Paralkar et al., 1991; Fava and McLure, 1987; Massagué and Like, 1985; Cheifetz et al., 1992; Yamaguchi et al., 1990). Our results, however, indicate that the association of TGF- β 1 to the extracellular matrix after secretion do not require a prior activation step. Active TGF- β 1 is a highly hydrophobic and basic protein, and is rapidly lost from solution (Brown et al., 1990); it therefore binds to many proteins with a relatively high affinity. However, the physiological significance of some of these interactions remains to be shown. Matrix components binding active TGF- β 1 could have a role in

rendering nascently activated TGF- β 1 more soluble and in delivering it to the cell surface receptors (see Wang et al., 1991; López-Casillas et al., 1993), as in the case of basic fibroblast growth factor (Flaumenhaft et al., 1990; Yayon et al., 1991).

Olofsson et al. (1992) found that glioblastoma cells secrete three forms of latent TGF- β 1. The small latent TGF- β 1, contains only β 1-LAP and TGF- β 1, while of the two large latent complexes, one contains LTBP and the other a protein immunologically distinct from LTBP. These high molecular weight binding proteins could target latent TGF- β 's to other extracellular localizations (for immunolocalization of TGF- β 's, see Thompson et al., 1989; Heine et al., 1990; Pelton et al., 1991). Our results indicate that the association of TGF- β 1 to the fibroblast form of LTBP results in targeting of latent TGF- β 1 to the ECM. Also TGF- β 2 and TGF- β 3 have been found in complex with LTBP (Olofsson et al., 1992). These two TGF- β isoforms thus probably bind to fibroblast-type extracellular matrices via LTBP. In addition, our model cells, human lung fibroblasts and HT-1080 fibrosarcoma, seem to produce an excess of LTBP which could serve as a matrix receptor for small latent TGF- β s (LAP·TGF- β).

Fibroblasts synthesize a high molecular mass LTBP (170–190 kD), while platelet α -granules contain smaller LTBP (140 kD) that lacks amino- and/or carboxy-terminal sequences (Kanzaki et al., 1990; Tsuji et al., 1990). Cleavage of large LTBP by plasmin (and trypsin, see Miyazono et al., 1991) results in the formation of components very similar in size to the platelet form. Our data suggests that plasmin cleavage results in the formation of a soluble "platelet-like" LTBP fragment and a domain that may remain associated to the ECM. Similar cleavages of LTBP could also occur on the regulated secretory pathway or in the α -granules of platelets (see legend to Fig. 7). Platelet degranulation would thus result in the formation of a large but transient pool of activatable TGF- β to the vicinity of damaged tissues, while latent TGF- β 1, stably incorporated to the ECM, could serve a more general purpose, possibly acting as a feedback regulator in cell invasion and ECM formation (see Saksela and Rifkin, 1988; Keski-Oja et al., 1991). Interestingly, mice defective in both TGF- β 1 alleles die due to invasion of inflammatory cells in various internal organs (Shull et al., 1992; Kulkarni et al., 1993). It is possible that the lack of TGF- β 1 in the extracellular matrix, resulting in the lack of inhibitory feedback to matrix degradation, contributes to the phenotype of these mice. Defective feedback by TGF- β 1 can also be important in tumor invasion, since some tumor cells respond to TGF- β 1 aberrantly, by increasing proteolytic activity (Keski-Oja et al., 1988a,b).

The interrelationships between matrix association and activation of TGF- β 1 remain to be determined. Recombinant latent TGF- β 1 and TGF- β from fibroblast conditioned medium can be activated by plasmin (Lyons et al., 1988, 1990). However, in the studies using fibroblast conditioned medium, only 30% of total acid activatable TGF- β could be activated by plasmin. Recombinant β 1-LAP is degraded by plasmin (Lyons et al., 1990), indicating that the small latent TGF- β 1 complex might be the target for direct plasmin activation. LTBP could mask plasmin sensitive bonds of LAP allowing more rigorous control for large latent TGF- β activation.

Recombinant β 1-LAP contains mannose 6-phosphate

(Purchio et al., 1989; Kovacina et al., 1989), and the activation of latent forms of TGF- β in co-culture of endothelial cells and smooth muscle cells requires binding to the cation independent mannose 6-phosphate/IGF II receptor (Dennis and Rifkin, 1991). Mannose 6-phosphate is a lysosome specific targeting signal (for review see Kornfeld, 1992), which should result in eventual transport of latent TGF- β 1 to the lysosomal compartment. Latent TGF- β 1 is, however, a secreted protein. This apparent discrepancy can be resolved by the possibility that in the absence of LTBP latent TGF- β 1 is retained in the *cis* aspect of the Golgi apparatus (Miyazono et al., 1992), while sorting of vesicles containing proteins targeted to the lysosomes occurs in the *trans*-Golgi network, later in the secretory pathway (see Rothman and Orci, 1992; Pryer et al., 1992; Kornfeld, 1992). In addition, some proteins containing mannose-6-phosphate are secreted prior to transport to lysosomes (Chao et al., 1990; Kornfeld, 1992). LTBP could act as an override targeting signal, either by masking the mannose-6-phosphate of the small latent TGF- β 1 complex, or by blocking postsecretion transport by association to the ECM. Cleavage of LTBP by proteinases releasing latent TGF- β 1 from the extracellular matrix could possibly result in "unmasking" of the mannose 6-phosphate and retargeting of latent TGF- β 1 to cell surface or to lysosomal compartment for activation.

A second possibility is also consistent with available data. It has been shown that cation independent mannose 6-phosphate receptor participates in extracellular matrix degradation by transferring lysosomal hydrolases to the cell surface (Brauker et al., 1986; see also Chao et al., 1990). Rather than acting directly, the inactivation of the mannose 6-phosphate receptor by antibodies or competing ligand could thus inhibit TGF- β activation by restricting the supply of matrix-derived latent TGF- β . The importance of proper matrix assembly to TGF- β 1 activation is also demonstrated in a study showing that inhibitors of a matrix cross-linking enzyme, transglutaminase, inhibit TGF- β 1 activation in retinol treated endothelial cells (Kojima et al., 1993).

The association of latent TGF- β complexes with the pericellular matrices and their release by proteinases implies that these events participate in a number of biological events where enhanced or focal proteolysis takes place, including cell invasion, tissue remodeling and wound healing.

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