Latent TGF-β binding protein LTBP-2: assembly to the extracellular matrix and effects on cell adhesion

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ACADEMIC DISSERTATION

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LTBP-2 assembly and effects on cell adhesion

Original publications

This thesis is based on the following original publications, referred to by their roman numerals in the text:


## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>8-Cys domain</td>
<td>Eight cysteine domain (also called TB or CR domain)</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>GDF</td>
<td>Growth and differentiation factor</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>His-tag</td>
<td>Protein tag consisting of six histidine residues</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>LAP</td>
<td>Latency associated peptide</td>
</tr>
<tr>
<td>LL-TGF-β</td>
<td>Large latent transforming growth factor β</td>
</tr>
<tr>
<td>LTBP</td>
<td>Latent TGF-β binding protein</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAGP</td>
<td>Microfibril associated glycoprotein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimal essential medium</td>
</tr>
<tr>
<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>RGD</td>
<td>Tripeptide consisting of arginine-glycine-aspartic acid residues</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel</td>
</tr>
<tr>
<td>SL-TGF-β</td>
<td>Small latent transforming growth factor β</td>
</tr>
<tr>
<td>TB-domain</td>
<td>TGF-β binding domain, also 8-Cys domain</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine isothiocyanate</td>
</tr>
</tbody>
</table>
Summary

Latent TGF-β binding proteins, LTBP, are components of the extracellular matrix. While LTBP 1-3 are real TGF-β binding proteins, LTBP-2 is not. We have therefore focused this work to finding other functions for LTBP-2.

LTBP-2 was recombinantly expressed in Chinese hamster ovary cells and purified to apparent homogeneity. The purified protein was then used for biochemical characterization. LTBP-2 was found to bind calcium and was glycosylated at its central, proteolytically stable domain. Polyclonal antibodies recognizing LTBP-2 were generated. In immunofluorescence assays, LTBP-2 was localized in fibrillar structures in the extracellular matrix of fibroblasts. LTBP-2 was proteolytically processed from its intact 240-kDa form to 160-120 kDa forms, resulting in the release of the processed fragments from the fibroblast extracellular matrix. Proteolytic degradation sites were determined by N-terminal sequencing of the fragments, and they were localized to proline rich hinge regions in the N-terminal part of LTBP-2. Purified, soluble LTBP-2 could be assembled to the extracellular matrix of fibroblasts, implicating soluble intermediates in the assembly process of LTBP-2 containing structures.

To assess the biological activities of its protein domains, we expressed and purified partially overlapping Ig-tagged fragments of LTBP-1 covering the whole protein. The association of these proteins with the extracellular matrix of fibroblast cultures was then analysed by immunofluorescence and enzyme-linked immunoassays. We identified three different regions of LTBP-1 that interacted with the extracellular matrix of fibroblasts. Two of those were located at the N-terminal part of the protein, whereas the third one was at the C-terminus of LTBP-1. Each of them inhibited the incorporation of endogenous LTBP-1 to the extracellular matrix. All of the interacting fragments contained an 8-Cys or hybrid domain, suggesting a role for those domains in the ECM interactions of LTBP-1.

We analysed the abilities of several cell lines to adhere to full length LTBP-2. Melanoma cell lines were the only ones that did. The adhesion was accompanied by cell spreading and focal adhesion formation. The melanoma cell adhesion was dependent on α3β1 and α6β1 integrins, and the site was located to the N-terminal part of LTBP-2. The adhesion to LTBP-2 was also inhibited by soluble heparin, implicating heparan sulphate proteoglycans in the process. In addition to supporting melanoma cell adhesion, LTBP-2 supported also melanoma cell migration.

Although full length LTBP-2 did not mediate the adhesion of other cell types, a proline rich N-terminal fragment of LTBP-2 supported lung fibroblast attachment. However, the attaching cells did not spread or form actin stress fibers when binding to LTBP-2 coated dishes. In contrast, the binding-mediating fragment, as well as full length LTBP-2 had dominant disrupting effects on actin stress fibers of fibroblasts attaching to fibronectin. Actin was
instead localized to membrane ruffles. The formation of focal adhesions was also disturbed. LTBP-2 and its fragments having antiadhesive effects bound to fibronectin, but not to type I collagen as shown by enzyme immunoassays. The antiadhesive fragments colocalized with the extracellular fibronectin fibers. LTBP-2 did not exhibit antiadhesive effects on cells adhering to type I collagen, suggesting an antiadhesive role for LTBP-2 bound to fibronectin.

These studies implicate LTBP-2 as an important ECM glycoprotein with roles in the modulation of cell adhesion in specific locations during embryogenesis, tissue repair as well as melanoma cell invasion.
Review of literature

Extracellular matrix (ECM) structure

Cells are surrounded in tissues by extracellular matrix (ECM), which is synthesized by the cells themselves (Aumailley and Gayraud, 1998; Zagris, 2001). ECM is composed of a multitude of different components, and the composition varies largely depending on the tissue. Some components are present ubiquitously in tissues, whereas some have very restricted expression pattern localizing only to specialized structures. ECM is classified to basement membranes (BM) and interstitial ECM. BM is a macromolecular structure giving support and lining epithelial and endothelial cells. Interstitial ECM is a stromal network of more heterogenous composition. Some individual components of the ECM are distributed in both ECM types. The ECM types are further classified according to their structure, function and components they contain.

Collagenous matrix

Collagens are the most abundant components of the interstitial ECM (Linsenmayer, 1991; Prockop and Kivirikko, 1995). The main definition for collagens is their characteristic triple helical structure. Three polypeptide chains form this structure. Within each type of collagen, these chains may be identical, two may be identical and the third chain different, or all three chains may be different. There are altogether 27 different types of collagens identified (Myllyharju and Kivirikko, 2001; Heino, 2003). Different collagen chains are called α-chains. For example, collagen type I is composed of α1(I) and α2(I) chains (type of collagen is indicated by roman numerals in parenthesis, number of α chain by arabic numerals), whereas collagen type XI is composed of α1(XI), α2(XI) and α3(XI) chains.

The chains form usually triple helices within their own type only. Collagen type I forms typically triple helices composed of type I collagen chains only, but there seems to be heterogeneity in the composition of triple helices as well (Hendrix et al., 1982; Birk et al., 1988; Linsenmayer et al., 1990). Collagens have been divided according to the structures they form. They have been classified to fibrillar (types I, II, III, V and XI) and non-fibrillar collagens (Prockop and Kivirikko, 1995). Non-fibrillar collagens have been further grouped to fibril associated, FACIT or FACIT-like collagens (types IX and XII, XIV, XVI and XIX), transmembrane collagens (types XIII, XVII, XXIII, XXV) network forming collagen type IV, beaded filament collagen type VI, short chain collagens (types VIII and X) and anchory fibril collagen type VII (Linsenmayer, 1991; Heino, 2003). As can be expected from the names of collagen subfamilies or groups, different collagens have distinct properties in
matrix formation. Fibrillar collagens are distributed as striated fibers widely in tissues providing mechanical strength. Type I collagen is the most abundant collagen, which exists as striated fibers in adult connective tissues such as bone, skin and tendon (Linsenmayer, 1991). Non-fibrillar collagens form only interrupted fibrillar structures and are a relatively heterogeneous group. Type IV collagen forms network-like structures in basement membranes (see below). Type XIII, XVII, XXIII and XXV collagens in turn are targeted to the cell surfaces by their transmembrane domain (Prockop and Kivirikko, 1995). Numerous proteins can associate with collagen fibers, such as small leucine rich proteoglycans (Iozzo, 1997). These kinds of proteins may also play a role in collagen fiber formation.

Fibronectin matrix

Fibronectin (FN) (Ruoslhti and Vaheri, 1974) is an abundant 250 kDa ECM glycoprotein (Fig. 1), which also forms soluble dimers in vivo. It exists at relatively high concentrations as circulating protein in blood plasma, and as a fibrillar component in the ECM especially in provisional matrices during wound healing (Mosher, 1995).

It is composed primarily of cysteine-rich type I, type II and type III fibronectin repeats (Magnusson and Mosher, 1998). FN is subject to alternative splicing. FN produced in liver and present in plasma usually lacks two extra FN type III modules, as well as some variable regions (V regions), whereas FN made by other tissues contains these sequences. Soluble, secreted fibronectin forms a dimer, which is sensitive to disulfide bond reduction (Chen and Mosher, 1996). Fibronectin binds to numerous proteins at the cell surface as well as in the extracellular matrix (Couchman et al., 1990; Schwarzbauer, 1991). Some of the binding sites are cryptic, and are exposed to interactions only after the fibronectin molecules assemble to the ECM (Schwarzbauer and Sechler, 1999).

![Fig. 1. Domain structure and locations of different functions of fibronectin.](image)

FN is composed mainly of three types of repeating domains, type I, II and II FN repeats. Type I repeats are indicated with white rectangles, type II repeats with black ovals and type III repeats with circles. Alternatively spliced type III repeats are shown with shaded circles. Alternatively spliced variable regions are shown with shaded rectangle (V120). Site important for FN assembly is indicated, as well as interacting sites for some of the ligands. The cysteine residues important for dimer formation are indicated with "S". Modified from (Magnusson and Mosher, 1998).
Proteoglycans in the ECM

Proteoglycans are a diverse group of highly glycosylated ECM components (Iozzo, 1998). They constitute the amorphous appearing material of the ECM and as highly hydrophilic molecules are important for retaining water in the ECM, and in that way maintain the volume of the tissues. However, proteoglycans have also more specialized functions, such as regulation of growth factor availability (Iozzo, 1998). In proteoglycans, sulphated polymeric sugar structures, glycosaminoglycans (GAGs) are attached to the core protein. There are different types of GAG chains attached to core proteins, namely chondroitin/dermatan sulphate, keratan sulphate and heparan sulphate/heparin (Wight et al., 1991). The GAGs are composed of repeating disaccharide subunits, and the chains may be thousands of sugar units long. The sugar chains are attached to their core proteins usually via O-linked glycosylation to serine or threonine residue (Wight et al., 1991). The core proteins are often secreted proteins of varying molecular mass. The core proteins include perlecan, agrin, aggrecan, serglycin, versican, biglycan and decorin. In addition, there are cell surface anchored proteoglycans such as syndecans and glypicans (David, 1993; Bernfield et al., 1999).

Basement membranes

Basement membranes (BM) separate epithelial and endothelial cell layers and the basal stromal connective tissues from each other. This ECM type is composed of electron dense and electron poor layers in electron microscopic images lining the endothelial or epithelial cells (Timpl, 1996). Epithelial/endothelial cells produce BM components, and are found practically in all organs. BMs are composed mainly of collagen type IV, entactin/nidogen, different laminins and heparan and chondroitin sulphate proteoglycans (Yurchenco and O'Rear, 1994; Timpl, 1996). BMs consist of two major independent networks; one formed of collagen type IV and another composed of laminins. These two networks are connected by nidogen. The other BM components have less well characterized role (Erickson and Couchman, 2000).

Type IV collagen forms independent networks. Like the other collagens, it is a triple helical molecule of molecular mass of 550 kDa. Two of its three \( \alpha \)-chains are identical, whereas third one is a different gene product. There are a total of six different type IV collagen \( \alpha \)-chain coding genes which are differentially employed in different tissues (Yurchenco and O'Rear, 1994). Collagen type IV forms covalently crosslinked structures in BMs. Laminins, in contrast, form mainly non-covalent networks. Laminins are also trimeric protein complexes. They are composed of three different polypeptide chains; \( \alpha \), \( \beta \) and \( \gamma \)-chains. The molecular masses of the complexes vary between 400 and 900 kDa. There are several different \( \alpha \), \( \beta \) and \( \gamma \)-chains, which form more than 10 different isoforms. Laminin chains are twisted around each other forming a coiled-coil structure with the central domains of each chain. N-terminal parts of each chain are not associated with each other, and form a cross- or T-shaped structure (Timpl, 1996). Each laminin chain has a different tissue distribution.
Nidogen, which connects the collagen type IV and laminin networks, is secreted from the cells as monomeric protein of molecular mass of 150 kDa.

Elastic fibers

Elastic fibers contribute to elasticity and resilience of tissues and provide ability to endure stretch in connective tissues such as blood vessels. Elastic fibers are composed of two distinct entities; elastin and 10-12 nanometer (diameter) microfibrils (Mecham and Heuser, 1991). Elastin is the major component of elastic fibers.

Elastin (Indik et al., 1989) forms amorphous appearing material deposited to the ECM of connective tissues. It is produced in the cell as a soluble precursor form, tropoelastin. After secretion from the cells, this form is converted to highly insoluble elastin, which forms the core of the elastic fiber. Polymerized elastin is highly stable as seen from the fact that the elastic fiber usually lasts for the whole lifetime of the organism. The stability of elastin is further implicated by the purification procedure. Boiling the elastic fibers in 0.1 M NaOH hydrolyses other ECM components. Elastin is relatively stable under these conditions (Mecham and Heuser, 1991).

![Electron microscopy image of elastic fibers](image)

*Fig. 2. Electron microscopy image of elastic fibers.* Fetal bovine ligamentum nuchae was subjected to tannic acid treatment, which stains elastin (E) and electron microscopy. Fibrillin microfibrils (MF) are located at the periphery of growing elastic fiber. Bar 0.3 µm. Modified from (Mecham and Heuser, 1991).

Elastic fibers are surrounded by microfibrils, which are thought to guide the assembly of elastin to its insoluble fibrillar form. Microfibrils are mainly composed of fibrillins (Sakai et al., 1986; Zhang et al., 1994). The other known components of microfibrils include microfibril associated glycoproteins (MAGPs) (Gibson et al., 1989) and latent TGF-β binding proteins (LTBPs) (Saharinen et al., 1999). Several other proteins associate with microfibrils, such as the proteoglycans decorin, biglycan and versican (Trask et al., 2000a; Isogai
et al., 2002; Reinboth et al., 2002), fibrulins -1, -2 and -5 (Kostka et al., 2001; Tsuda et al., 2001; Nakamura et al., 2002) and emilins 1-2 (Dolianova et al., 1999; Doliana et al., 2001). Fibrillins and MAGPs are considered integral components of microfibrils, whereas data on the roles of LTBP-2 to the structure of microfibrils is just accumulating.

There are three different fibrillins, fibrillin-1 (Sakai et al., 1986), -2 (Zhang et al., 1994) and -3 (Nagase et al., 2001). Fibrillins are 350 kDa glycoproteins composed mainly of cysteine rich repeat structures; epidermal growth factor (EGF) –like repeats and so called eight-cysteine (8-Cys) repeats (also called TB domains). In addition, there are two hybrid domains, which have characteristics from both EGF and 8-Cys domains (Handford et al., 2000). Fibrillin-1 is considered to be the major structural protein of microfibrils (Kielty et al., 2002). Fibrillin-1 is ubiquitously expressed in elastic tissues, whereas the expression of fibrillin-2 is more regulated during development (Zhang et al., 1995), suggesting specific roles for fibrillin-2 during development. Mutations in fibrillins-1 and –2 lead to connective tissue disorders such as Marfan syndrome and congenital contractural arachnodactyly, respectively (Dietz et al., 1991; Lee et al., 1991; Maslen et al., 1991).
Assembly and proteolysis of the ECM

ECM components are produced in cells as monomeric, secreted proteins. After or during the secretion, these components are polymerised to yield fibrillar high molecular mass network in the ECM. There are in principle two ways to accomplish the assembly process. Individual matrix components can self-polymerize to yield ECM fibers, or the process can be cell mediated, so that that the assembly process occurs at the cell surface driven by the machinery located at the plasma membrane.

During various occasions such as the invasion of the immune system or cancer cells to tissues, or wound healing ECM needs to be degraded. Numerous proteolytic enzymes mediate the degradation of the ECM.

Collagen matrix assembly

Purified collagen type I, II, III, V and XI monomers assemble under suitable conditions to fibrillar structures in vitro (Kadler et al., 1996), suggesting that collagen fibers self-assemble in the ECM. However, as collagens are synthesized as pro-forms, the propeptides need to be removed by specific proteases to obtain molecules forming proper fibers. This offers one step to regulate collagen fiber formation. Forming collagen fibers are usually detected near cell surfaces, suggesting that the collagen assembly is at least directed by the cells. In addition, recent results with fibronectin null cell lines suggest that other ECM components are needed also for collagen fiber formation (Velling et al., 2002). Once collagen fibers are assembled, they are crosslinked by tyrosine-, quinone-, aldimine-, ketoimine- and cysteine-derived crosslinks (Kadler et al., 1996).

Fibronectin assembly

FN assembly is clearly a cell-mediated process (Mosher et al., 1992; Schwarzbauer and Sechler, 1999). Assembly involves various domains of FN, as well as several cell surface proteins (see Fig. 1). FN is an abundant plasma protein, but it is assembled to the ECM only at locations where it is needed. This further strengthens the concept of cell mediated assembly process. FN assembly occurs stepwise involving several FN-cell surface interactions. As a first step, soluble FN binds to its integrin receptor, usually α5β1. Integrins then cluster at the specific assembly sites, which causes integrin-bound FN to concentrate at these spots. The assembly process involves five amino terminal type I domains, N-terminal second type III repeat and C-terminal heparin binding domain of FN (Schwarzbauer and Sechler, 1999; Sechler et al., 2001). Some of the interaction sites are cryptic; they are exposed only in the FN molecules that are getting assembled to the ECM. The exposed interaction sites act as binding sites for additional soluble FN molecules. Ability of the cells to stretch the initial FN assembly sites using integrins is essential for FN fibril formation to continue (Zhang et al., 1997; Pankov et al., 2000), and assembled FN fibers contribute to the cells ability to stimulate cell contractility (Hocking...
 LTBP-2 assembly and effects on cell adhesion

et al., 2000). The stretching is thought to expose cryptic interaction sites (Baneyx et al., 2002) and provides the force needed for fibronectin assembly.

Initial FN assembly spots are detergent soluble. However, they are gradually converted to detergent insoluble form. This process involves crosslinking of the FN molecules, probably by disulfide exchange (Langenbach and Sottile, 1999) and transglutaminase crosslinks (Vaheri et al., 1978; Barsigian et al., 1991). Transglutaminase has also a positive effect of FN assembly not dependent on its crosslinking activity (Akimov and Belkin, 2001).

Assembly of basement membranes

BMs are composed of two relatively independent networks (see above), collagen type IV and laminin. The formation of Type IV collagen network involves mainly three types of interactions; covalent association of four N-terminal segments of collagen type IV triple helices, which form a four-armed structure; the formation of disulfide linkages between C-terminal parts; and non-covalent side-by-side interactions (Yurchenco and O'Rear, 1994). No cell surface receptors have been implicated in these processes. The assembly of type IV collagen is thought to take place on assembled laminin network (Li et al., 2003). Laminin assembly was originally considered to occur as a self-assembly process as well. Purified laminin chains assemble to network-like structures in vitro. The network forming activity is driven by ionic and hydrophobic interactions within each chain. Cell surface receptors, however, are involved in the formation of laminin sheets. Dystroglycan is a cell surface receptor complex that has a role in skeletal muscle. In addition, it associates with laminin in epithelial cells (Hemler, 1999). Dystroglycan null mice do not form proper BMs (Henry and Campbell, 1998). In addition, β1 integrins have been implicated in BM assembly (Aumailley et al., 2000). The exact role of these cell surface receptors in BM assembly is not, however clear. One alternative is that the cell surface receptors concentrate and target laminin complexes to the cell surfaces to yield a critical concentration for self-assembly to occur (Li et al., 2003).

Nidogen is an important crosslinker of collagen and laminin networks in BMs. Nidogen binds with its N-terminus to the collagen type IV network, and via its C-terminus to laminin γ1 subunit. The BM proteoglycan perlecan also binds to nidogen, as well as to laminin (Li et al., 2003).

Assembly of elastic fibers

Elastin is produced as tropoelastin in fibroblasts. Tropoelastin is secreted as soluble protein. It has several lysine residues, which are subject to oxidative deamination, a reaction catalysed by the enzyme lysyl oxidase. Oxidated lysines form then rapidly stable covalent crosslinks with the other elastin molecules. This process plausibly takes place at the pre-existing microfibrils (Wirtschafter et al., 1967; Ross and Bornstein, 1969; Cleary and Gibson, 1983). The fibrillin containing microfibrils thus act as a scaffold for elastin deposition to the ECM. More recent results have indicated that tropoelastin
actually interacts with fibrillins-1 and -2 (Trask et al., 2000b) strengthening this hypothesis.

Fibrillins and MAGPs are the main structural components of microfibrils. These proteins can be found in the cell conditioned medium as soluble proteins, and soluble epithelial cell derived fibrillin-1 can get assembled to the ECM by fibroblastic cells (Dzamba et al., 2001a), suggesting that microfibril assembly is a cell mediated process. Current understanding of fibrillin-1 assembly to ECM microfibrils is as follows (Handford et al., 2000); monomeric fibrillin secreted from the cells is processed at its C-terminal furin processing site (Ritty et al., 1999), which is needed for fibrillin-1 ECM association. Shortly after secretion, or already during secretion, fibrillin-1 is dimerized by disulfide linkage at its N-terminal domain (Ashworth et al., 1999; Trask et al., 1999; Reinhardt et al., 2000a). Dimerization is considered to be the first step in microfibril formation. After this, fibrillin-1 is stabilized by transglutaminase crosslinks (Qian and Glanville, 1997). MAGPs –1 and –2 are thought to be involved in microfibril assembly as well (Brown-Augusburger et al., 1996; Penner et al., 2002). MAGP-1 is also a substrate for transglutaminase (Brown-Augusburger et al., 1994), implicating a role for this enzyme in the assembly process. MAGP-1 and fibrillin-1 interact with the chondroitin sulphate proteoglycan decorin, suggesting a role for this GAG in microfibril assembly (Trask et al., 2000a). Detailed steps in microfibril assembly are not well understood. However, fibrillins form a beaded fibrillar microfibril structure. In this fiber, fibrillin monomers are aligned parallelly with each other, and MAGP molecules form evidently the beads on the structure (Handford et al., 2000).

**ECM degradation**

ECM needs to be degraded during numerous physiological events such as the invasion of immune system cells to tissues, as well as cancer invasion and metastasis (Sternlicht and Werb, 2001). This task is generally committed by secreted or plasma membrane associated proteolytic enzymes. They are a large group of proteins, which degrade peptide bonds in target proteins. Proteolytic enzymes are classified according to their substrate specificities, and include serine, cysteine, aspartic and metalloproteinases (Woessner, 1998). Proteinases generally degrade crosslinked ECM protein targets to yield space for migrating and invading cells, but they have also more specialized roles. Proteinases can, for example, cleave and modulate the activity of ECM associated cytokines and growth factors, their cell surface receptors, growth factor binding proteins and proteinase inhibitors (Stamenkovic, 2000). Vice versa, growth factors can modulate pericellular proteolytic activity (Laiho and Keski-Oja, 1989). Cleaved and solubilized degradation products of the ECM may possess biological activity of their own, as observed of proteolytic processing of collagen type XVIII to obtain endostatin (Ramchandran et al., 2002). In addition, peptides of FN may act as chemoattractants for certain cell types (Trial et al., 1999).
Functions of the ECM

Traditionally the structural role of the ECM has been emphasized. In that vision ECM offers an architectural, rigid scaffold for cells. While ECM certainly has the architectural role, it has multiple other roles as well.

Structural role

ECM molecules maintain tissue structures as macromolecular aggregates. The most important molecules for maintaining tissue integrity are collagens and heparan sulphate proteoglycans. Collagens form a filamentous network in the ECM, which is important for structural integrity of the ECM in basically tissues such as cartilage, bone, skin and tendon. Heparan sulphate proteoglycans are important especially in the sense that as highly hydrophilic and water binding molecules they constitute the volume in tissues and resist compressive load.

Tissues must also be resistant to stretching. Elastin and elastic fibers are especially important in this aspect. Blood vessels are the best examples of such tissues. Elastic fibers are able to stretch and fold back repeatedly.

Epithelium and endothelium are important structures in lining surfaces of organs in basically all tissues. While tight cell-cell contacts are crucial for the integrity of these structures, basement membranes give support for these cells.

Effects on cell adhesion and intracellular signalling

Cells bind avidly to their environment. Cells adhere to all classes of ECM molecules and structures, such as collagen fibers, basement membranes, elastic fibers and fibronectin. Numerous additional adhesion molecules contribute to cell adhesion as well. Cells adhere to the ECM usually via their cell surface receptors, for example integrins (see below). Cell adhesion has a crucial role in cell behaviour and survival. Cell growth and survival is usually dependent on cell adhesion under physiological situations (Frisch and Screaton, 2001), and the loss of cell adhesion often leads to apoptosis (Gomez et al., 1998). This anchorage-dependent cell growth control is called anoikis. Cell adhesion, and especially adhesion to certain ECM molecules can also have an effect on cell differentiation during development (McNeill, 2000; Jamora and Fuchs, 2002).

Cell adhesion leads to changes in intracellular signalling (Keely et al., 1998; Braga, 2002; Matter and Balda, 2003). For example, integrin ligation to extracellular ligands leads to the activation of certain intracellular signalling pathways (Coppolino and Dedhar, 2000), which has implications on cell morphology and migration, as well as cellular survival, proliferation and differentiation. Cell-cell adhesion has implications on intracellular signalling as well (Braga, 2002; Matter and Balda, 2003). The response of cells depends on the molecule it is attaching to (Christofori, 2003), and especially on via which receptor the binding occurs. For example, melanoma cell attachment to FN via $\alpha_5\beta_1$ integrin leads to stress fiber formation, but simultaneous binding via
α4β1 to a different epitope of FN leads to less adherent cell morphology (Mostafavi-Pour et al., 2003; Moyano et al., 2003).

The changes caused by cell adhesion are in several respects similar to those caused by growth factors. For example, cell adhesion to FN leads to cell proliferation and protection from apoptosis. Therefore, several ECM proteins classified as structural proteins have growth factor-like properties (Danen and Yamada, 2001). ECM contains also numerous classical growth factors (Taipale and Keski-Oja, 1997). For example, fibroblast growth factors (FGFs) bind to heparan sulphate chains of proteoglycans in the ECM (Ornitz, 2000), and this binding enhances FGF-FGF receptor complex formation (Esko and Selleck, 2002).

Cell migration is a crucial step during development. Cells must adhere to their environment in order to be able to migrate (Lauffenburger and Horwitz, 1996). However, too strong interaction of cells with their environment decreases cell migration (Moyano et al., 2003). Migrating cells need therefore to control their adhesive strength. This may be carried out via controlling their intracellular signalling, but there are also adhesion-regulating proteins in the extracellular environment.

Antiadhesive functions

While several ECM components support cell adhesion, there are a number of proteins with antiadhesive functions (Chiquet-Ehrismann, 1995; Orend and Chiquet-Ehrismann, 2000). In this context "antiadhesive" means that a particular antiadhesive protein prevents/decreases cell adhesion either to other adhesive ECM component or to other cells. This can be detected as decreased number of attaching cells, or more prominently, as altered morphology and cytoskeletal structures in attaching cells. The antiadhesive function is often related to cell migration. Cell migration needs cells to attach to the substratum, but too firm attachment results in decreased cell motility (Lauffenburger and Horwitz, 1996; Maheshwari et al., 2000).

The phenomenon is most widely studied in neurons. The antiadhesive function is linked to cell/axon migration and is sometimes associated with repulsion. However, whether axon repulsion is tightly associated with antiadhesiveness, or whether there are additional mechanisms mediating repulsion is unclear at present.

Both adhesive and antiadhesive structures play important roles in axon migration, (Dickson, 2002). Axons migrate towards the adhesive, attractive structures, and away from antiadhesive, repulsive cues. Examples of repulsive cues for neuronal migration include the slit -proteins (Wong et al., 2002) and ephrin-Eph receptor system (Robinson et al., 1997; Holmberg and Frisen, 2002). These proteins have been mostly studied in neuronal migration, but may also be involved in adhesion modulation of other cell types as well (Wong et al., 2002).

The other antiadhesive proteins include tenascins (Chiquet-Ehrismann et al., 1988; Chiquet-Ehrismann, 1995), thrombospondins (Sage and Bornstein, 1991; Goicoechea et al., 2000), secreted protein acidic and rich in cysteine, SPARC (Sage et al., 1989) and fibulin-1 (Twal et al., 2001).
Tenascin-C has a dual role in cell adhesion. It has one epitope which supports cell adhesion, whereas another one decreases it (Spring et al., 1989). Tenascin-C decreases at least fibroblast and glioblastoma cell spreading (Chiquet-Ehrismann et al., 1988; Huang et al., 2001) when bound to FN. This binding prevents the binding of syndecan-4 to FN. Syndecan-4 has a prominent role in the formation of focal adhesions (Woods and Couchman, 2001), and tenascin-C binding to FN decreases focal adhesion formation by this mechanism (Huang et al., 2001).

Thrombospondin-1 (Adams, 2001) has a dual role in cell adhesion as well. Endothelial cells adhere to thrombospondin-1 (Murphy-Ullrich and Höök, 1989). However, it induces reorganization of actin cytoskeleton and disassembly of focal adhesions in endothelial cells as well (Goicoechea et al., 2002). Adhesion to thrombospondin-1 modulates focal adhesion formation, and involves fascin microspike formation (Adams, 1995; Fischer et al., 1997). Microspike formation seems to be dependent on syndecan-1 (Adams et al., 2001), whereas focal adhesion disassembly is reported to take place via calreticulin signalling (Goicoechea et al., 2002). Antiadhesive functions of TSP-1 may be partially related to its anti-angiogenic function (Lawler, 2002).
**Adhesive cell structures**

Cells sense and attach to their ECM environment mainly via cell surface receptors. These receptors are localized to specific structures at the cell surface, which are classified according to their structure and function.

**Focal adhesions**

Focal adhesions are electron dense cell surface structures connecting intracellular actin cytoskeleton to extracellular fibrillar structures. They are composed of several plasma membrane and cytoplasmic proteins (Petit and Thiery, 2000; Peterson and Burridge, 2001). The major class of adhesive proteins localizing to focal adhesions are integrins (van der Flier and Sonnenberg, 2001). Integrins are plasma membrane proteins having extracellular ligand binding domains and short cytoplasmic tails, which bind to intracellular proteins. Integrins are dimeric protein complexes composed of one \( \alpha \)- and one \( \beta \)-chain. To date, 18 \( \alpha \)-chains and 8 \( \beta \)-chains have been identified (Dzamba et al., 2001b). These chains dimerize by non-covalent interactions. Some \( \alpha \)- or \( \beta \)-chains form dimers with several different \( \beta \)- or \( \alpha \)-chains, whereas some form dimers following a more restricted pattern. To date, 24 different combinations have been detected. For example, \( \alpha_\text{v} \) integrin forms dimers with five different \( \beta \)-subunits, whereas \( \alpha_5 \) integrin forms dimers only with the \( \beta_1 \) subunit. Different integrins recognize different ECM ligands, with variably restricted pattern. For example, \( \alpha_\text{v}\beta_1 \) integrin recognizes mainly FN, whereas \( \alpha_\text{v}\beta_3 \) integrin recognizes several ECM substrates, for example FN, tenascin, vitronectin and fibrinogen (Dzamba et al., 2001b). Some integrins recognize arg-gly-asp (RGD) amino acid sequences in their extracellular ligands, while the other ones have other recognition sequences for binding (Ruoslahti, 1996).

In addition to integrins, there are some other transmembrane proteins localizing to and having functions in focal adhesions including heparan sulphate proteoglycan syndecan-4 (Woods and Couchman, 2001), members of the transmembrane 4 superfamily or tetraspanins (Hemler, 1998) and integrin associated protein IAP (Petit and Thiery, 2000).

Although the cytoplasmic tails of integrins are relatively short, multiple cytoplasmic proteins associate with them (Fig. 3). Some proteins, such as calreticulin and calnexin, interact with the cytoplasmic tail of \( \alpha \)-subunit (Petit and Thiery, 2000). However, a larger number of proteins interact with the cytoplasmic tail of \( \beta \)-subunit. Talin, \( \alpha \)-actinin and filamin act as structural proteins and as a link between integrin cytoplasmic tails and actin fibers (Dzamba et al., 2001b). The other group of \( \beta \)-subunit interacting proteins are regulatory proteins, for example focal adhesion kinase (FAK) and paxillin (Petit and Thiery, 2000). In addition, in focal adhesions, there are more associated proteins, which do not necessarily interact with cytoplasmic tails of integrins, but with integrin associated proteins. These proteins may be
Integrin ligation to its extracellular ligand leads to integrin activation. This results in conformational changes in integrin structure (Giancotti, 2003; Shimaoka et al., 2003). This leads, in turn, to the activation of intracellular signalling, in which focal adhesion associated regulatory molecules are involved (Brakebusch and Fassler, 2003). The phosphorylation mediated signalling induces small GTPase RhoA mediated focal adhesion and actin stress fiber formation (Etienne-Manneville and Hall, 2002). These changes are important for example in the regulation of cell proliferation and migration (Coppolino and Dedhar, 2000). Intracellular signalling can also function to the opposite direction; signalling cascades can promote the binding of integrins to their ligands in the absence of cell adhesion. This is called inside-out signalling.

**Adherens junctions**

Adherens junctions are structures forming cell-cell connections mainly in epithelial and endothelial cells, but they are present for example in synactic junctions as well. Adherens junctions are localized to sites where epithelial/endothelial cells are in contact with each other. The main components of adherens junctions are cadherins. Cadherins are a group of
transmembrane proteins mediating homotypic cell-cell interaction (Radice and Takeichi, 2001). They consist mainly of extracellular cadherin repeats, which bind homotypically to cadherins in neighboring cells, and short cytoplasmic tails which bind to intracellular catenins. Cadherin binding catenins include p120 catenin and β-catenin (plakoglobin). An additional catenin, α-catenin, links the cadherin complex to actin cytoskeleton by binding to both β-catenin and filamentous actin (Gottardi et al., 2001; Radice and Takeichi, 2001).

Several signalling molecules localize to adherens junctions, and the assembled adherens junctions have evidently similar signalling functions as focal adhesions. As RhoA is important signalling molecule for focal adhesions (Etienne-Manneville and Hall, 2002), another small GTPase, Rac is important for adherens junction formation (Braga et al., 1997; Takaishi et al., 1997). In addition to structural role in adherens junctions, β-catenin has a signalling function. β-catenin exists in cells as adherens junction associated protein and as a soluble pool. As β-catenin gets phosphorylated, it is rapidly ubiquitinylated and degraded by proteasome-mediated process. In the absence of β-catenin phosphorylation, it is transported to the nucleus, where it has activities in controlling transcriptional activity (Gottardi et al., 2001).

Desmosomes and hemidesmosomes

Desmosomes and hemidesmosomes are another type of adhesive structures present in endothelial and epithelial cells. While adherens junctions connect cell-cell boundaries to actin cytoskeleton, desmosomes are involved in connecting intracellular intermediate filament network to adhesive structures. This occurs especially in epidermal keratinocytes, where keratin intermediate filament network is connected to desmosomes and hemidesmosomes. Desmosomes are involved in cell-cell connections, whereas hemidesmosomes mediate cell adhesion to basement membranes (Bannon et al., 2001).

Desmosomes consist of desmosomal cadherins desmoglein and desmocollin, which are transmembrane proteins. Desmoglein and desmocollin bind to extracellular domains of each others and mediate homotypic cell-cell adhesion. Plakoglobin, plakophilin and desmoplakin can be regarded as desmosomal catenins. These intracellular proteins connect desmoglein and desmocollin intracellular tails to keratin cytoskeleton (Bannon et al., 2001).

Hemidesmosomes are present in basal keratinocytes, and connect these cells to the underlying BM. The major adhesive receptor in hemidesmosomes is the α6β4 integrin. This integrin binds via its extracellular domain to laminin-5 present in BMs. β4 subunit has an exceptionally long cytoplasmic tail, compared to the other β subunits. This cytoplasmic tail interacts with plectin, an intracellular hemidesmosome component. Plectin connects α6β4 integrin to intermediate filaments. In addition to α6β4 integrin, there is another transmembrane protein in hemidesmosomes, namely bullous pemphigoid antigen BP180 (type XVII collagen). Its extracellular domain interacts with laminin-5 (Franzke et al., 2003), and associates laterally with α6β4 integrin. It also binds to another intracellular hemidesmosomal protein, BP230, which links the complex to intermediate filaments (Bannon et al., 2001).
Other adhesive structures or adhesion molecules

Cells have numerous other adhesion-modulating molecules, which mediate or modulate cell adhesion in the above-mentioned structures, or in other structures. These include selectins, which are involved in low-affinity leukocyte adhesion to blood vessel walls in circulation. (Patel et al., 2002). Cell surface heparan sulphate proteoglycans involve glypicans and syndecans. Syndecan-4 is for example involved in focal adhesion formation (Saoncella et al., 1999; Woods and Couchman, 2001). Immunoglobulin superfamily cell surface receptors (Volkmer, 2001) are involved in neural cell migration and guidance. Receptor type protein tyrosine phosphatases are interesting in the sense that they have their own phosphatase domains. These proteins are involved in at least axon pathfinding (Johnson and Van Vactor, 2003).
Transforming growth factor β, TGF-β

Transforming growth factor (TGF) was first discovered from cell culture supernatant of sarcoma virus transformed mouse fibroblasts as an activity inducing anchorage independent growth of nonmalignant cells (de Larco and Todaro, 1978). The original activity has been found to derive from two structurally and functionally distinct growth factors, transforming growth factors -α and -β, TGF-α and TGF-β (Anzano et al., 1982; Anzano et al., 1983).

Since the cloning of TGF-β1 (Derynck et al., 1985), two other close family members have been found, and named TGF-β2 and TGF-β3 (de Martin et al., 1987; Derynck et al., 1988). TGF-βs 1-3 are secreted polypeptide growth factors. They belong to a large superfamily of growth factors, which are implicated in a multitude of developmental processes (Kingsley, 1994). This family comprises, in addition to TGF-βs themselves, the family of bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs) as well as inhibins and activins. TGF-β is a prototype member of this family. All family members share the similar structure; they are disulfide linked dimeric growth factors.

Active TGF-βs 1-3 are dimeric 25 kDa proteins. They are derived from a 55-kDa precursor polypeptide, which gets dimerized shortly after synthesis. After dimerization, the 55 kDa protein is proteolytically processed by furin protease to yield the mature growth factor, and N-terminal propeptides. The complex of the two disulfide linked propeptides is called latency associated peptide (LAP). LAP -part binds noncovalently with the mature growth factor, which is essential for maintaining TGF-β latent. The complex composed of active TGF-β and LAP is called small latent TGF-β (SL-TGF-β). SL-TGF-β gets associated with other protein, latent TGF-β binding protein (LTBP). LTBP binds covalently to the LAP –part of the SL-TGF-β complex. The complex composed of SL-TGF-β and LTBP is called large latent TGF-β (LL-TGF-β, see Fig. 4), which is the form of the growth factor secreted from the cells. After secretion, LTBP targets the complex to the ECM, TGF-β remaining in the complex in a latent form (Saharinen et al., 1999). Latency means in this context that the mature growth factor cannot bind to its cell surface receptors, and mediate its specific effects. Therefore, mature TGF-β needs to be released from this complex for its effects to take place.

The release of mature TGF-β from the complex of LAP and LTBP is called activation of TGF-β. There are several different mechanisms for TGF-β activation. In vitro, TGF-β can be activated with extremes of pH, heat, chaotropic agents or detergents (Brown et al., 1990). In vivo, TGF-β can be activated by plasmin mediated mechanism (Flaumenhaft et al., 1993b). Plasmin cleaves LAP peptide (Lyons et al., 1988), and destabilizes the complex so that the active TGF-β is released from the complex. In thrombospondin mediated mechanism (Murphy-Ullrich and Poczatek, 2000) TSP-1 gets bound to the LAP peptide, and changes the conformation of the complex so that active TGF-β is released from the complex. In addition, integrin α,β6 can recognize the
RGD sequence of the LAP peptide, and cause the activation of TGF-β (Munger et al., 1999; Annes et al., 2003). After TGF-β is activated, the active growth factor can bind to its cell surface receptors, and initiate activation of intracellular signaling pathways involving Smad protein phosphorylations (illustrated in Fig. 5.)

![Diagram of TGF-β activation and complex formation](image)

**Fig. 4.** TGF-β is produced in cells as monomeric polypeptide, which is dimerized and proteolytically processed shortly after synthesis to yield mature growth factor. Active TGF-β remains complexed to its N-terminal propeptides (LAP) to yield small latent TGF-β, SL-TGF-β. SL-TGF-β is further bound to an LTBP-protein, in this case LTBP-1, to yield large latent TGF-β, LL-TGF-β. Some interacting/active sites of LTBP-1 are shown.

TGF-β was originally identified as cell growth promoting factor for fibroblasts. However, TGF-β inhibits the proliferation of most cells, or even leads to the apoptosis of affected cells (Laiho and Keski-Oja, 1992). The
The growth inhibitory effect is most potent on cells of endothelial or epithelial origin (Moses, 1992). Interestingly, the cells of several advanced malignant tumors have lost the ability to respond to TGF-β growth inhibition (Cui et al., 1996; Pasche, 2001). TGF-β is a potent regulator of a multitude of enzymes, and prominently increases the expression of proteinase inhibitors (Laiho and Keski-Oja, 1992). The most notable example of those is the induction of expression of plasminogen activator inhibitor (PAI-1) (Laiho et al., 1986). The induced expression of PAI-1 downregulates plasminogen activation, which leads to ECM accumulation.

Additionally, TGF-β induces several ECM proteins. It upregulates both the genes of so called provisional matrix proteins, such as fibronectin (Keski-Oja et al., 1988), and aged matrix proteins, such as collagens (Roberts et al., 1986). It also modulates the expression of ECM degrading enzymes.
In addition to inducing the expression of ECM components in cells, TGF-β regulates how cells recognize ECM. TGF-β induces the expression of several integrin subunits, at least in some cell types (Heino et al., 1989; Nejjar et al., 1999; Thibault et al., 2001; Giannelli et al., 2002). In general, the increased expression of integrins leads to increased cell adhesion. Thus the net effect of TGF-β induced expression of both adhesive ECM molecules and their integrin receptors is substantially increased cell adhesion.

In addition, TGF-β inhibits activity of the immune system (Letterio and Roberts, 1998).

**LTBP family of ECM components**

LTBPs (Kanzaki et al., 1990; Tsuji et al., 1990; Morén et al., 1994; Gibson et al., 1995; Yin et al., 1995; Giltay et al., 1997; Saharinen et al., 1998) are 125-240 kDa extracellular proteins belonging to the fibrillin/LTBP-gene family. They were originally identified as binding proteins for the small latent TGF-β. They are mainly composed of characteristic cysteine pattern containing EGF-like, 8-Cys and hybrid domains. EGF domains have sequence similarity to epidermal growth factor, whereas 8-Cys repeats are present in LTBP and fibrulins only. Hybrid domains in turn have sequence similarity to both EGF and 8-Cys domains. In LTBP and fibrulins, there is only one such proline-rich region (domain structures of LTBP and fibrulins are illustrated in Fig. 6).

LTBP-1 was the first member of the LTBP-family to be discovered. LTBP-1 was identified and purified as a binding protein for TGF-β1 from platelets (Miyazono et al., 1988; Kanzaki et al., 1990). LTBP-1 is ubiquitously expressed in various tissues. Of the tissues analyzed, the highest expression levels have been observed in heart, placenta, lung, spleen, kidney and stomach (Tsuji et al., 1990). LTBP-1 mRNA is alternatively spliced at several sites. These forms have been suggested to have a role for example in the protection of the protein from proteolysis (Gong et al., 1998; Michel et al., 1998; Öklü et al., 1998). LTBP-1 has also an N-terminal alternatively spliced variant LTBP-1L, which associates more efficiently with the ECM (Olofsson et al., 1995). The importance of this splice variant is emphasized by the fact that the expressions of these two N-terminally different forms are driven by their own promoters (Koski et al., 1999).

LTBP-1 augments the secretion of SL-TGF-β from cells (Miyazono et al., 1991), and targets the latent complex to the ECM (Taipale et al., 1994). Antibodies against LTBP-1 can inhibit the activation of latent TGF-β (Flaumenhaft et al., 1993a), at least in certain activation mechanisms, suggesting a role for LTBP-1 in TGF-β activation. LTBP-1 associates with SL-TGF-β at its C-terminal 8-Cys repeat, and binds to the ECM mainly via its N-terminus (Gleizes et al., 1996; Saharinen et al., 1996; Nunes et al., 1997).

LTBP-1 has been found to localize in the ECM with latent TGF-β. However, matrix bound LTBP is not always associated with TGF-β (Taipale et al., 1994; Saharinen et al., 1996; Taipale et al., 1996). LTBP-1 colocalizes in cell cultures with fibronectin as well as with fibrillin-1 (Taipale et al., 1996).
The colocalization is dependent on the stage of cell culture. Shortly, a few days after plating the cells, LTBP-1 colocalizes with fibronectin (Taipale et al., 1996), whereas after extended periods (more than week) of cell culture, LTBP-1 colocalizes with fibrillin-1 (Dallas et al., 2000). This suggests that LTBP-1 containing matrix undergoes remodelling during maturation.

LTBP-1 has been found to colocalize with fibrillin-1 also in tissues (Sinha et al., 2002; Isogai et al., 2003). LTBP-1 physically interacts with fibrillin-1 at its C-terminal domain. However, LTBP-1 was not found in microfibril extracts purified from tissues. In addition, the major ECM interacting sites in at least LTBP-1 are located at the N-terminus of the proteins (Saharinen et al., 1996), whereas LTBP-1 – fibrillin interaction site is in the C-
LTBP-2 assembly and effects on cell adhesion

terminus (Isogai et al., 2003). These results suggest that LTBP-1 is not an integral component of microfibrils, but more like an associated protein. LTBP-1 has an alternatively spliced form in which it has an N-terminal extension, which has been suggested to aid the ECM localization of LTBP-1.

LTBP-1 has been implicated in TGF-β driven endothelial-mesenchymal transformation during mouse heart development (Nakajima et al., 1997), whereas the expression of LTBP-1 during in vitro differentiation of embryoid bodies suggests it to have a role in endothelial cell differentiation (Gualandris et al., 2000).

<table>
<thead>
<tr>
<th>LTBP-1</th>
<th>Not done.</th>
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<tbody>
<tr>
<td>LTBP-2</td>
<td>Very early embryonic lethal.</td>
</tr>
<tr>
<td></td>
<td>(Shipley et al., 2000)</td>
</tr>
<tr>
<td>LTBP-3</td>
<td>Defects in bone formation.</td>
</tr>
<tr>
<td></td>
<td>(Dabovic et al., 2002a)</td>
</tr>
<tr>
<td>LTBP-4</td>
<td>Colorectal cancer, lung emphysema.</td>
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<td></td>
<td>(Sterner-Kock et al., 2002)</td>
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LTBP-2 is expressed in developing cartilage perichondrium and blood vessels (Fang et al., 1997), as well as in lung, dermis, large arterial vessels, epicardium, pericardium, and heart valves (Shipley et al., 2000) during mouse development. LTBP–2 colocalizes with fibrillin in bovine tissues (Gibson et al., 1995). It was initially identified based on sequence similarity to LTBP-1 (Morén et al., 1994). Further analysis showed that it does not bind to TGF-β (Saharinen and Keski-Oja, 2000). In Northern hybridization analysis LTBP-2 appeared as two distinct transcripts suggesting that like LTBP-1, also LTBP-2 has two alternatively spliced amino terminal variants (Morén et al., 1994). The so far characterized LTBP-2 cDNA is similar to the long version of LTBP-1. It is unclear whether the cloned cDNA corresponds to the larger or smaller mRNA species detected in the Northern hybridization analysis. LTBP-2 associates in bovine tissues with elastic fibers (Gibson et al., 1995). Mice deficient in LTBP-2 die very early during embryogenesis, possibly during implantation (Shipley et al., 2000). This suggests a crucial role for LTBP-2 in mouse development.

LTBP-3 was identified on the basis of sequence similarity to LTBP-1. It is widely expressed during mouse development (Yin et al., 1995). The highest expression of LTBP-3 in human is detected in skeletal muscle, heart, prostate and ovaries (Penttinen et al., 2002). LTBP-3 is peculiar among the other
LTBPs in the sense that while the other LTBPs are generally secreted in excess to TGF-βs from the cells, LTBP-3 is not secreted from cells without making a complex with SL-TGF-β (Chen et al., 2002; Penttinen et al., 2002). Mice deficient in LTBP-3 have a skeletal phenotype (Dabovic et al., 2002a; Dabovic et al., 2002b). These mice develop shortly after birth cranio-facial defects, and at the age of several months, osteosclerosis and osteoarthritis (Dabovic et al., 2002a). These changes are consistent with the phenotype of mice with defects in TGF-β signaling (Erlebacher and Derynck, 1996; Filvaroff et al., 1999; Janssens et al., 2000) suggesting a role for LTBP-3 in mediating the effects of TGF-β in bone. In addition, these mice have growth retardation as well as spleen and thymus involution (Chen et al., 2003).

LTBP-4 was identified on the database homology searches for 8-Cys repeats (Saharinen et al., 1998). LTBP-4 is a component of the ECM of cultured cells (Saharinen et al., 1998) (K. Koli and J. Keski-Oja, unpublished results). LTBP-4 has the highest mRNA expression levels in aorta, heart, small intestine and ovary (Giltay et al., 1997; Saharinen et al., 1998). LTBP-4 has two alternative N-termini, which originate from alternative splicing (Saharinen et al., 1998). LTBP-4 binds and mediates the secretion of TGF-β1, whereas binding of TGF-βs 2 and 3 is negligible (Saharinen and Keski-Oja, 2000; Lack et al., 2003). LTBP-4 has a splice variant, void of the TGF-β binding 8-Cys repeat (Koli et al., 2001). Mice deficient in LTBP-4 develop cardiomyopathy, pulmonary emphysema and colorectal cancer (Sterner-Kock et al., 2002) indicating an important role for LTBP-4 in the negative regulation of cell growth. TGF-β signaling was decreased in affected tissues, showing direct dependence of TGF-β signaling on LTBP-4.

The main matrix interacting domains of LTBPs are located at the N-terminus of LTBPs. Proteolytic cleavage by for example plasmin of LTBP leads to the release of LTBP central domain from the matrix to soluble pool (Taipale et al., 1994). The central domain of LTBP is the site for association for the SL-TGF-β (Saharinen et al., 1996). Thus, the cleavage of LTBP leads to the release of latent TGF-β. At least some proteases of the serine protease family, such as plasmin, can release latent TGF-β complex from the ECM (Taipale et al., 1994; Taipale et al., 1995). Accordingly, matrix metalloproteinases have been implicated in this process as well (Maeda et al., 2002). The release is considered the first step in some mechanisms of TGF-β activation (Taipale and Keski-Oja, 1997). However, since there are several mechanisms for TGF-β activation, the release or shedding of latent TGF-β complexes from the ECM may even negatively regulate the availability of latent TGF-β. For example, as integrin αvβ6 recognizes ECM bound LLTGF-β complex, the shedding of LTBP from the matrix may negatively regulate the availability of TGF-β.
Aims of the present study

LTBP-2 was chosen the subject of the current studies to analyze the similarities and differences between LTBP-1 and LTBP-2, in relation to its potential role in the biology of latent TGF-β. During the course of the studies, it was found that LTBP-2 does not, in fact, bind latent TGF-β (Saharinen and Keski-Oja, 2000). However, LTBP-2 has important, albeit yet unidentified, functions during mouse embryogenesis (Shipley et al., 2000). These findings led us to search for new functions for LTBP-2. We first set the goal to biochemically characterize LTBP-2 and analyze its assembly to the ECM. In addition, we decided to characterize the ECM assembly of LTBP-1 to understand differences between these proteins.

Cell adhesion is a central phenomenon during development. Therefore, we used recombinant LTBP-2 and its fragments for cell adhesion studies. We searched for cell lines that would adhere to LTBP-2, and found so far that only melanoma cells had this ability. During the course of these studies we found that LTBP-2 acts as an antiadhesive protein for fibroblasts, which inspired us to further characterize the mechanism of this antiadhesion.

In summary, the specific aims of this work were:

I. To recombinantly express and purify LTBP-2 protein, study its basic biochemical properties, proteolytic processing and matrix assembly.

II. To localize the ECM interacting domains of LTBP-1, and to analyze the kinetics of the ECM association of these fragments.

III. To characterize the effects of LTBP-2 on melanoma cell adhesion, to elucidate the mechanism of adhesion and to identify the cell surface receptors involved.

IV. To study the effects of LTBP-2 on fibroblast adhesion and characterize mechanism of antiadhesiveness.
Materials and Methods

Only the materials and methods developed during this work have been explained in detail here. Standard materials and methods have been described in original publications or in their references. They have been referred to in tables below with Roman numerals.

Expression constructs

Constructs of LTBP-1

Diverse fragments spanning the entire sequence of short splice variant of human LTBP-1 (see Fig. 7 for designations) containing appropriate restriction endonuclease recognition sites at their 5’ and 3’ ends were generated by PCR. The design of the primers was based on the LTBP-1S sequence published under the Genbank accession number M34057 (Kanzaki et al., 1990). The PCR products were ligated into a Signal pIg plus vector (R&D Systems, Minneapolis, MN) as XbaI-BamHI fragments (I-II), XbaI-BglII fragment (III-V) or HindIII-BamHI fragments (VI-X) to obtain fusion proteins containing an Ig-tag in the C-terminus of the fusion protein.

![Diagram of LTBP-1 constructs](image)

Fig. 7. Schematic representation of Ig-tagged LTBP-1 fragments used for ECM association studies in (II). Modified from (II).
Constructs of LTBP-2

SapI-HindIII fragment of LTBP-2 cDNA (bp 350-6317; Genbank Z37976) containing the full length open reading frame (Morén et al., 1994) was blunted with Klenow polymerase and ligated to EcoRV restriction site in pcDNA3 mammalian expression vector (Invitrogen, Oxon, UK) to obtain native full length LTBP-2.

LTBP-2 fragments were expressed in stably transfected CHO cells as dimeric N-terminal fusion proteins with Fc portion of immunoglobulin G as follows. Fragments of LTBP-2 cDNA were amplified by PCR with primers designed to maintain the open reading frame after the signal sequence and to continue to Fc tail in signal plg+ (R&D Systems, Minneapolis, MN) vector. Primer sequences contained HindIII (5’ primers, all constructs except L2-X) or BamHI (3’ primers, all constructs except L2-VII and L2-VIII) restriction enzyme recognition sites in the 5’ end of the primers. Construct L2-VII and L2-VIII 3’-primers contained NotI recognition sites, and construct L2-X 5’-primer contained KpnI recognition site (see Fig. 8). Amino acid numbering is according to the translated cDNA sequence in Genbank (accession number Z37976). Control protein containing only the constant region of human IgG was obtained by expression of vector plg+.

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<th>Construct</th>
<th>Amino Acid Range</th>
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<tr>
<td>L2-VII</td>
<td>221-621</td>
</tr>
<tr>
<td>L2-VIII</td>
<td>221-621</td>
</tr>
<tr>
<td>L2-V</td>
<td>429-621</td>
</tr>
<tr>
<td>L2-X</td>
<td>429-621</td>
</tr>
<tr>
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<td>161-843</td>
</tr>
<tr>
<td>L2-C</td>
<td>429-550</td>
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<tr>
<td>L3-N</td>
<td>161-843</td>
</tr>
<tr>
<td>L3-C</td>
<td>429-550</td>
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Fig. 8. Schematic representation of Ig- and his-tagged LTBP-2 fragments used for cell adhesion studies in (III and IV). Modified from (IV).
Histidine tagged monomeric fragments were produced utilizing pSecTagA vector (Invitrogen). cDNA fragments were produced by PCR with the primers containing HindIII (5’) and NotI (3’) restriction enzyme recognition sites. L2-N* contains amino acids 161-843, L2-V* amino acids 429-550 and L2-X* amino acids 728-843 (illustrated in Fig. 8).

Production of antibodies

Polyclonal antibodies (Ab-L22) against full length recombinant LTBP-2 protein were raised in rabbits. Briefly, rabbits were immunized with ~10 µg of recombinant purified LTBP-2 using complete Freund’s adjuvant. Subsequent boosts were carried out at four weeks intervals using ~10 µg of LTBP-2 in incomplete Freund’s adjuvant. For immunoblotting, specific IgG was immunoaffinity purified from the serum as follows. Purified LTBP-2 was coupled to CNBr-activated Sepharose (Pharmacia) in PBS (10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl). The antiserum was passed three times through the column, and the column was washed with 20 bed volumes of PBS. Bound IgG was eluted first with 1% CH3COOH, and subsequently with 20 mM NaOH. Eluted IgG was immediately neutralized with 1.5 volumes of 1 M Tris-Cl, pH 7.0. Ab-L22 is specific for LTBP-2 in immunoblotting and immunoprecipitation.

Expression of recombinant proteins in CHO cells

CHO cells were transfected using calcium phosphate transfection kit (I) or lipofectamine (II-IV) according to manufacturer’s instructions (Life Technologies, Gaithersburg, MD). One day after transfection the cells were changed to medium containing 0.8 mg/ml (I) or 1.5 mg/ml (II, IV) G418, or 0.5 mg/ml zeocin in the case of pSectag vector (IV) and the selection was maintained for 2 weeks. Expression levels of individual clones obtained by dilution cloning were estimated by immunoblotting. The cells were maintained after this in a medium containing 0.4 mg/ml G418 or 0.5 mg/ml zeocin.

For the collection of conditioned medium the cells were washed twice with serum free medium. The serum free conditioned medium was then collected for two successive 2-day periods. If the conditioned medium was used for LTBP-2 purification, aminoethylbenzene sulphonylfluoride (AEBSF; 1 mM, final concentration; Calbiochem, La Jolla, CA) was added to prevent processing by endogenous serine proteases.

Purification and quantification of recombinant LTBP-2

Conditioned medium from CHO cells expressing LTBP-2 (CHO-L2 cells) (400 ml in each purification) was first precipitated with (NH$_4$)$_2$SO$_4$ at room temperature. The precipitates were collected by centrifugation and redissolved in 50 mM Tris-Cl buffer, pH 7.0, followed by dialysis overnight against the same buffer.
The dialysate was applied to MonoQ HR 5/5 column (Pharmacia) equilibrated with 50 mM Tris-Cl buffer, pH 7.0. The column was washed with 5 bed volumes of 50 mM Bis-Tris-Cl buffer, pH 6.0, followed by 20 bed volumes of the same buffer containing 210 mM NaCl. The bound proteins were then eluted with a NaCl gradient (210-700 mM). Finally, LTBP-2 containing fractions were fractionated on a Superdex 200 gel filtration column (Pharmacia) equilibrated with PBS.

The amounts of LTBP-2 in the various fractions obtained during the purification were quantitated by dot immunoblotting and chemiluminescence detection of several dilutions of the samples from each fraction with the anti LTBP-2 antibodies (Ab-178). The dot blots were quantitated by scanning X-ray films, and measuring relative intensities of dots by NIH Image program (U.S. National Institutes of Health, available on the Internet at http://rsb.info.nih.gov/nih-image/). The amount of LTBP-2 present in the conditioned medium was considered as 1 unit/ml (1 U/ml). Total protein concentrations of the conditioned medium and redissolved ammonium sulphate precipitations were quantitated by the Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. After this point the protein amounts were estimated by measuring \( A_{280} \) (\( A_{280}=1 \) equals 1 mg/ml of protein). Protein concentrations were estimated by calculating the areas of the LTBP-2 containing peaks (ÄktAExplorer/Unicorn software; Pharmacia) in anion exchange or gel filtration chromatogram and assuming \( A_{280}=1 \) to correspond to 0.82 mg/ml (value calculated from the amino acid sequence of LTBP-2).

Purification protocol was altered (III and IV) to increase the yield of LTBP-2 as follows. The proteins of cell conditioned medium from a CHO clone overexpressing LTBP-2 were precipitated with 30 % (NH₄)₂SO₄. The precipitate was dissolved in 50 mM Tris-HCl buffer, pH 7.0, and dialyzed against the same buffer. The dialyzed solution was filtered and applied to MonoQ HR 5/5 column (Amersham Pharmacia Biotech, Uppsala, Sweden) in 50 mM Bis-Tris, pH 6.0, and the bound proteins were eluted with a gradient of 210-700 mM NaCl in the same buffer. LTBP-2 containing fractions were combined, and 4 M urea was added to achieve the final concentration of 2 M. Mono Q 5/5 column was equilibrated in 50 mM Bis-Tris-HCl, pH 7.0, containing 2 M urea, and the sample was applied to the column. Proteins were eluted with 0-1 M NaCl gradient. Gel filtration column Superdex 200 (Amersham Pharmacia Biotech) was equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 2 M urea, and LTBP-2 containing fractions were gel filtrated in the same buffer. Finally, the buffer of LTBP-2 containing fractions was changed to PBS with Fast Desalting column HR 10/10 (Amersham Pharmacia Biotech). Addition of urea to the purification buffers increased the yield of LTBP-2 at least ten fold, while the purity was at the same level as earlier. LTBP-2 obtained with this protocol behaved in cell adhesion assays in a manner similar to the protein purified under non-denaturing conditions.

**Purification of recombinant tagged protein fragments**

Purification of Ig-tagged proteins was carried out by affinity chromatography, using HiTrap protein A columns (Amersham Pharmacia Biotech).
Biotech, Uppsala, Sweden). The fusion proteins were eluted with 0.58% acetic acid, 140 mM NaCl. Affinity chromatography was followed by gel filtration with a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) with phosphate buffered saline (PBS; 0.14 M NaCl in 10 mM sodium phosphate buffer, pH 7.4) as the buffer system.

Histidine tagged proteins were first precipitated with 35 % (NH₄)₂SO₄. The precipitate was dissolved in the wash buffer (2 M urea, 300 mM NaCl, 50 mM Tris-HCl buffer, pH 7.2). The sample was loaded to Talon metal chelate (Clontech, Palo Alto) column. The column was washed with the wash buffer, and the bound proteins were eluted with the same buffer containing 100 mM imidazole. The buffer of the fractions containing the fusion proteins was then exchanged to PBS with Fast Desalting column HR 10/10 (Amersham Pharmacia Biotech).

### Antibodies

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LTBP-2 assembly and effects on cell adhesion

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<td>Purification of Ig and His-6-tagged proteins</td>
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Results

LTBP-2 expression, purification, biochemical characterization and assembly to the ECM (I)

To study the properties and effects of LTBP-2, we first recombinantly expressed and purified LTBP-2 protein. For this purpose we employed Chinese hamster ovary (CHO) cells. We constructed a full length LTBP-2 cDNA construct using a commercially available mammalian expression vector, transfected this construct to CHO cells, and selected stable LTBP-2 expressing cell clones. The migration of recombinantly expressed LTBP-2 in SDS-PAGE was identical with that made by human fibroblasts. We chose the clone with the highest expression of LTBP-2, collected serum-free conditioned medium, and purified LTBP-2 to apparent homogeneity, as judged by SDS-PAGE and Coomassie blue staining. The purification procedure of LTBP-2 included ammonium sulphate precipitation, anion exchange chromatography and gel filtration. The procedure was later (III, IV) optimized to increase the yield of LTBP-2.

So far tested members of fibrillin-LTBP family (Colosetti et al., 1993; Corson et al., 1993), and more generally proteins containing EGF-like repeats bind calcium (Davis, 1990). We therefore tested whether LTBP-2 can also bind calcium. By using radioactive calcium isotope, we noted that LTBP-2 does bind calcium. By using chemical deglycosylation, we observed that recombinantly expressed LTBP-2 is glycosylated. By using a procedure specifically tagging carbohydrate structures and proteolytic enzymes, we detected that most if not all of the carbohydrate structures are located at the central, EGF-like repeat containing the proteolytically stable region of LTBP-2.

We used recombinantly expressed LTBP-2 as a substrate to characterize its proteolytic processing. We noted that serine proteases plasmin and elastase process LTBP-2 in a manner very similar to that of LTBP-1 (Taipale et al., 1994; Taipale et al., 1995). By using purified LTBP-2, its proteolytic fragments and subsequent N-terminal amino acid sequencing, we determined the proteolytic cleavage sites in LTBP-2.

We also noted that recombinantly expressed and purified, soluble LTBP-2 gets incorporated into the ECM of cultured fibroblasts. Proteolytic processing resulted in the release of ECM bound LTBP-2.

Identification of ECM binding regions in LTBP-1 (II)

Previously it was known that the N-terminal regions of LTBP-1 are important for ECM association (Saharinen et al., 1996; Nunes et al., 1997). We next wanted to confirm the earlier results, to narrow down the ECM interacting areas and identify other possible ECM interacting domains. For this purpose
we generated recombinant fragments of LTBP-1 as Ig-tagged proteins in CHO cells. The reason for employing this technology was that Ig-tagged protein fragments were easy to produce in relatively large quantities and the purification of the fragments was efficient.

We identified three different regions of LTBP-1, which interacted with the ECM of cultured human lung fibroblasts. Two of the regions were located at the N-terminus of the protein, and one in the C-terminus of LTBP-1. Two of the ECM associating regions contained an 8-Cys repeat and one of them a hybrid domain (the most N-terminal ECM associating region). The efficiency and kinetics for the fragment association with the ECM varied between the fragments. The N-terminal fragments bound more rapidly to the ECM. Detectable levels bound to the ECM within hours. Association of the C-terminal fragment with the ECM was not detectable until after 24 h incubation. The binding of this fragment to the ECM was more efficient in the presence of cell-derived factors in the conditioned medium of the cells. However, all three fragments bound also to cell-free ECM preparations. Incubation of all these three fragments in culture medium of fibroblasts prevented incorporation of endogenous full length LTBP-1 to the ECM, confirming the specificity of fragment binding to the ECM.

LTBP-2 is an adhesion protein for melanoma cells (III)

Cell adhesion is a critical requirement for cell survival and locomotion in tissues (Gumbiner, 1996). Knowing the vital importance of LTBP-2 during development (Shipley et al., 2000) we wanted to determine whether LTBP-2 would have a role in cell adhesion. The role for LTBP-2 in cell adhesion was also suggested by the presence of an RGD sequence (Ruoslahti, 1996) in the N-terminus of the protein. Therefore, we investigated with several different cell types, such as melanoma, fibrosarcoma (III), fibroblast (IV), keratinocyte, endothelial and myoblast cells (data not shown), whether they would adhere to LTBP-2.

Of the tested cells, only melanoma cells were able to adhere to full length LTBP-2. The result was confirmed with several independent melanoma cell lines (Bowes, G361, WM793, WM852, WM163, WM164 and WM239). Cell adhesion was dependent on the concentration of LTBP-2 used. Adhesion was accompanied by cell spreading and focal adhesion formation, which indicated that LTBP-2 is a real adhesion protein, not just a binding protein for melanoma cells. Additionally, LTBP-2 acted as substratum bound haptotactic attractant and supported melanoma cell migration.

Function blocking antibodies against \( \beta_1 \) and to a lesser extent \( \alpha_3 \) and \( \alpha_6 \) -integrins prevented cell adhesion to LTBP-2. In addition, heparin decreased melanoma cell adhesion to LTBP-2 implicating hepan sulphate proteoglycans in the process. In contrast, RGD peptide had negligible effects on cell adhesion, and the area of LTBP-2 mediating cell adhesion was localized to a fragment not containing the RGD sequence, excluding this integrin recognition motif from the adhesive event.
LTBP-2 has antiadhesive effects on fibroblastic cells (IV)

While firm cell adhesion is crucial for cell survival and functionality, cells need to control and decrease their adhesion to their extracellular ligands, for example during cell migration (Lauffenburger and Horwitz, 1996). The regulation/decrease of adhesion may originate from cells themselves and take place by negative regulation of cell surface receptors (Hynes, 2002). However, there are negative regulators of cell adhesion among ECM components (Chiquet-Ehrismann, 1995). Since the melanoma cells were the only examples of cells, which could adhere to full length LTBP-2, we wanted to explore whether LTBP-2 would have antiadhesive effects for other cell types.

We produced several partially overlapping Ig-tagged fragments of LTBP-2, and identified within these fragments a region of LTBP-2, which supported fibroblast binding. The binding was less efficient than cell binding to fibronectin (~60%), and cells adhering to this fragment did not form actin stress fibers or spread to the same extent as cells adhering to FN. Histidine-tagged fragments of LTBP-2 covering the cell-binding region supported cell adhesion as well. However, the adhesion-supporting effect was bell-shaped; after reaching optimal concentration for maximal cell binding, the number of attached cells decreased.

We noted that LTBP-2 had drastic effects on cells adhering to fibronectin. LTBP-2 present in the cell-attaching substratum together with fibronectin prevented cells from forming actin stress fibers and focal adhesions. In addition, cell spreading was decreased. The effects were most prominent with full length LTBP-2, but both of the fragments mediating fibroblast binding had similar effects. LTBP-2 and the histidine-tagged fragments having these effects bound fibronectin, and the cytoskeleton disturbing effects were concentration-dependent. Higher concentrations had more prominent effects on actin cytoskeleton, and especially with histidine-tagged fragments, a saturation point could be reached (increasing the concentration of fragment bound to fibronectin-coated coverslip after a certain concentration did not further increase the effects). This suggested that LTBP-2 binding to fibronectin is necessary for the antiadhesive effect. LTBP-2 had no effect on cells adhering to type I collagen. LTBP-2 fragments with the cytoskeleton-disturbing effects associated with the ECM of cultures cells and colocalized with fibronectin fibers.

**Fig. 9.** Schematic representation of the domain structures of LTBP-1 and LTBP-2. The active sites affecting on LTBP-1 ECM association, LTBP-2 proteolytic processing sites and region having effects on cell adhesion are underlined.
Discussion

ECM molecules have several different roles, such as maintenance of tissue architecture and providing proper environment for cell survival and proliferation. Growth factors have a vital role in regulating these functions. They may reside in the ECM (Taipale and Keski-Oja, 1997), implicating the indirect role of the ECM in this process. LTBP-proteins are involved in the process by targeting the effects of TGF-β (Saharinen et al., 1999).

LTBP-2 was originally identified as a protein mediating the effects of TGF-β (Morén et al., 1994), by transporting it from the cells, like LTBP-1 does (Miyazono et al., 1991). However, further examination of the binding between LTBP-2 and TGF-β did not reveal a binding (Saharinen and Keski-Oja, 2000; Lack et al., 2003). LTBPs are relatively similar to each other and form a compact subgroup within fibrillin-LTBProtein family, as judged by sequence analysis. Although the sequence similarity with other proteins gives clues, it does not necessarily give proof about protein function. Therefore, the specific function(s) of LTBP-2 are largely unknown.

Analysis of the functions of LTBP-2 by knockout mice (Shipley et al., 2000) showed the vital importance of LTBP-2 during embryogenesis. These mice died very early, possibly due to a defect in implantation. However, the specific function of LTBP-2 was still shrouded, because no LTBP-2 deficient embryos could be recovered from heterozygous pregnant mice.

We have approached these problems by studying the role and functions of LTBP-2. We have employed cell culture models, and have characterized some plausible effects of LTBP-2. For this purpose we have generated and purified recombinant LTBP-2 protein as well as its fragments. We have investigated the biochemical properties of the protein, analyzed its proteolytic processing and assembly to the ECM. We have then tested recombinant LTBP-2 in cell culture, specifically analyzing its effects on cell adhesion, which is often crucial for affecting cell functions.

LTBP-2 is a calcium binding ECM glycoprotein

EGF-like repeats of several ECM proteins bind calcium. Fibrillins and LTBP-2 have several EGF-like repeats, which have a consensus sequence for calcium binding (Handford et al., 1991). Calcium-ion is critical for maintaining the structure in these proteins (Downing et al., 1996), and it may have a role in protein-protein interactions mediated by EGF-like repeats (Rao et al., 1995). Calcium binding in fibrillin-1 appear also to protect it from proteolytic processing (Reinhardt et al., 1997; Reinhardt et al., 2000b). We have noted in our studies that LTBP-2 binds calcium (I). We speculate that the EGF-like repeats are responsible for calcium-binding in LTBP-2 and that calcium binding may have a protein stabilizing role for LTBP-2. We noted also that LTBP-2 is glycosylated. Most, if not all, of the glycosylation sites were located...
to the central, proteolytically stable region of LTBP-2 containing EGF-like repeats (I).

LTBP-2 was susceptible to proteolytic processing by serine proteases (I). The processing resembled that of LTBP-1 (Taipale et al., 1994; Taipale et al., 1995), and processing released proteolytically stable central domain of LTBP-2 from the ECM. This suggests that the major ECM interacting domains of LTBP-2 are located at the N-terminal half of LTBP-2. Recently, we have characterized the ECM association of Ig-tagged fragments of LTBP-2 (M. Hyytiäinen, unpublished results). These studies have yielded similar results (see also II).

By using purified protein, we localized proteolytic processing sites to the N-terminal half of the protein, to the proline rich hinge regions of the protein. We could not detect C-terminal processing of LTBP-2, although there is a similar proline-rich region in the C-terminus of the protein. The experimental settings we employed left the possibility that such processing can occur. By using purified proteins in the processing studies, we confirmed that LTBP-2 is a direct substrate for plasmin and elastase, and excluded the possibility that these proteases would initiate a cascade in which other proteases activated by plasmin and elastase would cleave LTBP-2. However, the proteases acting in vivo on LTBP-2 may still be different from the ones tested.

In principle, there are two ways by which ECM proteins may assemble to the ECM. ECM protein may either self-assemble to form insoluble fibers, or the assembly may be a cell-mediated process, in which soluble or cell-derived ECM components localize to the surface of cells where the polymerisation occurs. Collagen fiber assembly is thought to happen via self-assembly, whereas fibronectin assembly is a classical example of cell-mediated polymerisation. However, recent results have shown that even collagen assembly is not strictly a self-assembly process (Velling et al., 2002).

We noted that the purified, soluble LTBP-2 could get assembled to the ECM (I). This excludes the possibility that only cells, which produce it, could assemble LTBP-2 to the ECM. However, we have later noticed in transfection and immunofluorescence studies that LTBP-2 is preferentially located in the proximity of the cells, which produce it (M. Hyytiäinen, unpublished results). This, on the other hand, suggests that LTBP-2 producing cells also assemble it to the ECM.

**LTBP-1 has three potential ECM binding regions**

We studied next the ECM association of LTBP-1 (II). It was previously known that LTBP-1 associates with the ECM at its first 400 N-terminal amino acids (Saharinen et al., 1996). In addition, amino acid residues 294-441 of LTBP-1 are substrates for transglutaminase (Nunes et al., 1997), an enzyme making covalent crosslinks between glutamine residues. Transglutaminase also co-localizes with LTBP-1 at the cell surface (Verderio et al., 1999). The crosslinking activity of transglutaminase is at least in part responsible for ECM association of LTBP-1.

By using Ig-tagged fragments of LTBP-1 as well as related immunofluorescence and enzyme immunoassays, we found evidence for three
ECM associating domains/areas of LTBP-1. Two of the ECM binding areas contain an 8-Cys repeat, and one contains a hybrid domain, suggesting that these domains may act as ECM binding regions. The functions of the 8-Cys repeats have been poorly known; the only function so far has been that the third 8-Cys repeat of LTBP-1, -3 and -4 bind latent TGF-β (Gleizes et al., 1996; Saharinen et al., 1996; Saharinen and Keski-Oja, 2000). We propose here that the 8-Cys repeats may have another, ECM binding function. However, the SL-TGF-β binding 8-Cys repeat did not bind to the ECM.

Recently, ECM association of LTBP-1 was characterized with monomeric, HA-tagged fragments of LTBP-1 (Dallas et al., 2000). The ECM association was not observed with the fragments lacking N-terminus of LTBP-1. This may be due to the fact that they used only relatively long C-terminal fragments of LTBP-1 containing a stretch of EGF-repeats. The ECM binding region may be cryptic which could explain the result. The fact that the C-terminal fragment bound to the ECM with slower kinetics than N-terminal fragments is also in favor of this hypothesis.

We have made similar ECM binding studies with the corresponding fragments of LTBP-2. The results are very similar to the studies with LTBP-1 (M. Hyytiäinen, unpublished results), at least with the N-terminal fragments of LTBP-2. In addition, we have localized yet another ECM associating fragment of LTBP-2 to the proline-rich region located N-terminally to the hybrid domain (IV).

**Effects of LTBP-2 on cell adhesion**

We noted that melanoma cells have an ability to attach to LTBP-2 (III). The adhesion was accompanied with cell spreading and actin stress fiber formation, in a manner similar to cells adhering to laminin. The adhesion was dependent on integrins α3β1 and α6β1. These integrins were first identified as receptors for laminin (Belkin and Stepp, 2000). Adhesion could be prevented to some extent with heparin, implicating cell surface heparan sulphate proteoglycans in the process. HT-1080 fibrosarcoma cells, which express integrins α3β1 and α6β1 and adhere to laminin via these receptors, did not adhere to LTBP-2. This indicates that the adhesion is not entirely dependent on integrins α3β1 and α6β1. Cell adhesion was not dependent on the RGD sequence present in the N-terminus of LTBP-2. The partial adhesion-mediating domain was located to another proline-rich region in the N-terminal part of LTBP-2. This domain was unexpectedly noticed to possess antiadhesive functions for fibroblasts (see below).

Soluble LTBP-2 had only minor inhibitory effect on melanoma cell adhesion to LTBP-2. This suggested that the adhesive region of LTBP-2 is cryptic on full length soluble LTBP-2. The binding of LTBP-2 to the plastic surfaces used in the assays would then expose adhesive sites in the protein, thus potentially mimicking the situation in which LTBP-2 is assembled to the ECM.

Melanocytes are progenitors of melanoma cells, and melanocytes are developmentally derived from neural crest (Misugi et al., 1965). Neural crest is an important embryonic structure, from which for example different types of
neurons are derived from, and where active and strictly coordinated cell migration is taking place (Gilbert, 1997). ECM plays an important role in guiding neural crest cell migration (Perris and Perissinotto, 2000). LTBP-2 supported melanoma cell migration. Therefore, it is tempting to speculate that LTBP-2 could have a more general role in neural crest cell migration.

Even though full length LTBP-2 was not detected to mediate the adhesion of other cell lines than melanoma cells, we detected a 120 amino acids long fragment of LTBP-2, which had a fibroblast binding property (IV). We assume that this cell-binding activity is cryptic in full length LTBP-2, and therefore we can observe fibroblast binding to recombinant fragment of LTBP-2 only.

The antiadhesive fragment appeared to be the same, which mediated melanoma cell adhesion. This result leads to apparent discrepancy between results of cell adhesion with melanoma cells and fibroblasts. Full length LTBP-2 mediated melanoma cell adhesion, but not fibroblast adhesion. However, the same LTBP-2 fragment mediated adhesion/cell binding of both cell types. Therefore, why can we observe melanoma cell adhesion to a fragment, which seems to be cryptic in full length LTBP-2?

It is obvious that the proline-rich fragment in the N-terminus of LTBP-2 is not the only region of LTBP-2, which is involved in melanoma cell adhesion. Therefore, melanoma cells presumably recognize LTBP-2 in a different manner, and with different set of receptors. This recognition may give melanoma cells an ability to expose the cryptic adhesion site of LTBP-2. In addition, it is possible that there are several cell-adhesion mediating regions in the 120 amino acids long proline rich region, of which the fibroblast recognizable region is cryptic but the melanoma cell recognizable region is not. Furthermore, it is still possible that the adhesion site in LTBP-2 is not cryptic, but that the two cell types have different responses to this binding.

Fibroblasts in response to the binding to proline rich region spread only slightly, but did not form any stress fibers. In fact, the full length LTBP-2 had antiadhesive effects on fibroblasts. When plated on substratum containing FN together with LTBP-2, fibroblast spreading was decreased, and more prominently, actin stress fiber formation was inhibited. The effect was similar, but not identical with LTBP-2 cell binding fragment. The effect was more prominent with full length LTBP-2, suggesting that LTBP-2 contains some additional active antiadhesive epitopes.

LTBP-2 and its antiadhesive fragments bound to FN, and the antiadhesive effect was specific for FN. LTBP-2 had no effects on cells adhering to type I collagen. In addition, the antiadhesive effect was clearly concentration dependent and saturatable with LTBP-2 fragments: When keeping the concentration of FN constant, and increasing the fragment concentration in adhesion assays, the antiadhesive effects became more prominent to the point where the approximate molar concentrations of FN and the fragment were the same. When increasing the concentration of LTBP-2 fragment further, the antiadhesive effects did not increase further. These results suggest that LTBP-2 binds specifically to FN and blocks some adhesive epitopes in FN. This mechanism has been proposed for tenascin-C (Huang et al., 2001), another antiadhesive ECM molecule. However, since the antiadhesive effects of full length LTBP-2 were more prominent, and the molar concentrations of full
length LTBP-2 to obtain similar effect were smaller, it is probable that some additional epitopes participate in mediating the effects.

The fact that the cells bound to the antiadhesive LTBP-2 fragment suggest that there may be a cell surface receptor for LTBP-2. A related antiadhesive protein, thrombospondin, has been suggested to exert its antiadhesive effects via binding to cell surface calreticulin (Goicoechea et al., 2000; Goicoechea et al., 2002). This binding has been suggested to affect intracellular signalling and cause actin cytoskeleton disassembly.

We have not been able to detect any cell surface proteins in fibroblasts recognizing the antiadhesive LTBP-2 fragment. However, melanoma cells bound to LTBP-2 via α3β1 and α6β1 (III), and melanoma cell adhesion to fragment of LTBP-2 was also inhibitable with antibody blocking integrin α3 function (Vehviläinen, unpublished results). Therefore, an obvious question exists whether this (these) integrin(s) might have some role in the fibroblast antiadhesive function. However, the cell-binding activity does not necessarily have a role in antiadhesive function.
Perspective

Cell adhesion is a critical and crucial event in developmental processes during embryogenesis. Mice deficient in LTBP-2 die very early during embryogenesis (Shipley et al., 2000), which suggests that LTBP-2 has an important role in developmental processes, possibly in regulation of cell adhesion.

The current results establish a novel role for LTBP-2 as an adhesive protein for melanoma cells, and on the other hand as an antiadhesive ECM component for fibroblastic cells. These cell adhesion-regulating effects give clues for future experiments on explaining the phenotype of LTBP-2 knockout mouse.

Melanocytes, the progenitors of melanoma cells are originally derived from the neural crest, a developmentally important early fetal structure. Therefore, it will be of interest to learn whether other cell types of neural crest adhere to LTBP-2, and what is the expression pattern of LTBP-2 in that specific structure during development.

We found that melanoma cells adhere to LTBP-2 via integrins α3β1 and α6β1. In addition, we found that fibroblasts bind to LTBP-2 fragment, but do not show specific signs of real adherence. α3β1 integrin has been noted to regulate cell adhesion and other functions of integrin (Kreidberg, 2000). Therefore, it will be of interest to evaluate the role of α3β1 in relation to the antiadhesive functions of LTBP-2.
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LTBP-2 assembly and effects on cell adhesion


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