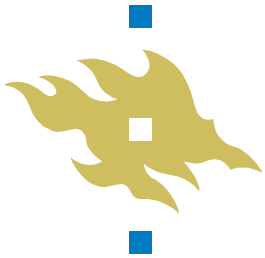
INTERACTIONS OF SMALL LATENT TRANSFORMING GROWTH FACTOR-BETAS WITH THEIR BINDING PROTEINS, LTBPs

JUHA SAHARINEN



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HAARTMAN INSTITUTE
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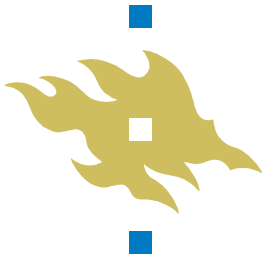

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ACADEMIC DISSERTATION

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HELSINKI 2000

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1 CONTENTS

2 ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to by their Roman numerals in the text.

I. Saharinen, J., Taipale, J. and Keski-Oja, J.
Association of the small latent transforming growth factor-β with an eight cysteine repeat of its binding protein LTBP-1.
EMBO Journal, **15**, 245-253, 1996.

II. Saharinen, J. and Keski-Oja, J.
Specific sequence motif of 8-Cys repeats of TGF-β binding proteins, LTBPs, creates a hydrophobic interaction surface for binding of small latent TGF-β.
Molecular Biology of the Cell, **11**, 2691-2704, 2000.

III. Saharinen, J., Taipale, J., Monni, O. and Keski-Oja, J.
Identification and characterization of a new latent transforming growth factor-β binding protein, LTBP-4.
Journal of Biological Chemistry, **273**, 18459-18469, 1998.

IV. Koski, C., Saharinen, J. and Keski-Oja, J.
Independent promoters regulate the expression of two amino terminally distinct forms of latent transforming growth factor-β binding protein-1 (LTBP-1) in a cell type-specific manner.
Journal of Biological Chemistry, **274**, 32619-32630, 1999.

3 ABBREVIATIONS

8-Cys	eight cysteine protein domain, also known as TB (TGF-β binding protein like) domain and CR (cysteine-rich) domain
α_2M	α_2 macroglobulin
BAMBI	BMP and activin membrane-bound inhibitor
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic protein
CDMP	cartilage-derived morphogenetic protein
cDNA	complementary deoxyribonucleic acid
CNTF	ciliary neurotrophic factor
ECM	extracellular matrix
EGF	epidermal growth factor
EST	expressed sequence tag
FAST	forkhead activin signal transducer
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GDF	growth and differentiation factor
GDNF	glial cell line-derived neurotrophic factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HGF	hepatocyte growth factor (also known as scatter factor)
HHT-1	hereditary hemorrhagic telangiectasia
IFN-γ	interferon-γ
IGF	insulin-like growth factor
IL	interleukin
kb	kilobase
kDa	kilodalton
LAP	latency associated protein / peptide
LIF	leukemia inhibitory factor
LTBP	latent TGF-β binding protein
MFS	Marfan syndrome
MH	Mad homology
MIS/AMH	Müllerian inhibiting substance/anti-Müllerian hormone
MMP	matrix metalloproteinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NT	neurotrophin
PA	plasminogen activator
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
SARA	SMAD anchor for activation
SBE	SMAD binding element
SCID	Severe combined immunodeficiency
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
TGF-β	transforming growth factor beta
TβRI, TβRII	TGF-β type I and II receptors
TIE	TGF-β inhibitory element
TIMP	tissue inhibitor of metalloproteinases
TNF-α	tumor necrosis factor-α
tPA	tissue-type plasminogen activator
TSP	thrombospondin
uPA	urokinase-type plasminogen activator
uPAR	receptor for urokinase-type plasminogen activator
VEGF	vascular endothelial growth factor

4 SUMMARY

Transforming growth factor-betas (TGF-βs) are multipotent peptide growth factors, whose main effects are to enhance the synthesis of extracellular matrix (ECM) components, to decrease ECM proteolysis, to inhibit cell proliferation and to act as immunosuppressive agents. TGF-β is secreted from cells as a latent complex, consisting of a TGF-β dimer and its N-terminal latency associated propeptide (LAP) dimer. TGF-β and LAP are proteolytically cleaved apart during secretion, but remain associated via non-covalent forces. In TGF-β activation, this interaction is disrupted or modified so that TGF-β can bind to its cell surface signaling receptors. Frequently, a latent TGF-β binding protein (LTBP) is covalently attached to the LAP part of the TGF-β complex. LTBPs are required for the correct folding and efficient secretion of the complex. LTBPs are ECM components, directing the large latent TGF-β complex to the ECM structures.

In the current work, the protein domains of LTBP-1 responsible for both the deposition of LTBP-1 to ECM and for the covalent association with TGF-βLAP were first identified. Using stably co-transfected cell lines, TGF-βLAP was found to be secreted as the large latent complex, whenever LTBP-1 or endogenous LTBP proteins were present. The ECM binding function was localized to the N-terminus of LTBP-1S protein.

All mammalian TGF-βLAP isoforms were found to associate with the 3rd 8-Cys repeat of LTBPs -1 and -3, providing the first biological function for these protein domains. Fibrillins -1 and -2, as well as LTBP-2, were found to be incapable of covalent or non-covalent association with LAP, while LTBP-4 could associate very weakly with TGF-β1LAP isoform. Molecular modeling of multiple 8-Cys repeats of LTBPs revealed increased hydrophobic surfaces in all of the TGF-β binding type 8-Cys repeats as well as to some extent a more relaxed structure. These results suggest that hydrophobic interaction(s) may well be involved in the association of LTBPs with TGF-βLAP, enabling the formation of a large latent TGF-β complex and its efficient secretion.

In addition, a new member of the LTBP-fibrillin family was cloned and named as LTBP-4. The human LTBP-4 gene is located in the chromosomal position 19q13.1-13.2. The LTBP-4 protein has a domain structure similar to the other known LTBPs. LTBP-4 was found to be efficiently incorporated into the ECM and is susceptible to specific proteolytic release from the ECM.

Cloning of the genomic region covering parts of the LTBP-1 gene revealed that the two LTBP-1 isoforms, LTBP-1S and LTBP-1L, have their own independent promoters, which are regulated in a cell-type specific manner. Independent promoters may provide a more specific regulation of expression in response to different stimuli during e.g. development or tissue repair. The generation of the two LTBP-1 transcripts was found to employ a rare intra-exonic splice acceptor site, in which the same genomic sequence can be used as an exon for transcription of LTBP-1S, while a part of it will be spliced out as an intron, when the LTBP-1L is transcribed from its own promoter

The current work emphasizes the controlled localization as well as the rapid and targeted responses acquired by the activation of latent growth factors deposited to ECM structures. The results aid our understanding of the biological functions of LTBP proteins in ECM accumulation and activation of latent TGF-βs.

5 INTRODUCTION

5.1 Transforming growth factor-β superfamily

The transforming growth factor-β superfamily consists of more than 30 different genes (reviewed by Kingsley, 1994, Roberts and Sporn, 1996, Massague, 1998). This growth factor superfamily has distinctive subfamilies, like the TGF-βs, activins/inhibins, bone morphogenetic proteins (BMPs) as well as growth and differentiation factors (GDFs). However, the boundaries between these subfamilies have at least partly vanished due to the cloning of new family members. The members of TGF-β superfamily have very diverse and profound effects at various stages of development as well as in regulating tissue function and integrity during adult life (**Table 1**).

Table 1. Transforming growth factor-β superfamily and their representative activities (modified from Massague, 1998)

Name [Homologue]	%	Activities and references
BMP2 subfamily		
BMP2 [Dpp^{D}]	100	Gastrulation, neurogenesis, chondrogenesis, interdigital apoptosis; in frog: mesoderm patterning; in fly: dorsalization, eyes, wings (Harland, 1994, Hogan, 1996, Mehler *et al.*, 1997).
BMP4	92	
BMP5 subfamily		
BMP5 [60 A^{D}]	61	Along with BMPs 2 and 4, this subfamily participates in the development of nearly all organs; many roles in neurogenesis (Hogan, 1996, Mehler *et al.*, 1997).
BMP6/Vgr1	61	
BMP7/OP1	60	
BMP8/OP2	55	
GDF5 subfamily		
GDF5/CDMP1	57	Chondrogenesis in developing limbs (Kingsley, 1994, Hogan, 1996).
GDF6/CDMP2	54	
GDF7	57	
Vg1 subfamily		
GDF1 [Vg1^{X}]	42	Vg1: axial mesoderm induction in frog and fish (Kingsley, 1994).
GDF3/Vgr2	53	
BMP3 subfamily		
BMP3/osteogenin	48	Osteogenic differentiation, endochondral bone formation, monocyte chemotaxis (Cunningham *et al.*, 1992).
GDF10	46	
Intermediate members		
Nodal [Xnr 1 to 3^{X}]	42	Axial mesoderm induction, left-right asymmetry (Beddington, 1996, Hogan, 1996).
Dorsalin	40	Regulation of cell differentiation within the neural tube (Basler *et al.*, 1993).
GDF8	41	Inhibition of skeletal muscle growth (McPherron *et al.*, 1997).
GDF9	34	

Activin subfamily		
Activin βA	42	Pituitary follicle-stimulating hormone (FSH) production, erythroid cell differentiation; in frog, mesoderm induction (Vale *et al.*, 1990, Harland, 1994, Gaddy-Kurten *et al.*, 1995).
Activin βB	42	
Activin βC	37	
Activin βE	40	
TGF-β subfamily		
TGF-β1	35	Cell cycle arrest in epithelial and hematopoietic cells, control of mesenchymal cell proliferation and differentiation, wound healing, ECM production, immunosuppression (Massague, 1990, Roberts and Sporn, 1990, Roberts and Sporn, 1993, Alexandrow and Moses, 1995).
TGF-β2	34	
TGF-β3	36	
Distant members		
MIS/AMH	27	Müllerian duct regression (Cate *et al.*, 1990, Josso *et al.*, 1993).
Inhibin	22	Inhibition of FSH production and other actions of activin (Gaddy-Kurten *et al.*, 1995, McPherron *et al.*, 1997).
GDNF	23	Dopaminergic neuron survival, kidney development (Massague, 1996a).

The sequence identities for the mature growth factor regions are shown in percentages, using BMP-2 sequence as the reference. All members listed have been identified in human and/or mouse. Important homologues from Drosophila (D) and Xenopus (X) are listed in brackets. CDF, growth and differentiation factor. CDMP, cartilage-derived morphogenetic protein. MIS/AMH, Müllerian inhibiting substance/anti-Müllerian hormone. GDNF, glial cell–derived neurotrophic factor.

The different isoforms of TGF-β have been cloned from various sources, including mammalian TGF-βs -1, -2 and -3 (Derynck *et al.*, 1985, de Martin *et al.*, 1987, Derynck *et al.*, 1988, Hanks *et al.*, 1988, ten Dijke *et al.*, 1988), chicken TGF-β4 (Jakowlew *et al.*, 1988) and *Xenopus* TGF-β5 (Kondaiah *et al.*, 1990). TGF-β is a dimeric growth factor, and while homodimers are most prevalent, also biologically active heterodimers, like TGF-β 1.2 and TGF-β 2.3 have been identified (Cheifetz *et al.*, 1987, Ogawa *et al.*, 1992). Other heterodimeric growth factors of the TGF-β superfamily have also been characterized. These include activins βA and βB homo- and heterodimers as well as inhibins, which are heterodimers of inhibin α-chains and activin β-chains and function as antagonists of activins (reviewed by Mathews, 1994) as well as BMP 4/7 and 2/7 heterodimers (Israel *et al.*, 1996, Nishimatsu and Thomsen, 1998).

5.2 Biological effects of TGF-β

TGF-βs are multipotent growth modulators. The effects of TGF-βs are usually categorized by the enhancement of ECM production and suppression of ECM proteolysis, growth inhibitory actions for epithelial and endothelial cells and strong immunosuppressive effects (**Fig. 1**; reviewed by Massague, 1990, Laiho and Keski-Oja, 1992, Kingsley, 1994, Lawrence, 1996, Moses and Serra, 1996). The major sources of TGF-β are the platelets, bone and serum.

An essential piece of information about the biological effects of different TGF-β isoforms has been obtained from mice lacking the corresponding functional gene. The immunosuppressive effects of TGF-β1 are well exemplified in TGF-β1 deficient mice (Shull *et al.*, 1992, Kulkarni *et al.*, 1993, Christ *et al.*, 1994, Diebold *et al.*, 1995, Letterio and Roberts, 1996). About half of the mice die before birth because of defects in vasculogenesis and hematopoiesis (Dickson *et al.*, 1995). The born pups die shortly after weaning due to a massive infiltration of cells of the immune system to different tissues (Boivin *et al.*, 1995, Dickson *et al.*, 1995). It has been suggested that maternal supply of TGF-β1 would contribute to the development of the born mice (Letterio *et al.*, 1994). When TGF-β1 gene deficiency was introduced in SCID (severe combined immune deficiency) mice, they could live untill adulthood (Diebold *et al.*, 1995). TGF-β -2 and -3 deficient mice have different, but not overlapping developmental defects (Kaartinen *et al.*, 1995, Proetzel *et al.*, 1995, Sanford *et al.*, 1997). TGF-β2 deficiency causes perinatal lethality and a number of developmental malformations in various organs, including the heart, lung, eye and ear as well as in the urogenital system (Sanford *et al.*, 1997). Homozygous TGF-β3 deficient mice suffer from delayed pulmonary development and cleft palate and die soon after birth (Kaartinen *et al.*, 1995, Proetzel *et al.*, 1995). The different phenotypes of the various TGF-β isoform null mice might indicate low levels of isoformic redundancy *in vivo*. Also the expression patterns of different TGF-β isoforms as well as their promoter regions are unique, contributing to the phenotypic differences of the various TGF-β gene deficient mice (reviewed by Roberts and Sporn, 1990, Roberts *et al.*, 1991).

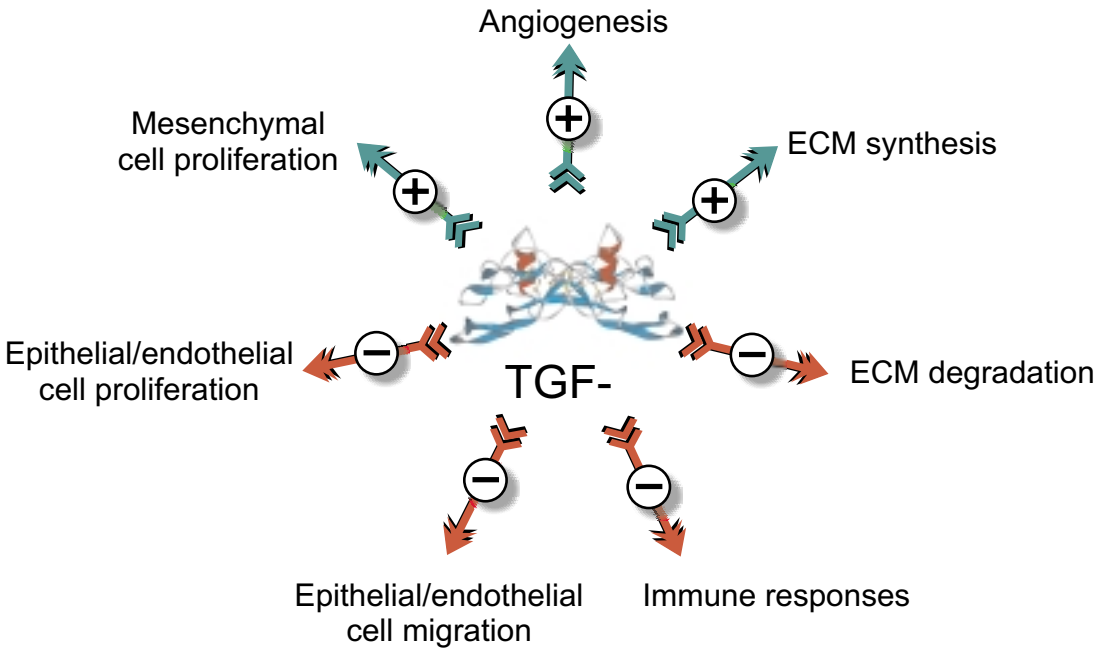

Figure 1. The main effects of TGF-β
The main biological effects of TGF-β are presented. TGF-β increases the events indicated by the + symbol and decreases the events indicated by the - symbol.

5.2.1 Effects of TGF-β on extracellular matrix and skeletal system

TGF-β has very strong effects on the regulation of ECM synthesis and degradation. TGF-β induces the expression of multiple ECM components, including collagen, fibronectin, tenascin, thrombospondin, vitronectin, elastin and proteoglycans. TGF-β decreases extracellular proteolysis by decreasing the expression of proteases and their activators like plasmin, plasminogen activators and metalloproteinases and by increasing expression of protease inhibitors, like plasminogen-activator inhibitor (PAI-1) and type 1 tissue inhibitor of metalloproteinase (TIMP-1). TGF-β also regulates the expression of certain cell surface receptors for ECM proteins (integrins) (reviewed by Laiho and Keski-Oja, 1992, Noble *et al.*, 1992, Roberts and Sporn, 1996, Taipale *et al.*, 1998).

TGF-β has important functions in wound healing. Platelets are a very rich source of TGF-β and release a part of their TGF-β load, complexed with the latent TGF-β binding protein -1 (LTBP-1), into the wound from their α-granules upon activation (Assoian and Sporn, 1986, Grainger *et al.*, 1995; see below section *5.4 Large latent TGF-β complex*). The rest of the TGF-β from platelets remains in the clot and can be released by peptides containing the RGD sequence motif, providing a long-lasting TGF-β reservoir during wound healing (Grainger *et al.*, 1995). TGF-β is chemotactic for e.g. fibroblasts, macrophages and leukocytes, and capable of enhancing platelet aggregation (Roberts *et al.*, 1986, Postlethwaite *et al.*, 1987, Wahl *et al.*, 1987, Hoying *et al.*, 1999) and recruiting them to the injured area. By increasing the production of several ECM components and by inducing angiogenesis, the net effect of TGF-β function in wound healing is increased maturation and strength of the wounds (reviewed by Roberts and Sporn, 1996, O'Kane and Ferguson, 1997). The importance of an intact TGF-β signaling pathway for generation and maintenance of blood vessels is demonstrated by the TGF-β1 as well as TGF-β type II receptor null mice (Dickson *et al.*, 1995, Oshima *et al.*, 1996). These mice suffer from weak blood vessels and inadequate capillary vessel formation. The negative effect of the enhanced wound healing by TGF-β is increased scarring. This has been observed for exogenously added TGF-β1 in adult rodent wounds, while anti-TGF-β1 antibodies reduce scarring (Shah *et al.*, 1992, Shah *et al.*, 1994). TGF-β1 is the prevalent isoform in wound fluids of adults, whereas in the wounds of fetuses, TGF-β2 predominates (Longaker *et al.*, 1990, Whitby and Ferguson, 1991a, Whitby and Ferguson, 1991b). The latter is a phenomenon, which may contribute to the lack of scarring of fetal wounds (reviewed by Mast *et al.*, 1992, Adzick and Lorenz, 1994).

TGF-β is also centrally involved in bone formation. Because of its large mass, the bone tissue is the richest source of TGF-β1 (reviewed by Bonewald and Dallas, 1994, Bonewald, 1996). Osteoblasts are stimulated by TGF-β (Centrella *et al.*, 1987, Pfeilschifter and Mundy, 1987, Pfeilschifter *et al.*, 1987), which is in agreement with TGF-β induced bone formation. In addition, TGF-β1 gene deficient mice suffer from a decreased bone mass and elasticity (Geiser *et al.*, 1998). During bone degradation, latent TGF-β, stored in bone matrix, is activated and inhibits bone-degrading osteoclasts.

5.2.2 TGF-β in regulation of cell proliferation

Despite its name, TGF-β is a potent suppressor of growth for many cell types. TGF-β inhibits epithelial, endothelial and hematopoietic cell proliferation in a reversible manner (Tucker *et al.*, 1984, Heimark *et al.*, 1986 , Shipley *et al.*, 1986, Müller *et al.*, 1987, Silberstein and Daniel, 1987, Takehara *et al.*, 1987, Sato and Rifkin, 1989). The inhibition is caused by arresting the cell cycle at the G_1-phase (reviewed by Ravitz and Wenner, 1997). However, the effects of TGF-β on the proliferation of cells of mesenchymal origin are quite opposite. TGF-β can stimulate the growth of e.g. fibroblasts under certain culture conditions (Shipley *et al.*, 1985, Soma and Grotendorst, 1989). Mesenchymal cell proliferation caused by TGF-β is believed to be indirect. Namely, TGF-β increases its own expression as well as that of several other growth factors like platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which are considered to be responsible for TGF-β induced cell proliferation and angiogenesis in an auto/paracrine fashion (Leof *et al.*, 1986, Battegay *et al.*, 1990, Bronzert *et al.*, 1990, Pertovaara *et al.*, 1993, Pertovaara *et al.*, 1994, Kay *et al.*, 1998).

5.2.3 TGF-β as an immunosuppressive agent

TGF-β is a very potent immunomodulatory factor (reviewed by McCartney-Francis and Wahl, 1994, Letterio and Roberts, 1997, Letterio and Roberts, 1998, de Visser and Kast, 1999). The immunomodulatory functions of TGF-β are quite diverse. TGF-β can induce both differentiation and growth of precursors of many hematopoietic cells. TGF-β is also chemotactic for e.g. macrophages. At the same time, TGF-β exhibits strong immunosuppressive effects, as clearly demonstrated by the TGF-β1 null mice. These mice die after weaning due to a multifocal infiltration of inflammatory cells, especially to the heart, lungs and salivary glands (Shull *et al.*, 1992, Kulkarni *et al.*, 1993, Christ *et al.*, 1994).

TGF-β is expressed by many leukocyte lineages (Assoian *et al.*, 1987). E.g. activation of resting T lymphocytes results in the upregulation of TGF-β and its signaling receptors (Kehrl *et al.*, 1986b). In general, TGF-β is considered to be growth inhibitory as well as an apoptotic factor for both T and B lymphocytes (Kehrl *et al.*, 1986b, Kehrl *et al.*, 1986a, Ranges *et al.*, 1987, Kehrl *et al.*, 1989, Kehrl *et al.*, 1991, Holder *et al.*, 1992, Lomo *et al.*, 1995). TGF-β also suppresses natural killer (NK) cells (Rook *et al.*, 1986). However, TGF-β promotes the growth of naive T cells (Cerwenka *et al.*, 1994). In addition, TGF-β decreases the expression of MHC class II proteins (Czarniecki *et al.*, 1988), an effect that may attenuate host responses against tumors. TGF-β has a strong influence on inflammation as well as on fibrosis caused by chronic inflammation. Monocytes/macrophages, centrally involved in these processes, are regulated by TGF-β (reviewed by Wahl, 1992, Bogdan and Nathan, 1993, Border and Noble, 1994). Macrophages are also capable of activating TGF-β (see below, *Activation of latent TGF-β).*

5.3 Synthesis and processing of TGF-β

The TGF-β cDNA codes for both an N-terminal pro-domain, LAP (TGF-β latency associated protein; Lawrence *et al.*, 1984, Gentry *et al.*, 1988) and the C-terminal mature TGF-β. TGF-β is cleaved from its LAP propeptide by a furin-like endoproteinase at an RR[KA][RKL] sequence during the secretion of the TGF-β-LAP complex (Dubois *et al.*, 1995). LAP is required for the correct folding, disulphide bond-mediated dimerization and secretion of TGF-β (Gray and Mason, 1990). The secreted TGF-β is dimerized via a single disulphide bridge between the monomers, while dimerization of the LAP part involves two disulphide bridges (Gentry *et al.*, 1988). The LAP part is substantially larger than the TGF-β part. β1LAP is 279 amino acids long and has a calculated molecular mass of about 33 kDa, while TGF-β1 is 112 amino acids long, and has a calculated molecular mass of about 14 kDa. Recombinant LAP, is glycosylated, whereas TGF-β is not (Brunner *et al.*, 1988, Purchio *et al.*, 1988). The correct glycosylation of LAP is required for the secretion of the protein complex (Sha *et al.*, 1989).

The LAP propeptide dimer remains associated with the TGF-β dimer by non-covalent interactions after secretion (Gentry *et al.*, 1988, Gentry and Nash, 1990). The interaction of TGF-β with LAP renders TGF-β biologically latent, i.e. unable to bind to its signaling receptors on a cell surface. The latent TGF-β complex consisting of LAP and TGF-β is referred to as small latent TGF-β. The association between active TGF-β and its LAP propeptide is reversible (Gentry and Nash, 1990, McMahon *et al.*, 1996, Yang *et al.*, 1997). β1LAP is able to inactivate the mammalian TGF-βs -1, -2 and -3 (Gentry and Nash, 1990, Miller *et al.*, 1992, Bottinger *et al.*, 1996) suggesting that the inactivation of TGF-β via LAP is not TGF-β isoform-specific. However, it is not known whether the non-isoform-specific "neutralization", or reversible binding of active TGF-β to LAP is physiologically relevant. The 3-dimensional structures of TGF-βs -1, -2 and -3 have been determined, and they all involve four internal disulphide bridges as well as a disulphide link between the monomers (Daopin *et al.*, 1992, Schlunegger and Grutter, 1992, Archer *et al.*, 1993, Daopin *et al.*, 1993, Schlunegger and Grutter, 1993, Mittl *et al.*, 1996). TGF-βs are composed mainly of β-strands. An alpha helix and a beta sheet interact between the monomers, forming a hydrophobic core. In addition to the single disulphide bridge, the dimer is stabilized via multiple hydrogen bonds. The three-dimensional structure of LAP is not known, but it is predicted to be also rich in β-strands (McMahon *et al.*, 1996). The association of TGF-β with LAP results in extensive structural changes in LAP (McMahon *et al.*, 1996).

Similar regulation of the activity of the other members of the TGF-β superfamily by their propeptide parts is not known to exist. However, other proteins are known to be capable of inhibiting the activity of the activins and BMPs. Follistatin is a secreted protein that can bind to activin and BMPs and prevent their binding to cell surface signaling receptors (de Winter *et al.*, 1996, Iemura *et al.*, 1998). Similarly, noggin, chordin and members of the DAN family bind to BMPs and block their interaction with signaling receptors (Piccolo *et al.*, 1996, Zimmerman *et al.*, 1996, Hsu *et al.*, 1998, Piccolo *et al.*, 1999, Yokouchi *et al.*, 1999).

5.4 Large latent TGF-β complex

In platelets, which are a rich source of TGF-β, TGF-β was found to be in complex with a high molecular weight protein (Miyazono *et al.*, 1988, Wakefield *et al.*, 1988). This protein was cloned and named as latent TGF-β binding protein (LTBP or transforming growth factor type beta masking protein, later renamed as LTBP-1; Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990). After the characterization of LTBP-1, two other LTBP isoforms were cloned (Moren *et al.*, 1994, Gibson *et al.*, 1995, Yin *et al.*, 1995a). The interaction between TGF-β binding LTBP and TGF-β is covalent, involving disulphide bond(s) between LAP propeptide of TGF-β and LTBP. LTBPs are ECM components, which target the large latent complex rapidly after secretion to ECM structures, where TGF-β resides in a latent form (Taipale *et al.*, 1994, Dallas *et al.*, 1995, Nakajima *et al.*, 1997). In addition to LTBPs, also the cysteine-rich fibroblast growth factor receptor has been reported to function as a small latent TGF-β binding protein (Olofsson *et al.*, 1997).

LTBP-1 has been found to possess a central role in the processing and secretion of TGF-β1 (Miyazono *et al.*, 1991, Miyazono *et al.*, 1992), and the expression of LTBP-1 is in some cases found to be co-regulated with TGF-β1 (Miyazono *et al.*, 1991, Dallas *et al.*, 1994, Taipale *et al.*, 1994, Koli and Keski-Oja, 1995). The small latent TGF-β complex is secreted very slowly residing in the *cis*-aspect of the Golgi apparatus (Miyazono *et al.*, 1991, Miyazono *et al.*, 1992, Mizoi *et al.*, 1993). Furthermore, the secreted small latent TGF-β complex is not correctly folded (Miyazono *et al.*, 1991). LTBPs, in turn, are secreted rapidly, and the association of the small latent TGF-β with LTBP-1 is required for the correct folding and rapid secretion of TGF-β (Miyazono *et al.*, 1991, see also Taipale *et al.*, 1994). TGF-βs are secreted in the large latent complex in most studied cultured cell lines (Olofsson *et al.*, 1992, Dallas *et al.*, 1994, Taipale *et al.*, 1994, Taipale *et al.*, 1995). However, the major fraction of secreted LTBPs does not contain TGF-β (Miyazono *et al.*, 1991, Taipale *et al.*, 1994, Taipale *et al.*, 1995), and thus the association with TGF-β is optional for LTBPs. The only known cell types secreting both small and large latent TGF-β complexes are platelets, cells from malignant tumors as well as some osteoblast cell lines and primary osteoblasts (Bonewald *et al.*, 1991, Eklöv *et al.*, 1993, Mizoi *et al.*, 1993, Dallas *et al.*, 1994, Grainger *et al.*, 1995).

5.5 Activation of latent TGF-β

Activation of TGF-β involves the disruption or modification of the non-covalent interaction between LAP and TGF-β in a way that enables TGF-β to bind to its signaling receptors. Since TGF-β signaling receptors are ubiquitously expressed (reviewed by Massague, 1996b), the activation of TGF-β is a key step in the regulation of its biological effects. Cultured cells do not normally secrete active TGF-β or activate significant proportions of latent TGF-β. Only a few primary cells and established cell lines have been found to contain activated TGF-β in their conditioned culture medium. Cultured BSC-1 African green monkey kidney cells (Holley *et al.*, 1985, Hanks *et al.*, 1988, McPherson *et al.*, 1989) as well as certain

human glioblastoma cell lines (de Martin *et al.*, 1987, Olofsson *et al.*, 1992) have been found to secrete active TGF-β. In addition, certain tumor cells, like the human gastric cancer cell line Kato III, have been reported to have the capacity to activate secreted TGF-β1 (Ura *et al.*, 1991, Takiuchi *et al.*, 1992, Mahara *et al.*, 1994, Horimoto *et al.*, 1995). Multiple pathways resulting in the activation of TGF-βs have been described (**Table 2**).

Table 2. Mechanisms involved in activation of latent TGF-β (modified from Saharinen *et al.*, 1999)

Activation method	Reference
Physicochemical	
Acidic cellular microenvironment	Jullien *et al.*, 1989
Extremes of pH	Brown *et al.*, 1990
Gamma-irradiation	Barcellos-Hoff, 1993
Reactive oxygen species	Barcellos-Hoff and Dix, 1996
Enzymatic	
Proteases	
· plasmin, cathepsin G	Lyons *et al.*, 1988, Lyons *et al.*, 1990,Sato and Rifkin, 1989
· calpain	Abe *et al.*, 1998
· MMP-2, MMP-9	Yu and Stamenkovic, 2000
· Kato III cells (unidentified protease)	Horimoto *et al.*, 1995
Glycosidases	Miyazono and Heldin, 1989
Nonspecified protein interactions	
Thrombospondin-mediated	Schultz-Cherry and Murphy-Ullrich, 1993, Schultz-Cherry *et al.*, 1994a, Schultz-Cherry *et al.*, 1994b
Integrin a_vb_6-mediated	Munger *et al.*, 1999
Drug-induced (biochemical mechanism unknown)	
Antiestrogens	Knabbe *et al.*, 1987, Colletta *et al.*, 1990
Retinoids	Glick *et al.*, 1989, Kojima and Rifkin, 1993
Vitamin D3	Koli and Keski-Oja, 1993
Glucocorticoids	Oursler *et al.*, 1993, Boulanger *et al.*, 1995

5.5.1 Proteolytic activation of TGF-β

Proteolysis is the most studied activation mechanism of TGF-β. Proteolysis has been shown to target degradation of LAP propeptide *in vitro*, resulting in the liberation of active TGF-β (Lyons *et al.*, 1988). Protease inhibitors can abrogate activation of TGF-β in several cell culture models (Antonelli-Orlidge *et al.*, 1989, Sato and Rifkin, 1989, Sato *et al.*, 1990, Huber *et al.*, 1992, Chu and Kawinski, 1998). Proteolysis can also lead to conditions, where the effects of TGF-β are suppressed. For example, shedding of the TGF-β type III receptor, betaglycan, from the cell surface (LaMarre *et al.*, 1994, Lopez-Casillas *et al.*, 1994) results in the sequestration of TGF-β from its signaling receptors (see below section *5.6 TGF- signal transduction*).

Plasmin mediated TGF-β activation is the best-characterized proteolytic TGF-β activation model (Lyons *et al.*, 1988, Lyons *et al.*, 1990, Grainger *et al.*, 1995). It has been shown to require several other factors including urokinase plasminogen activator (uPA) and its receptor (uPAR), carbohydrates on LAP as well as mannose 6-phosphate / insulin-like growth factor II receptor (Kovacina *et al.*, 1989, Dennis and Rifkin, 1991b, Kojima and Rifkin, 1993, Sato *et al.*, 1993, Odekon *et al.*, 1994). The biological model for plasmin-mediated TGF-β activation is co-cultivation of endothelial and smooth muscle cells, requiring close proximity of the two cell types (Antonelli-Orlidge *et al.*, 1989, Sato and Rifkin, 1989, Sato *et al.*, 1990).

The ECM bound large latent TGF-β complex is susceptible to proteolysis that releases the complex still in a latent form. TGF-β activation in the co-cultivation assay can be prevented by exogenous LTBP-1, free of TGF-β, and by anti-LTBP-1 antibodies against either its N- or C-terminal regions (Flaumenhaft *et al.*, 1993, Kojima and Rifkin, 1993, Nunes *et al.*, 1997). Transglutaminase has been found to be essential for covalent binding of the large latent complexes to ECM (Nunes *et al.*, 1997, Verderio *et al.*, 1999). In the co-cultivation or retinoid stimulation mediated TGF-β activation models the blocking of transglutaminase activation results in inhibition of TGF-β activation (Kojima *et al.*, 1993, Kojima *et al.*, 1995, Nunes *et al.*, 1995, Nunes *et al.*, 1997). Thus, at least in the co-cultivation model, proper ECM deposition of the large latent complex has a distinctive role in TGF-β activation. This suggests that TGF-β activation, at least in the proteolytic activation models, is preceded by the release of large latent complex from the ECM. The activation itself would then take place at or near the cell surface by another step, which could involve also proteolysis or other activation mechanisms (presented schematically in **Fig. 2**). In addition to plasmin, also other proteases have been identified capable of releasing large latent complexes from the ECM. However, these proteases have not been found to activate TGF-β (Taipale *et al.*, 1995).

The proposed plasmin dependent TGF-β activation model is self-regulatory; TGF-β increases the expression of plasminogen activator inhibitor-1 (PAI-1), which inhibits the activation of plasminogen by plasminogen activators and subsequent TGF-β activation (Laiho *et al.*, 1986). However, the results from transgenic mice suggest alternative pathways for the activation of TGF-β. Mice defective either in plasminogen, receptor for urokinase-type plasminogen activator (uPAR, CD87), or both urokinase and tissue type plasminogen activators (uPA and tPA) do not have phenotypic overlap with TGF-β1 knockout mice, indicating that these factors are not indispensable for TGF-β1 activation (Shull *et al.*, 1992, Carmeliet *et al.*, 1994, Bugge *et al.*, 1995).

In addition to plasmin, also other proteases have been found to be involved in TGF-β activation. The gastric cancer cell line Kato III cells have been reported to activate secreted TGF-β1 from the conditioned medium (Horimoto *et al.*, 1995), by a serine protease other than plasmin. Abe and co-workers (Abe *et al.*, 1998) have reported that the calpain protease is capable of activating TGF-β. Also subtilisin-like endoproteases (Chu and Kawinski, 1998), thrombin (Benezra *et al.*, 1993) as well as matrix metalloproteinases MMP-2 and MMP-9 are

able to activate TGF-β (Yu and Stamenkovic, 2000). The MMP-mediated proteolytic activation model was been found to be TGF-β isoform specific. TGF-β3 was most readily activated with MMP-9, whereas the effect on latent TGF-β1 was negligible.

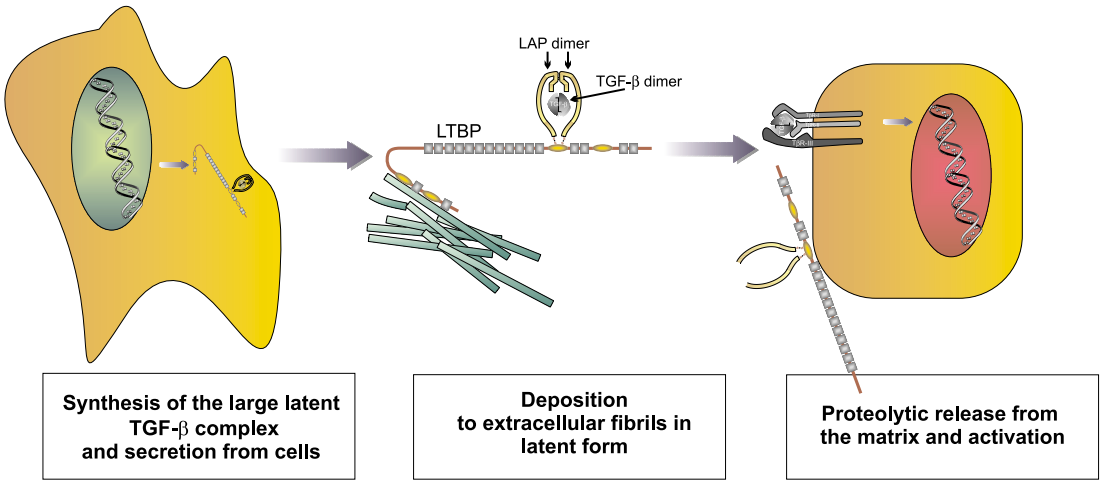

Figure 2. Proposed life course of the large latent TGF-β complex
TGF-β is secreted from the cells in a covalent complex with the TGF-β binding LTBPs. This complex is rapidly accumulated to ECM via the LTBPs, thus depositing TGF-β in a latent, ECM-bound form. The activation cascade of TGF-β can first involve a release of the large latent complex from the ECM by proteolytic cleavage(s) in the LTBP. The final activation of TGF-β requires conformational changes in the association of TGF-β with LAP, enabling TGF-β to bind to its signaling receptors. This can be a proteolytic cleavage of the LAP part or a change of the conformation of LAP via interaction with other proteins. The activated TGF-β can then induce signaling by binding to the cell surface TGF-β receptor system. The TGF-β signaling can also be prevented by sequestering of activated TGF-β with proteins not involved in TGF-β signaling. For detailed structure of the large latent complex, see **Fig. 11**.

5.5.2 Thrombospondin-1 as a latent TGF-β activating protein

Thrombospondins (TSPs) are large, trimeric proteins that are produced by many cell types (**Fig. 3**). The apparent molecular weight of the TSP monomer is about 160 kDa. TSPs are involved in cell adhesion and angiogenesis (reviewed by Mosher, 1990, Adams, 1997, DiPietro, 1997). The α-granules of platelets are a rich source of TSPs as well as large latent TGF-β1, from which they are released

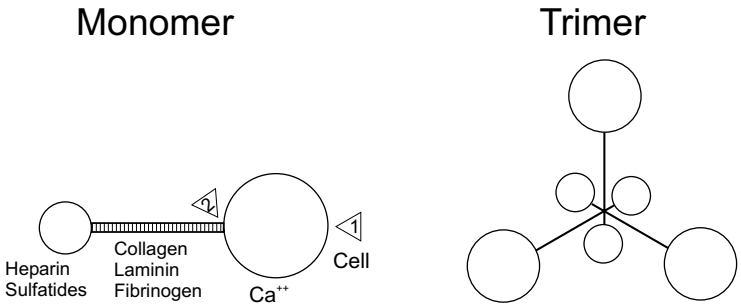

Fig 3. Structure of thrombospondin
TSP is a trimeric molecule, linked together by interchain disulphide bridges near the N-termini. The globular N-terminal domain of TSP binds heparin. The center part of TSP is composed of linear domain. The C-terminus contains a large globular domain, that binds Ca^{++}. TSP has three identified cell binding sites, two at the C-terminal domain and one in the N-terminal doman. Modified from Yamada, 1991.

during platelet activation. TSP-1 has been shown to activate both large latent TGF-β and recombinant small latent TGF-β (Schultz-Cherry and Murphy-Ullrich, 1993, Schultz-Cherry *et al.*, 1994b). On the contrary, TSP-2 is unable to activate TGF-β and appears to inhibit TSP-1 mediated TGF-β activation by competing for TGF-β binding (Melnick *et al.*, 2000). The TSP-1 mediated activation mechanism involves the N-terminal end of LAP (Schultz-Cherry *et al.*, 1994b, Yang *et al.*, 1997, Ribeiro *et al.*, 1999) and two specific sequences in the type I repeats of TSP-1 (Schultz-Cherry *et al.*, 1994a, Schultz-Cherry *et al.*, 1995). TGF-β activation by TSP-1 occurs most likely via a conformational change of LAP, which allows TGF-β to bind to its signaling receptors. Activation of TGF-β1 can be achieved also with small peptides containing the KRFK sequence, as in type I repeats of TSP-1 (Schultz-Cherry *et al.*, 1995), and this activation can be prevented by anti-LAP antibodies (Schultz-Cherry *et al.*, 1994b, Yang *et al.*, 1997). Recombinant LAP is shown to interact with purified TSP-1 (Yang *et al.*, 1997) and co-purify with TSP-1 (Ribeiro *et al.*, 1999). In addition, TSP-1 is found to be associated with TGF-β (Murphy-Ullrich *et al.*, 1992). However, the interaction between TSP-1 and TGF-β1TGF-β seems to be indirect, since in surface plasmon resonance experiments no direct interaction between the TSPs -1 or -2 and TGF-β1, β1LAP or TGF-β1LAP complex could be detected (Bailly *et al.*, 1997). TSP-1 has been localized to microfibrils between the basement membrane and connective tissue by electron microscopy (Arbeille *et al.*, 1991). A direct interaction between TSP-1 type I repeats and fibrillin-2, a member of LTBP-fibrillin family, has been reported (Aho and Uitto, 1998, reviewed by Dennis and Rifkin, 1991a, Fauvel-Lafeve, 1999; see below section *5.7 LTBP- fibrillin family*). However, whether TSPs directly associate with LTBPs remains to be studied. TSP-1 expression is stimulated by TGF-β, suggesting a positive feedback loop for TGF-β activation (Penttinen *et al.*, 1988). TSP associates with the cell surface via the CD36 protein (Asch *et al.*, 1987, Kieffer *et al.*, 1988, Imamura *et al.*, 1989, Silverstein *et al.*, 1989). Thus, one possible mechanism for the TSP-1 mediated TGF-β activation would be that the large latent TGF-β complex is first removed from the ECM by proteases. TGF-β in the soluble large latent complex would then be activated by the cell surface associated TSP-1.

Interestingly, TSP-1 deficient mice display many phenotypic alterations, similar to those seen in TGF-β1 deficient mice (Crawford *et al.*, 1998). The abnormalities in some tissues of the TSP-1 null mutant animals were even reverted by TSP-1 derived TGF-β activating peptides, further emphasizing the role for TSP-1 in TGF-β activation. However, no less TGF-β1 activation was observed in thrombin-treated platelets from TSP-1 null mice compared to the wild type animals, suggesting other activation methods for platelet derived TGF-β1 (Abdelouahed *et al.*, 2000).

5.5.3 Other mechanisms of TGF-β activation

The LAP parts of TGF-βs -1 and -3 contain RGD-motifs, which are recognized by integrins $\alpha_v\beta_1$ and $\alpha_v\beta_5$ (Munger *et al.*, 1998; see **Fig. 4**). In addition, integrin $\alpha_v\beta_6$ is also able to activate TGF-β1 (Munger *et al.*, 1999). This activation model is particularly interesting, because the $\alpha_v\beta_6$ integrin is expressed solely on epithelial cells, which are very sensitive to

TGF-β-mediated growth inhibition, and also because of the overlap of the phenotypes of TGF-β1 and integrin β_6 chain deficient mice. β_6 integrin deficient mice show increased inflammation and decreased fibrosis, processes which are strongly regulated by TGF-β (Huang *et al.*, 1996). It is not known, whether this integrin activation can directly utilize the ECM bound large latent complexes or, whether the complexes have to be first solubilized by proteolysis. TGF-β activation by the $\alpha_v\beta_6$ integrin could be a part of the proteolysis mediated TGF-β activation model observed in epithelial and smooth muscle cell co-cultivation. Thus, the final stages of the TGF-β activation, after release of large latent TGF-β complex from ECM by plasmin digest, would be carried out by the $\alpha_v\beta_6$ integrin at the surface of epithelial cells.

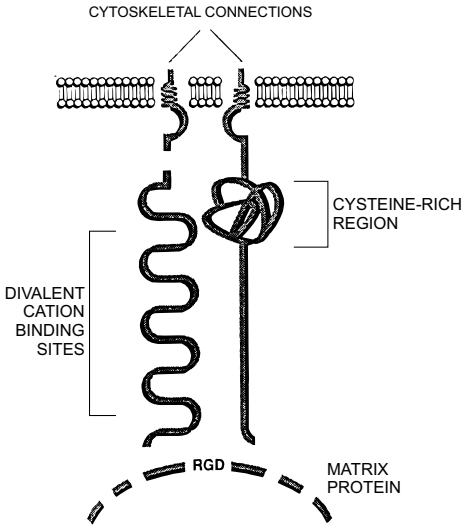

Figure 4. Structure of integrin
Integrins are composed of α- and β-chains, that are non-covalently linked together. Integrins have usually short cytoplasmic tails, which interact with the cytoskeleton. Integrins mediate cell-ECM as well as cell-cell interactions. Modified from Ruoslahti, 1991.

Glycosylation is predicted to be important for the non-covalent association between TGF-β and LAP. The removal of the glycosyl moieties from LAP by Endo F glycosidase can bring about the activation of TGF-β (Miyazono and Heldin, 1989). The authors speculated that sialidase, produced by activated macrophages, could activate TGF-β *in vivo*.

γ-radiation is causes a rapid TGF-β activation *in situ* (Barcellos-Hoff, 1993, Barcellos-Hoff *et al.*, 1994). Evidence for the role of TGF-β in irradiation-mediated induction of fibrosis and growth inhibition stems from the observation that the irradiation induced effects were attenuated by neutralizing antibodies against TGF-β (Ehrhart *et al.*, 1997, Burger *et al.*, 1998). Using recombinant latent TGF-β it was found that TGF-β activation is efficiently induced both by ionizing radiation and metal ion catalyzed ascorbate oxidation, both of which are systems producing reactive oxygen species (Barcellos-Hoff and Dix, 1996). The oxidation of LAP was suggested to lead a change in its conformation and thus TGF-β activation.

5.5.4 Binding of activated TGF-β to proteins not involved in signal transduction

After activation, TGF-β can bind either to its specific cell surface signaling receptors or to other proteins that can abrogate TGF-β signaling. Active TGF-β has a very short half-life in plasma (Coffey *et al.*, 1987), whereas the half-life of the LAP bound latent TGF-β is significantly longer (Wakefield *et al.*, 1990). The plasma protease inhibitor α_2-macroglobulin (α_2M) exists at high concentrations in plasma, and binds active TGF-β (O'Connor-McCourt and Wakefield, 1987, Huang *et al.*, 1988, Philip and O'Connor-McCourt, 1991). TGF-β binding to α_2M is enhanced by treatment of α_2M with proteases, involved also in TGF-β

activation (LaMarre *et al.*, 1991c). Thus, α_2M may have a TGF-β clearance function. α_2M complex can be endocytosed by its receptor (LaMarre *et al.*, 1991a, Moestrup, 1994). However, this requires a conformational change in α_2M (Gonias and Pizzo, 1983, Sottrup-Jensen, 1989), which is not induced by the bound TGF-β. In addition, the half-life of TGF-β in plasma is not affected by inhibition of the α_2M receptor (Philip and O'Connor-McCourt, 1991). Therefore, also a TGF-β carrier function for α_2M has been presented (Philip and O'Connor-McCourt, 1991, Borth, 1992). In addition to TGF-β, α_2M binds also other cytokines and growth factors, like NGF (nerve growth factor), CNTF (ciliary neurotrophic factor), NT-3 and -4 (neurotrophins), TNF-α (tumor necrosis factor-α), PDGF-BB, BDNF (brain-derived neurotrophic factor), IFN-γ (interferon-γ) and interleukins IL-1β, IL-2, IL-4, IL-6, IL-8 and IL-10 (LaMarre *et al.*, 1991a, James *et al.*, 1992, Liebl and Koo, 1993, Wolf and Gonias, 1994, Gonias *et al.*, 2000, Garber *et al.*, 2000; reviewed by LaMarre *et al.*, 1991b).

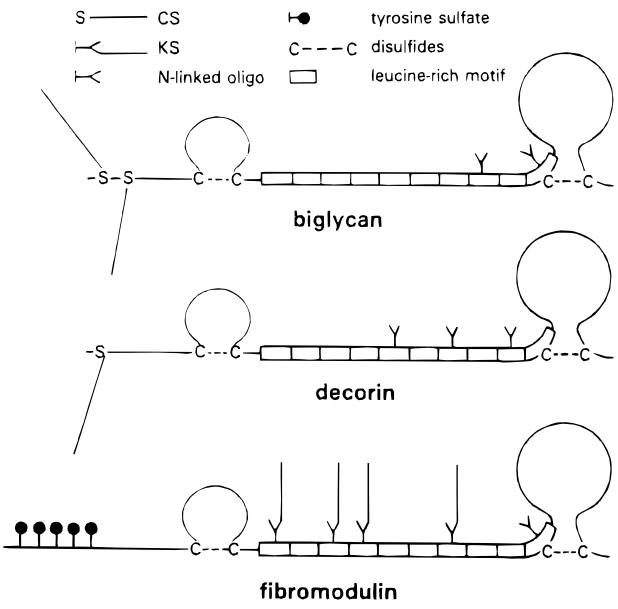

Figure 5. Biglycan, decorin and fibromodulin proteoglycans
These small proteoglycan contain protein backbone, about 45-60 kDa, to which the glycosaminoglycans are attached. These proteins conatin ten copies of leucine-rich repeats (about 25 amino acids long).
Key: CS = chondroitin sulphate; KS = keratan sulphate.
Modified from Hascall *et al.*, 1991.

TGF-β has also been found to associate with several ECM proteins, such as fibronectin (Fava and McClure, 1987), collagen IV (Paralkar *et al.*, 1991, Vukicevic *et al.*, 1992), fibromodulin, decorin and biglycan (Hildebrand *et al.*, 1994). Small proteoglycans decorin and biglycan have also the ability to neutralize (Yamaguchi *et al.*, 1990, Border *et al.*, 1992) or enhance (Takeuchi *et al.*, 1994) the activity of TGF-βs (**Fig. 5**).

5.6 TGF-β signal transduction

TGF-β signaling involves three different types of transmembrane receptors, two of which, namely TGF-β receptors I and II (TβR-I and TβR-II, respectively), have signaling capacities via their cytoplasmic serine/threonine kinase domains. The third receptor type, type III receptor (TβR-III), has only a short intracellular part with no known signaling motifs (**Fig. 6**). The TβR-III is speculated to function as an auxiliary transmembrane protein, associating with TGF-β and subsequently "serving" the active TGF-β to the actual signaling receptors (Cheifetz *et al.*, 1988). TGF-β signaling receptors are expressed in almost all cell types. Currently the best known downstream signaling molecules following the TGF-β receptors are

the SMAD proteins. TGF-β signal transduction has been reviewed by Massague, 1998, Christian and Nakayama, 1999, Piek *et al.*, 1999, Roberts, 1999, Zhang and Derynck, 1999, Massague and Wotton, 2000, ten Dijke *et al.*, 2000, Wrana, 2000.

5.6.1 TGF-β receptors

Two different transmembrane proteins, betaglycan and endoglin (CD105) can independently function as TβR-IIIs (Wang *et al.*, 1991, Lopez-Casillas *et al.*, 1991, Cheifetz *et al.*, 1992, Lopez-Casillas *et al.*, 1993). Betaglycan is more prevalent, while endoglin is expressed only on endothelial cells (St-Jacques *et al.*, 1994). TGF-β isoforms have varying affinities for the different TβR-IIIs, providing also a way for TβR-IIIs to modulate TGF-β signaling. TGF-β2 has a weaker affinity for TβR-II than other TGF-β isoforms. TβR-III betaglycan favors binding of TGF-βs -1 and -2, whereas TGF-β3 has a weaker affinity for betaglycan. The net overall effect is boosting of TGF-β2 signaling by betaglycan (Sankar *et al.*, 1995, Kaname and Ruoslahti, 1996). In certain cell types, which do not express betaglycan, TGF-β2 is less efficient in activating TGF-β signaling. Transfection of betaglycan to these cells enhances TGF-β2 activity. Endoglin can associate with TGF-βs -1 and -3, but not with TGF-β2 (Cheifetz *et al.*, 1992), further decreasing TGF-β2 signaling potency. In contrast to betaglycan, endoglin can also bind activin A, BMP-2 and BMP-7 in the presence of either type I or type II receptors (Barbara *et al.*, 1999). Mutations in endoglin can lead to the hereditary hemorrhagic telangiectasia type -1 disorder (HHT-1) (McAllister *et al.*, 1994).

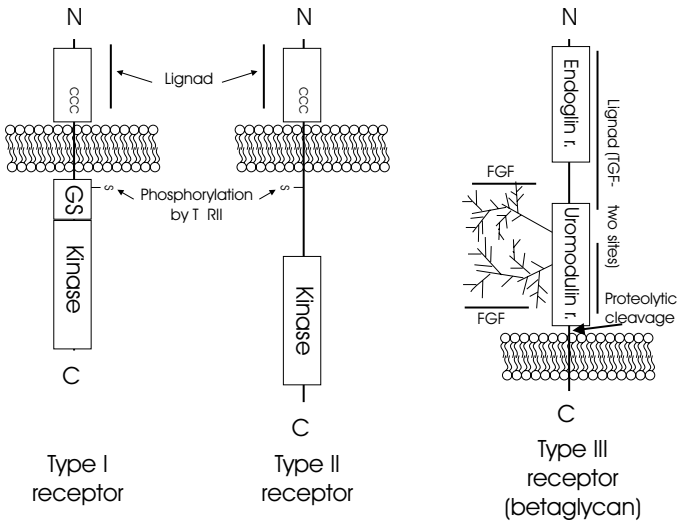

Figure 6. TGF-β receptors type I, II and III
The signaling type I and II recpetors have relatively short extracellular parts, with a charasteristic cysteine motif. The intracellular parts code for a serine/threonine kinase domain, which is preceeded in type I recpetor by a GS-domain. Constantly active type II receptor phosphorylates the GS-domain of type I receptor, leading to activation of type I receptor.
Type III receptor (here betaglycan) is a transmembrane protein, with two attached glycosaminoglycans. The type III receptor is suggested to serve the TGF-β to the actual type I and II signaling recpetors, boosting TGF-β signaling. Betaglycan has endoglin and uromodulin like domains, which both have a binding site for TGF-β. In addition, betaglycan also binds FGF. Betaglycan can be cleaved with plasmin, resulting in a soluble extracellular part, capable of binding TGF-β and sequestering it away from the signaling recpetors.

Betaglycan is a proteoglycan, which has attached heparin- and chondroitin-sulphate glycosaminoglycans (GAGs). These GAGs are required for binding of FGF, but not for TGF-β binding (Andres *et al.*, 1992). Betaglycan is also found as a soluble molecule (Andres *et al.*, 1989), where the TGF-β binding part has been proteolytically cleaved by plasmin (LaMarre *et al.*, 1994, Lopez-Casillas *et al.*, 1994). Soluble betaglycan is able to hinder TGF-β binding to its signaling receptors. A similar soluble form has been suggested also for endoglin (Li *et al.*, 1998).

The actual TGF-β signaling receptors TβR-I and TβR-II belong to their respective type I and II TGF-β receptor families, which include the receptors involved in the signaling of the different members of the TGF-β superfamily except the most diverse family member, GDNF. Currently twelve type I receptors, including orphan ALK7 receptor, and seven type II receptors are known.

Type I and II receptors are glycoproteins with molecular masses of about 55 and 70 kDa, respectively. Their extracellular parts are rather small, about 150 amino acids and include several cysteine residues, some of which form a characteristic motif near the transmembrane area. In type I receptors, a characteristic GS domain containing the sequence SGSGSG is located just before the serine/threonine kinase domain in the intracellular part.

In the absence of ligand, the type I and II receptors are found as monomers. TGF-β binds to TβR-II, which recruits TβR-I to the receptor complex (Attisano *et al.*, 1993, Franzen *et al.*, 1993, Chen *et al.*, 1995). TβR-I alone is unable to bind TGF-β in solution, but it can associate with the TβR-II – TGF-β complex (Wrana *et al.*, 1992, Wrana *et al.*, 1994). The sequential binding model applies in addition to TGF-βs, also to activins (see **Fig. 7**), whereas with BMPs, both receptor types I and II can bind it with weak affinity, but the actual signaling complex requires the presence of both receptor types. Type II receptor is a constitutively active kinase. Substrates for type II receptor are the receptor itself (autophosphorylation) and the GS motif of type I receptor. Phosphorylation of type I receptor by type II receptor activates type I receptor, which is assumed to be solely responsible for the subsequent downstream signaling events. The substrate specificity of type I receptor is determined by its kinase domain (Feng and Derynck, 1997, Persson *et al.*, 1998). Intracellular proteins FKBP-12 and BAMBI can inhibit activation of type II receptor by type I receptor (Wang *et al.*, 1996, Onichtchouk *et al.*, 1999). BAMBI (BMP and activin membrane-bound inhibitor) is a type I pseudoreceptor that lacks the kinase domain and prevents formation of functional receptor complex (Onichtchouk *et al.*, 1999). The immunophilin FKBP-12 binds to the GS motif of type I receptor, and by steric hindrance inhibits type II receptor mediated activation (Huse *et al.*, 1999). FKBP-12 has been suggested to function as a regulatory mechanism inhibiting signaling in the absence of the extracellular ligand (Chen *et al.*, 1997) and FKBP-12 has been shown to be dispensable for TGF-β signaling (Charng *et al.*, 1996, Bassing *et al.*, 1998, Shou *et al.*, 1998).

5.6.2 SMAD proteins as downstream signal transducers of TGF-β

The major downstream signaling proteins for the activated type I receptors are thought to be the SMADs (see **Fig. 7** and **Fig. 8**). SMADs can be divided into three different groups. The so-called receptor regulated SMADs (R-SMADs, namely SMADs -1, -2, -3, -5 and -8) are

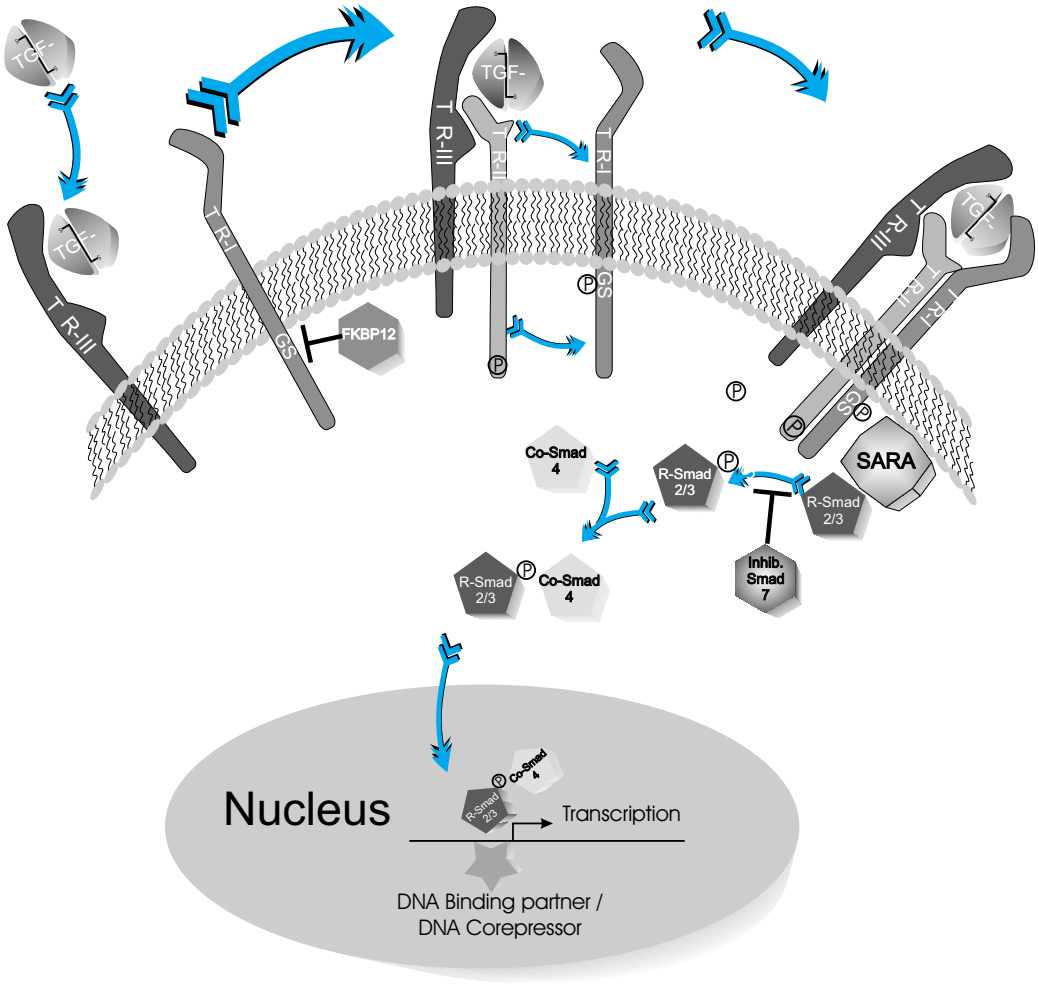

Figure 7. Initiation of the TGF-β signaling
TGF-β employs sequential signaling mechanisms, leading to activation of the SMAD proteins. Activated TGF-β can first associate with the type III receptor (TβR-III), which is thought to serve TGF-β to the actual signaling receptor TβR-II. The participation of TβR-III is optional and functions to boost TGF-β signaling. TβR-II is a constitutively active threonine/serine kinase receptor. Association of TβR-II with TGF-β recruits TβR-I to the receptor complex. TβR-I is phosphorylated at its GS domain by TβR-II, which dislocates the GS domain from the TβR-I kinase domain and hence activates TβR-I. TβR-I is thought to be solely responsible for the further downstream signaling events. Receptor-SMAD (R-SMADs 2 and 3) proteins are ligands for TβR-I. SARA (Smad anchor for activation), being a membrane protein, is assisting in the enrollment of R-SMADs to TβR-I. The ubiquitous immunophilin FKBP-12 blocks TβR-I basal activity by binding to GS domain. The ligand induced formation of the TβR-I - TβR-II receptor complex via is supposed to release FKBP12 from TβR-I. Once activated by phosphorylation, R-SMAD is dimerized with Co-SMAD (SMAD-4), and the heterodimeric SMAD complex is translocated to the nucleus, where it can function as a transcription factor. Inhibitory-SMAD-7 (Anti-SMAD-7) can dimerize with activated R-SMADs and block the signaling. Transcriptional activation by SMAD dimers is assisted by DNA binding partners and can be hindered by SMAD co-repressors, like TGIF, Ski and SnoN.

substrates for type I receptor. The recruitment of the R-SMADs to the receptor complex is enhanced by the SARA protein (Tsukazaki *et al.*, 1998). Receptor associated R-SMADs are phosphorylated, which allows them to heterodimerize with the Co-SMAD, SMAD-4. This complex is then translocated to the nucleus, where it functions as a transcription factor. SMADs -6 and -7 are inhibitory SMADs (anti-SMADs), which can block the SMAD signaling. SMAD-7 functions by occupying type I receptor and thus preventing activation of the R-SMADs. BMP signaling specific SMAD-6 can form heterodimers with activated R-SMADs, instead of SMAD-4, resulting in inactive SMAD-6 - R-SMAD heterodimers. The expression of anti-SMADs is rapidly enhanced after TGF-β stimulation, providing negative feedback for the TGF-β signaling cascades (Nakao *et al.*, 1997a, Afrakhte *et al.*, 1998, Takase *et al.*, 1998). SMADs -2, -3, -4 and -7 are involved in TGF-β signaling (Eppert *et al.*, 1996, Macias-Silva *et al.*, 1996, Zhang *et al.*, 1996, Nakao *et al.*, 1997b). Mutations of the SMAD proteins occur in many disorders (see below section *5.8.4 Role of TGF- in cancer*).

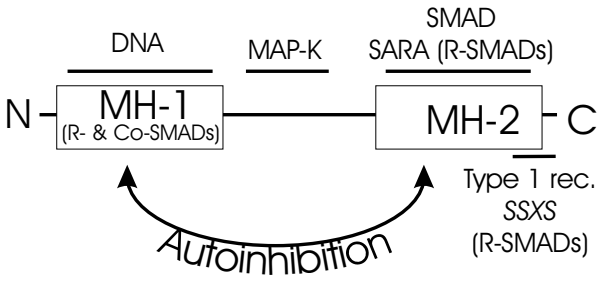

Figure 8. SMAD proteins
The SMAD proteins have two protein domains, N-terminal MH-1 (Mad homology 1) and C-terminal MH-2, connected with a linker region. The exception is the anti-SMADs 6 and 7, which lack most of the MH-1 domain. MH-1 domain has the DNA binding function, while MH-2 is required for the SMAD dimerization as well as for the interaction with SARA. In inactive SMADs, MH-1 and MH-2 are interacting, and phosphorylation of the SSXS motif at the end of MH-2 activates SMADs. The linker region is a substrate for many regulatory proteins, like the MAP-kinase.

5.7 LTBP-fibrillin family

The latent transforming growth factor-β binding protein (LTBP) – fibrillin family consists of LTBPs and fibrillins (**Fig. 9**). LTBPs and fibrillins are ECM proteins, often seen to assemble to long microfibrillar structures with a diameter of about 10 nm. Before the present study, three LTBPs had been cloned from human and other mammalian sources (Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990, Moren *et al.*, 1994, Gibson *et al.*, 1995, Yin *et al.*, 1995a, Fang *et al.*, 1997). LTBPs are large glycoproteins of about 120 to 220 kDa. Fibrillins are considerably larger glycoproteins than LTBPs with molecular masses of about 350 kDa. Fibrillins are found in diverse species, which include mammals, chicken and *Xenopus* (Sakai et al., 1986, Lee et al., 1991, Maslen et al., 1991, Zhang et al., 1994, Yin et al., 1995b, Kanwar et al., 1998, Masabanda et al., 1999, Yang et al., 1999, Zhou et al., 2000).

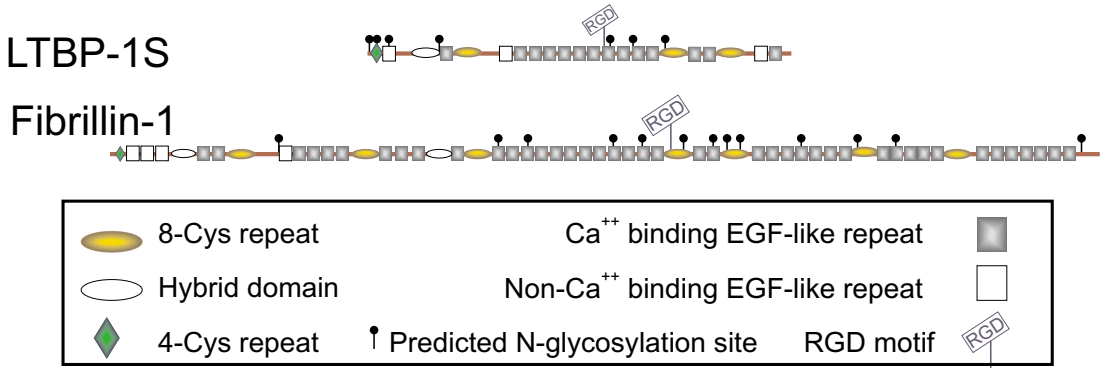

Figure 9. Domain structure of LTBPs and fibrillins
LTBPs and fibrillins are both composed mainly of multiple copies of EGF-like and 8-Cys repeats. Most of the EGF-like repeats are of calcium-binding type. Some of the 8-Cys repeats, namely the first ones in LTBPs, and the first and the fourth ones in fibrillins, are less conserved. These domains are often called also as hybrid domains. LTBPs have an apparent molecular mass between 120 - 170 kDa, whereas fibrillins are considerably larger, about 350 kDa in size. The N-terminal regions of LTBPs contain less repeated structures, and have a lower degree of similarity among the different LTBPs. A protease sensitive hinge region has been assigned to the non-repetitive area on the N-terminal side of the long, central cluster of repeated EGF-like domains in LTBPs.

5.7.1 LTBPs and fibrillins are mainly composed of EGF-like and 8-Cys repeat protein domains

LTBPs and fibrillins have a repetitive domain structure, consisting mainly of epidermal growth factor (EGF) like repeats and protein domains with a conserved pattern of eight cysteine residues, called eight cysteine repeats (8-Cys repeats/domains). LTBPs contain 15-20 EGF-like repeats and four 8-Cys repeats. The first 8-Cys repeat of LTBPs is often called also as the hybrid-domain, since it is divergent from the other 8-Cys repeats. Controversy has arisen about whether the hybrid domains should be considered as 8-Cys repeats or as distinct entities. In this review the hybrid domains are included in the 8-Cys repeats, and the numbering of 8-Cys repeats in LTBPs and fibrillins reflects this convention. Fibrillins have 47 EGF-like repeats and nine 8-Cys repeats, including two hybrid type 8-Cys repeats.

EGF-like repeats are conserved, approximately 45 amino acid long protein domains, with six cysteine residues, forming interdomain disulphide bridges in a 1-3, 2-4, 5-6 arrangement. In addition to LTBPs and fibrillins, EGF-like repeats are present in many extracellular and transmembrane proteins like fibulins -1 and -2, nidogen, protein S, uromodulin, thrombomodulin, low density

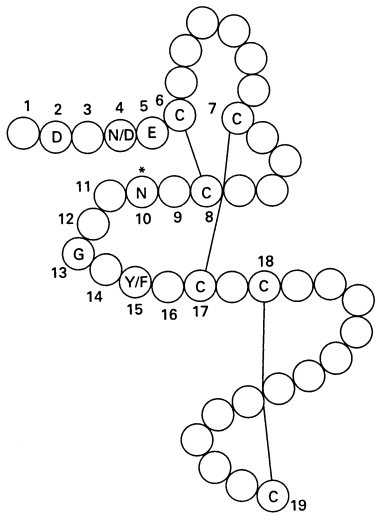

Figure 10. Calcium binding type EGF-like repeat
Sequence motif and disulphide bridging is shown. The conserved residues are numbered.

lipoprotein (LDL) receptor as well as *Drosophila*'s Notch, Delta and Serrate. EGF-like repeats function as structural domains and in mediating protein–protein interactions between e.g. Notch and Delta proteins (Rebay *et al.*, 1991) as well as between fibulin-1 and nidogen (Adam *et al.*, 1997).

EGF-like repeats are divided into two groups according to their ability to bind calcium. The majority of the EGF-like repeats in LTBPs and fibrillins are of calcium binding type (cbEGF repeats, **Fig. 10**), with the characteristic sequence motif [DN]-x-[DN]-[EQ]-C-x(6)-C-x(4)-C-x-[DN]-x(4)-[YF] at their N-termini. Calcium stabilizes the cbEGF repeats, and the cbEGF repeats are often found as tandem domains, in which the calcium binding is critical for providing the structural integrity (Werner *et al.*, 2000). The packing of EGF/cbEGF-cbEGF tandem repeats can either result in a very extended structure as in fibrillins and LTBPs (Downing *et al.*, 1996), or in more globular organization, like in factor IX and Notch / Delta / Serrate (Rao *et al.*, 1995). There are significant changes in the Ca^{++} binding affinities of the various cbEGF-like repeats (Smallridge *et al.*, 1999). Changes in the Ca^{++} saturation of cbEGF-like repeats may alter the protein flexibility. Numerous mutations in the cbEGF domains have been identified, causing disorders like hemophilia (factor IX mutations), familial hypercholesterolemia (LDL receptor mutations) and the Marfan syndrome (MFS; fibrillin-1 mutations. See below section *5.8.1 Connective tissue disorders related to LTBP-fibrillin family*).

8-Cys repeats have been found only in LTBPs and fibrillins. They are approximately 55 amino acids long protein domains with a characteristic cysteine pattern. 8-Cys repeats contain eight cysteine residues, except the hybrid domains, that contain usually seven cysteine residues. In the hybrid domains one of the three adjacent cysteine 3-5 residues is missing. The N-terminal halves of the hybrid domains are well conserved, while the C-terminal regions of the hybrid domains are divergent. The 8-Cys repeats are essential for the function of fibrillin microfibrils, as demonstrated by mutations in 8-Cys repeats causing Marfan syndrome (see below section *5.7.2 LTBP-fibrillin microfibrils and tissue distribution of LTBPs* and *5.8.1 Connective tissue disorders related to LTBP-fibrillin family*). However, before the current study, no specific functions were assigned for the 8-Cys repeats.

5.7.2 LTBP-fibrillin microfibrils

Fibrillins are known to be integral components of the so-called fibrillin microfibrils with a diameter of 8-12 nm. They have a characteristic "beads on a string" appearance, with a regular periodicity (Keene *et al.*, 1991, Sakai *et al.*, 1991). Fibrillin-containing microfibrils are found abundantly in all areas of the body, often in the vicinity of elastin, like in the aorta and ligaments. Fibrillin microfibrils are extensible structures, possibly strengthening the elastic tissue (Keene *et al.*, 1991, Lillie *et al.*, 1998). The role of fibrillin-1 in these microfibrils seems to be to maintain tissue integrity, as the mice with fibrillin-1 underexpression revealed that fibrillin-1 containing microfibrils are not critical for elastic fiber construction (Pereira *et al.*, 1997, see also Raghunath *et al.*, 1996). Instead fibrillin-2, which appears earlier in the development, is suggested to provide the scaffolding upon which the elastic fibers are assembled in early embryogenesis (Zhang *et al.*, 1995, Rongish *et al.*, 1998).

There is considerable evidence that also LTBPs are deposited to the fibrillin containing microfibrils. LTBP-1 from fibroblast cultures has been found to be deposited to both 50 nm thick fibronectin fibrils in the vicinity of the cell surface, and to 10 nm microfibrillar structures of cultured fibroblasts (Taipale *et al.*, 1996). These microfibrils were devoid of collagen VI, which also forms similar microfibrillar structures, suggesting that the observed microfibrils are fibrillin microfibrils. In cultured osteoblasts LTBP-1 has also been localized both to fibronectin fibers and fibrillin microfibrils (Dallas *et al.*, 2000). Deposition of LTBP-1 to fibronectin fibers took place earlier, while during prolonged culture, LTBP-1 no longer co-localized with fibronectin, but was instead detected in microfibrillar structures only. Also other reports confirm the association of LTBP-1 with collagen and fibronectin (Olofsson *et al.*, 1995, Verderio *et al.*, 1999). In the developing mouse heart, LTBP-1 has been found to co-localize with 40-100 nm fibers as well as in 5-10 nm microfibrils surrounding the endocardial cushion (Nakajima *et al.*, 1997, Nakajima *et al.*, 1999). Interestingly, LTBP-1 antibodies were able to prevent endothelial-mesenchymal transformation in that model. In both fibroblast cultures and developing mouse heart, TGF-β1 co-localized with LTBP-1 in fibrillar structures (Taipale *et al.*, 1996, Nakajima *et al.*, 1997). In addition, LTBP-1 has been localized to the fibrillin microfibrils in the skin (Raghunath *et al.*, 1998, see also Karonen *et al.*, 1997). Bovine LTBP-2 has been localized to developing elastin associated microfibrils in bovine aorta and nuchal ligament, with biochemical data supporting these microfibrils being the fibrillin microfibrils (Gibson *et al.*, 1995).

The role of LTBPs in the fibrillin microfibrils structures is unclear. It is not known, whether LTBPs are just "accessory" proteins "decorating" the microfibrils, and some of the LTBPs being able to deposit TGF-β to these structures, or whether LTBPs are more integral components, required for the assembly of the microfibrils. The very early lethality of the LTBP-2 null mice suggests a crucial role for LTBPs in the ECM structures (Shipley *et al.*, 2000).

Most of the EGF-like repeats in fibrillins and LTBPs are of the calcium binding type, and calcium has been found to be required for the stability and lateral packaging of these microfibrils (Kielty and Shuttleworth, 1993, Handford *et al.*, 1995, Wu *et al.*, 1995, Downing *et al.*, 1996, Reinhardt *et al.*, 1997a, Reinhardt *et al.*, 1997b, Wess *et al.*, 1998). The long stretches of EGF-like repeats are assumed to form the extended areas between the beads in the microfibrils (Cam *et al.*, 1997, Cardy and Handford, 1998). In addition, some of the asparagines in certain EGF-like repeats in the fibrillin-LTBP family proteins are hydroxylated (Glanville *et al.*, 1994).

The length of a single fibrillin molecule is about 148 nm (Sakai *et al.*, 1991). This is considerably longer than the observed about 55 nm periodicity of fibrillin molecules in microfibrils (Wess *et al.*, 1997). Based on the dimensions of a cbEGF dimer (14.5 x 2 nm), the microfibrils are apparently built up from 50% overlapping, parallel fibrillin molecules (Downing *et al.*, 1996). The use of Ca^{++}-chelation lead to a relaxed, distorted structure of the microfibrils, with a decreased length of the interbead areas and increased flexibility (Cardy and Handford, 1998). Observed mutations in the cbEGF domains of fibrillin-1, resulting in impaired Ca^{++} binding ability, can result in Marfan syndrome, possibly due to weakened

microfibril structure and hence increased susceptibility for proteolysis (Dietz *et al.*, 1993, Handford *et al.*, 1995, Reinhardt *et al.*, 1997b; see below section *5.8.1 Connective tissue disorder related to LTBP-fibrillin family*).

In addition to fibrillins and LTBPs, the fibrillin-microfibrils contain a number of other proteins, often localized to the "bead" regions (see **Table** 3). These other proteins can connect the microfibrils to other ECM structures and have cell-adhesive properties via their RGD-motifs.

Table 3. Components of fibrillin microfibrils and the location of their genes in the human genome (modified from Pyeritz, 2000, Robinson and Godfrey, 2000)

Protein	Map locus	Reference
Confirmed		
Fibrillin-1 (FBN1)	15q21.1	Sakai *et al.*, 1986
Fibrillin-2 (FBN2)	5q23.q31	Lee *et al.*, 1991, Zhang *et al.*, 1994
LTBP-1	2p12-q22	Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990
LTBP-2	14q24	Moren *et al.*, 1994, Gibson *et al.*, 1995
Possible		
Microfibril-associated protein-1 (MFAP-1)	15q15-q12	Horrigan *et al.*, 1992
LTBP-3	11q12	Yin *et al.*, 1995a, Saharinen *et al.*, manuscript 2000
LTBP-4	19q13.1-13.2	**III**, Giltay *et al.*, 1997
Fibulin-2	3p24.2-p25	Reinhardt *et al.*, 1996b
Laminin β2	3p21.2-21.3	Rupp and Maslen, 1996
Emilin	2p23.2-23.3	Bressan *et al.*, 1993
Versican	5q12-5q14	Zimmermann *et al.*, 1994
Chondroitin sulphate proteoglycans		Kielty *et al.*, 1996
Immunolocalized only		
Microfibril-associated protein-2 (MFAP-2, also known as microfibril-associated glycoprotein-1, MAGP-1)	1p36.1-p35	Gibson *et al.*, 1986
Microfibril-associated glycoprotein-2 (MAGP-2)	12p13.1-p12.3	Gibson *et al.*, 1996
Microfibril-associated protein-3 (MFAP-3)	5q32-q33.3	Abrams *et al.*, 1995
Microfibril-associated protein-4 (MFAP-4)	17p11.2	Kobayashi *et al.*, 1989
Lysyl oxidase (LOX) (found in elastic fibers but not in isolated microfibrils)	5q23.3-q31.2	Kagan *et al.*, 1986

5.7.3 Connective tissue disorders related to LTBP-fibrillin family

Several disorders of microfibrils have been described (Table 4). The best studied is the Marfan syndrome (MFS), a genetic disorder with an autosomal inheritance pattern (Lee *et al.*, 1991, Maslen *et al.*, 1991). MFS is caused by mutations in the fibrillin-1 gene. In MFS the structure of the fibrillin-containing microfibrils in the connective tissue is perturbed leading to diverse effects on various organs. The disorder affects the heart, lungs, eyes, the skeletal tissue and blood vessels. The most serious complications of MFS are the mitral valve collapse in the heart, and aortic dilatation and disruption. The skeletal symptoms include scoliosis and a generally tall stature, including long digits, arms and legs as well as chest alterations. Other hallmarks of MFS are the lens dislocation and myopia. There are numerous reports on the different mutations of the fibrillin-1 gene causing MFS (reviewed by Ramirez, 1996, Child, 1997, Pyeritz, 2000). Currently 137 different MFS causing mutations are known in the Marfan database, available on the Internet at http://www.umd.necker.fr. MFS is quite a frequent hereditary disorder, affecting about one out of 10.000 people.

Table 4. Human disorders of fibrillin microfibrils and their Online Mendelian Inheritance in Man database accession numbers (modified from Pyeritz, 2000)

Disorder	OMIM #	Reference
Confirmed		
Familial aortic aneurysm/dissection	132900	Francke *et al.*, 1995
Familial ectopia lentis	129600	Kainulainen *et al.*, 1994, Lönnqvist *et al.*, 1994
Marfan syndrome (MFS)	154700	Lee *et al.*, 1991, Maslen *et al.*, 1991
Severe neonatal Marfan syndrome (nMFS)	(154700)	Kainulainen *et al.*, 1994
Isolated skeletal features of the MFS	(154700)	Milewicz *et al.*, 1995
MASS phenotype/familial mitral valve prolapse / familial myxomatous valvular disease	604308	Dietz *et al.*, 1993
Congenital contractural arachnodactyly (CCA)	121050	Lee *et al.*, 1991
Shprintzen-Goldberg syndrome (SGS)	182212	Sood *et al.*, 1996
Familial arachnodactyly	121050	Hayward *et al.*, 1994
Possible		
Homocystinuria	236200	Reviewed by Finkelstein and Martin, 2000
Bicuspid aortic valve/coarctation/ascending aortic aneurysm	109730	
Weill-Marchesani syndrome	277600	Wirtz *et al.*, 1996
Scleroderma	181750	Tan *et al.*, 1998
Ectopia lentis et pupillae	225200	Colley *et al.*, 1991
Marfanoid mental retardation syndrome	248770	Fragoso and Cantu, 1984

Online Mendelian Inheritance in Men (OMIM) database is available on the Internet at http://www.ncbi.nlm.nih.gov/omim. Diseases with their OMIM numbers in parenthesis do not have their own OMIM entries, and the number refers to the more general disease classification.

The most severe form of MFS is the neonatal MFS (nMFS, Kainulainen *et al.*, 1994). All the known mutations causing nMFS are located in fibrillin-1 exons 24-27 and 31-32. nMFS is usually diagnosed right after birth, and the death occurs within one to two years due to congestive heart failure.

A disease association for fibrillin-2 has also been found. Mutations in fibrillin-2 cause a genetic disorder, namely congenital contractural arachnodactyly (CCA; Lee *et al.*, 1991), a disorder related to MFS. Both MFS and CCA are caused by various mutations in the fibrillin genes, resulting either in amino acid changes or shortening of the coded protein by the generation of STOP-codons. Notably, over 2/3 of the mutations in MFS are located in the calcium binding type EGF-like repeats, emphasizing the role for calcium in the microfibril integrity. MFS results supposedly from the interference of the microfibril construction by the mutated protein, thus giving this syndrome its dominant character.

The tight skin syndrome (TSK) in mice is another disorder resulting from modifications in fibrillin-1 gene. The heterozygous TSK phenotype includes thickened skin, increased growth of cartilage and bone (Green *et al.*, 1976). Homozygous TSK mice die between embryonic days 7 and 8. TSK results from a partial duplication (30-40 kbp) of the fibrillin-1 gene (Siracusa *et al.*, 1996). The fibrillin-1 protein encoded in the TSK mice has an increased molecular mass of approximately 450 kDa, compared to the 350 kDa of the wild type fibrillin-1. The TSK fibrillin-1 protein is expressed and secreted as wild type fibrillin-1. The fibrillin microfibrils in TSK mice are in two separate populations, those with a normal fibrillin morphology and those that have an altered structure specific to the TSK mice. These microfibrils have a longer interbead periodicity and more diffuse interbead regions than normal fibrillin microfibrils (Kielty *et al.*, 1998). Additional disorders which result, or have been suggested to result, from mutations in the fibrillin genes are listed in **Table 4**. While the LTBPs are structurally very similar to fibrillins, direct disease associations for LTBPs have not been detected yet. Mathews *et al.* have found two incidents, where the patient had partial phenotypic match to those of patients with MFS and a mutation in their LTBP-2 gene, suggesting that alterations in LTBPs could cause MFS-like disorder (Mathews and Godfrey, 1997). The importance of LTBPs is also clearly observed in LTBP-2 deficient mice. These mice have an embryonic lethal phenotype and appear to die very early, between E3.5 and E6.5, which coincides with the implantation period (Shipley *et al.*, 2000). The function of LTBP-2 during this time in development is not clear. The possible vital importance of LTBPs in general may be the reason why LTBPs have not been associated with diseases so far.

5.7.4 Modification of the expression of LTBPs in certain diseases

The expression of matrix-associated LTBP-1 is elevated in several fibrotic conditions, such as allograft arteriosclerosis, and tuberculosis pleurisy (Maeda *et al.*, 1993, Waltenberger *et al.*, 1993a, Waltenberger *et al.*, 1993b). In tuberculous pleurisy, the levels of LTBP-1 are highest in fibroblasts and mesothelia of immature fibrotic areas, while granulomas containing infiltrated T-cells and macrophages are no longer positive for LTBP-1 (Maeda *et al.*, 1993). In the skin, LTBP-1 immunoreactivity is increased in areas of solar damage suggesting an association with elastin (Karonen *et al.*, 1997). LTBP-1 was missing in areas of anetoderma, which are characterized by the absence of elastin. In immunohistology, LTBP-1 co-distributed

with elastic fibers, and co-distribution of TGF-β with LTBP-1 was identical indicating that these proteins are retained in similar structures also *in vivo* (Karonen *et al.*, 1997, see also Raghunath *et al.*, 1998).

The effects of secreted TGF-β can be modulated by the deposition of TGF-β to the matrix, and by activation. A failure of its paracrine growth control may be related to the expression and matrix deposition of LTBP-1. SV40 transformed fibroblasts lack ECM, which is known to contain LTBP-1 fibrils (Taipale *et al.*, 1996). In prostatic tumors, the tumor cells of various stages of differentiation, as well as the stromal cells, stain positively for TGF-β1. Cystectomized and benign prostatic tumors stain also positively for LTBP-1 (Eklöv *et al.*, 1993). However, the staining for LTBP-1 is lost in malignant prostatic cells (Eklöv *et al.*, 1993). Similarly, in gastrointestinal carcinomas, TGF-β1 is found in both tumor and stromal cells, while LTBP-1 is found only in stromal cells and in the stromal ECM (Mizoi *et al.*, 1992, Mizoi *et al.*, 1993), and in studied ovarian cancer cases, TGF-β expression was increased, while LTBP-1 was mainly detected only in normal epithelial cells (Henriksen *et al.*, 1995).

5.7.5 Role of TGF-β in cancer

TGF-β is often associated with various malignancies. However, there is no clear relation between TGF-β and its role as an inhibitor or activator of malignant cell growth. TGF-β increases the synthesis of ECM and decreases the proteolytic degradation of ECM. This is an obstacle for metastasing cancer cells, which need to penetrate ECM structures for invasion and intra- and extravasation. Transgenic mice overexpressing active form of TGF-β1 are resistant to chemically induced mammary tumors (Pierce *et al.*, 1995), while more tumors could be generated in TGF-$\beta1^{+/-}$ mice (Shida *et al.*, 1998, Tang *et al.*, 1998). Also keratinocytes from TGF-β1 null mice are more susceptible to malignant transformation (Glick *et al.*, 1994). However, TGF-β has also angiogenic effects, favoring neovascularization required for tumor growth, which supports TGF-β's role as an oncogenic growth factor (Ueki *et al.*, 1992, O'Mahony *et al.*, 1998, Wikström *et al.*, 1998; reviewed by Pepper, 1997). It has been proposed that in the initial stages of carcinogenesis, TGF-β is acting as tumor suppressor, inhibiting the growth of transformed cells. However, when these cells overcome the inhibitory effects of TGF-β, the endogenous expression and unresponsiveness to TGF-β can be favorable to cancer cells (reviewed by Reiss, 1999).

Several tumor cells endogenously express TGF-β (Terui *et al.*, 1990, Lotz *et al.*, 1994, Vanky *et al.*, 1997, Picon *et al.*, 1998, Wikström *et al.*, 1998, Junker *et al.*, 1996, Wunderlich *et al.*, 1997, Constam *et al.*, 1992; reviewed by Reiss, 1999), and TGF-β levels have been tested to be clinical markers of tumor progression (Kong *et al.*, 1995, Tsushima *et al.*, 1996, Perry *et al.*, 1997, Sminia *et al.*, 1998). Since most tumors are derived from epithelial or myeloid cells, which are sensitive for TGF-β induced growth inhibition, the proliferation of tumor cells would thus be inhibited by TGF-β. This is actually the case for some slowly progressing tumors, such as B-cell chronic lymphocytic leukemia (B-CLL) (Lotz *et al.*, 1994), where endogenously produced TGF-β suppresses the proliferation, but the B-CLL cells are still insensitive to TGF-β induced apoptosis (Douglas *et al.*, 1997). In contrast, B cell precursor acute lymphoblastic

leukemia cells (B-ALL) do not endogenously express TGF-β, but are sensitive to exogenous TGF-β, which both suppresses their growth and induces apoptosis (Buske *et al.*, 1997). Tumor cells can also have lost their responsiveness to TGF-β, by e.g. lack of functional TGF-β type I or II receptors (TβRI, TβRII) (DeCoteau *et al.*, 1997, Markowitz *et al.*, 1995, Kim *et al.*, 1996a, Kim *et al.*, 1998, Kimchi *et al.*, 1988) or the intracellular TGF-β signaling proteins SMAD2 or SMAD4 (see above section *5.6 TGF- signal transduction*; Eppert *et al.*, 1996, Hahn *et al.*, 1996, Kim *et al.*, 1996b, Riggins *et al.*, 1996, Schutte *et al.*, 1996, Uchida *et al.*, 1996, MacGrogan *et al.*, 1997). Humans homozygous for altered TβR-I have significantly higher risk for various neoplasms (Pasche *et al.*, 1998, Chen *et al.*, 1999). The TGF-β insensitivity together with TGF-β overexpression provides cancer cells a way to escape the host's immunosurveillance, which is efficiently prevented by TGF-β (see above section *5.2.3 TGF- as an immunosuppressive agent*).

5.7.6 TGF-β and fibrosis

Overproduction of activated TGF-β is known to lead to pathological conditions, in which the accumulation of ECM is exaggerated. Tissue specific overexpression of active TGF-β typically results in massive fibrosis of the organs where TGF-β is expressed, while overexpression of active TGF-β in a tissue type independent manner is lethal (reviewed by Bottinger and Kopp, 1998, McCartney-Francis and Wahl, 1994). The pathological situations, where TGF-β is overexpressed include disease states like cheloid formation, scleroderma and liver chirrosis (reviewed by Border and Noble, 1994). Underexpression of TGF-β can cause reduced bone mass, and a TGF-β1 gene variant has been associated with very low bone mass in osteoporotic women (Langdahl et al., 1997).

6 AIMS OF THE PRESENT STUDY

The different biological effects of TGF-β have been very extensively studied during the fifteen years since its discovery. However, a key step in the biology of TGF-βs, how these growth factors are deposited to the ECM in latent forms and subsequently activated, is much less known. In most cases, the latent TGF-β is covalently bound to the LTBPs proteins, which target it to the ECM. This study concentrated on elucidating the molecular level mechanisms involved in the TGF-β latency aspect namely:

1) How latent TGF-β interacts with LTBPs?

2) How LTBPs in turn are deposited to ECM?

3) What are the elements regulating the transcription of LTBPs?

4) Do new human LTBPs exist?

7 MATERIALS AND METHODS

7.0.1 Cell lines

The cell lines used are listed in the table below with their ATCC (American Type Culture Collection, Manassas, VA, USA) identification numbers:

Name	Description	Used in	Culture medium
CCL-137	Human embryonic lung fibroblasts. ATCC CCL-137.	**I**, **II**, **III**, **IV**	MEM + 10% FCS
CHO-K1	Chinese hamster ovary epithelial cells. ATCC CCL-61.	**I**, **III**	MEM + 0.2% BSA, + 10% FCS
COS-7	African green monkey kidney epithelial cells, SV40 transformed. ATCC CRL-1651.	**I**, **II**, **III**	D-MEM + 10% FCS
293T	Human kidney epithelial cells, expressing the transforming gene of adenovirus 5. ATCC CRL-1573.	**II**, **III**	D-MEM + 10% FCS
WI-38	Human lung fibroblasts. ATCC CCL-75.	**IV**	MEM + 10% FCS
WI-38 / VA13	SV40 transformed subline of WI-38. ATCC CCL-75.1.	**IV**	MEM + 10% FCS
HT-1080	Human fibrosarcoma cells. ATCC CCL-121.	**IV**	MEM + 10% FCS
HA	Human umbilical vein epithelial primary culture	**IV**	Medium 199 + 0.1% glucose, + 10% FCS

Cell culturing was carried out in the medium mentioned in the above table, supplemented with 100 IU/ml penicillin and 50 µg/ml streptomycin. All experiments were carried out under serum-free conditions. For the collection of conditioned medium, the cells were washed twice with serum-free medium, and the subsequently added serum-free medium was collected after specified periods of time.

7.0.2 Antibodies

Polyclonal antibodies against human LTBP-1 (Ab39) and LTBP-2, immunoprecipitating anti-human TGF-β1LAP (Lt2) and anti-human TGF-β3LAP (Ab95) antibodies were kind gifts of Dr. C.-H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden) and used as purified IgG. Mouse monoclonal anti-fibrillin-1 antibodies mAb 201 and mAb 69 were kind gifts of Dr. Lynn Sakai (Shriners Hospital, Oregon, USA). Affinity purified polyclonal anti-human TGF-β1 (#627) and TGF-β1LAP (#680) peptide antibodies as well as anti-human LTBP-2 antibodies have been described previously (Taipale *et al.*, 1992; Taipale *et al.*, 1995, Hyytiäinen *et al.*, 1998). Mouse monoclonal antibody 12CA5 against the hemagglutinin epitope was purchased from Berkeley Antibody Co. (Berkeley, CA, USA) and used as purified IgG. Polyclonal anti- human TGF-β2 sc-20 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

For the generation of LTBP-4 specific antibodies, synthetic peptides derived from LTBP-4 sequence at the beginning of the 3rd (YFDTAAPDACDNILARNVTWQE) and 4th (WQEVGADLVCSHPRLDRQATYTE) 8-Cys repeats, respectively, were coupled to keyhole limpet hemocyanin (KLH, Pierce, Rockford, IL, USA). The KLH-peptide complexes were used to raise antibodies #28-3 (against the 3rd 8-Cys repeat) and #33-4 (against the 4th 8-Cys repeat) in rabbits. Subsequently, the antibodies were affinity purified with the antigenic peptide. Both Ab #28-3 and Ab #33-4 were reactive in immunoblotting assays under both reducing and non-reducing conditions.

7.0.3 DNA constructs

The DNA constructs used are listed in their respective publications. Most LTBP and fibrillin-cDNA fragments were expressed from a vector, named pSignal, initially constructed for the publication **I**. pSignal is derived from InVitrogen's pcDNAIII (Oxon, UK). In the 5' end of the polylinker (HindIII-BamHI), a synthetic epitope coding for an optimized so-called Kozak translation initiation sequence, followed by in frame IgG heavy chain signaling sequence was inserted. In the 3'end of the polylinker (XhoI-XbaI), a synthetic epitope sequence was added, that codes for both IBI's FLAG and BabCO's HA-epitopes as well as an in-frame STOP codon. Both the signal sequence and the epitope were done by annealing two partially overlapping oligonucleotides, filling the ends with Klenow polymerase, restricting with mentioned restriction endonucleases and then cloned into pcDNAIII. pEpitope is a derivative of pSignal, which lacks the signal sequence. pEpitope was used to express N-terminal LTBP fragments containing their own native signal sequence.

7.0.4 Cloning of cDNA and genomic DNA

LTBP-4 cDNA was cloned from two human heart cDNA libraries, obtained from Clontech (Palo Alto, CA, USA). Library HL3005q is poly-T primed pCDM-8 plasmid library, and library HL3026a is both poly-T and random primed λ gt10 phage library. The library was screened with [^{32}P]dCTP labeled LTBP-4 cDNA probes, initially derived from the identified LTBP-4 EST, subsequently with probes derived from the newly cloned cDNA.

The genomic region coding for the LTBP-1S promoter was cloned by using a commercial Genome Walker Kit and nested oligonucleotides derived from the LTBP-1S cDNA. The amplification products obtained from different genomic restriction fragment pools were cloned in pGEM-T vector (Promega, Madison, WI, USA). Initially a total of 1.75 kbp of genomic DNA upstream of translation initiation site was obtained. A second round of genome walking produced a further 1.2 kbp genomic 5' flanking sequence.

The upstream genomic region of LTBP-1L was cloned from human placenta genomic λ phage library, using a [^{32}P]dCTP labeled LTBP-1L 5' cDNA fragment as a probe. Inserts from positive phages were cloned into Bluescript II KS vector (Stratagene, La Jolla, CA, USA).

7.0.5 Sequencing, sequence analysis and molecular modeling

DNA clones were sequenced using Amersham-Pharmacia's ALF Express (Amersham-Pharmacia Biotech, Uppsala, Sweden) and Perkin-Elmer's ABI 373, ABI 377 and ABI 310 automatic DNA-sequencers at the Haartman Institute and at Institute of Biotechnology, University of Helsinki.

All trace file analyses, restrictions, translations, primer design, creation of silent restriction sites etc. general sequence analyses were done using DNA Works sequence analysis package, developed during this thesis project for Microsoft Windows (Microsoft Corp., Redmond, WA, USA) using Borland Delphi software development environment (Inprise Corp., Scotts Valley, CA, USA). The assembly and analysis of contigs from overlapping reads were performed using the Staden sequence assembly package (Bonfield *et al.*, 1995) in a Linux workstation (Linus Torvalds, University of Helsinki, Finland).

The multiple sequence alignment was done by using the Clustal W 1.74 program (Thompson *et al.*, 1994) and corrected by hand. In the sequence alignments, the human LTBP-3 sequence was used. The molecular models were built using the Insight II version 98 (Molecular Simulations Inc., San Diego, CA, USA), using an SGI Origin 2000 computer (SGI Corp, Mountain View, CA, USA, located at Center for Scientific Computing, Finland). The NMR solved structure of the 8th 8-Cys repeat of human fibrillin-1 (PDB accession number 1APJ, 7th structure out of 21

structures in entry 1APJ) was used as a template. All the indels were modeled by searching from PDB-loop database. The preliminary models were soaked in a waterbox, extending at least 9 Å beyond the 8-Cys repeat. The energy minimizations were done by gradually diminishing the fixations of the model between successive minimization steps with the steepest descent followed by conjugate gradient algorithms using the Discover v. 2.98 module and Amber forcefield.

7.0.6 Northern hybridization analysis

The cDNA fragments used as probes in Northern blotting were [^{32}P]dCTP labeled using random priming kit (Amersham-Pharmacia Biotech). In multitissue Northern blots, β-actin probe was used as a control. Hybridization of both multitissue and RNA Master blots were carried out according to manufacturer's instructions (Clontech). The amounts of RNA in RNA Master blot have been equalized by the manufacturer by comparing the expression levels of 8 different housekeeping mRNAs. Radioactivity levels in hybridized RNA Master blots were quantitated with a BAS-1500 bio-imaging analyzer (Fuji Photo Film Co, Ltd., Tokyo, Japan).

7.0.7 Transfection of cell lines

Cells were transfected prior to confluency, except human lung fibroblasts CCL-137, which were transfected as confluent cultures. Transfections were carried out using a calcium phosphate transfection system (Gibco-BRL, Gaithersburg, MD, USA), lipofectamine (Gibco-BRL) or FuGENE6 (Roche Molecular Biochemicals, Palo Alto, CA, USA) according to manufacturer's instructions. After transfection, the cells were washed twice, fed with serum-free medium, and the conditioned medium was collected after specified time.

7.0.8 Isolation of the extracellular matrix

ECM was prepared by first washing cell cultures once with phosphate-buffered saline (PBS; 0.14 M NaCl, 10 mM sodium phosphate buffer, pH 7.4) and then treated three times with 0.5% sodium deoxycholate in 10 mM Tris-HCl buffer, pH 8.0, at 0°C for 10 min (see Hedman *et al.*, 1979). The plates were then washed again with PBS and allowed to dry overnight at room temperature. Cross linked components of the matrix were partially solubilized by digesting the sodium deoxycholate insoluble matrices by plasmin (0.3 CU/ml) in matrix digestion buffer (PBS containing 1 mM Ca^{++}, 1 mM Mg^{++} and 0.1% *n*-octyl-d-β-glycopyranoside) at 37°C for 1 hr. Finally, released ECM proteins were dissolved in non-reducing SDS-PAGE sample buffer (see Taipale *et al.*, 1994).

7.0.9 Proteinase digestion of fibroblast conditioned medium

Conditioned medium from confluent fibroblasts was collected for 3 days under serum-free conditions. Aliquots of the medium were treated with proteinases at 37°C for 1 hr. The following concentrations of proteinases were used: 50 nM plasmin; 10 nM chymase; 10 nM leukocyte elastase; 10 nM porcine pancreatic elastase; 100 nM cathepsin G; 500 nM cathepsin G; 500 nM cathepsin D (see Taipale *et al.*, 1995).

7.0.10 Interspecies genomic DNA blot

Zoo Southern blot (Clontech) contained 10 μg of *Eco*RI digested genomic DNA from various species. The Zoo-blot was hybridized using [^{32}P]dCTP labeled clone 1.1.1 A as a probe and washed first with 1xSSC, 0,1% SDS and subsequently with 0.1xSSC, 0,1% SDS at 42°C.

7.0.11 Fluorescence *in situ* hybridization

A fragment LTBP-1 or -4 cDNA was used as a probe to clone the genomic DNA from a human genomic PAC library (Genome Systems Inc, St Louis, MO, USA). The obtained PAC clone in vector pAd10SacBII was labeled with biotin-14-dATP by nick translation. The metaphase preparations made from human lymphocyte culture were pretreated with pepsin (0.2 mg/ml at 0.01 M HCl) for 10 minutes at 37°C, and chromosomes were denatured in 70% formamide in 0.3 M NaCl, 0.3 M Na-citrate, pH 7.0 (2xSSC) at 64°C for 2 minutes. Hybridization signals were detected by avidin-tetramethylrhodamine isothiocyanate (TRITC) and analyzed by Olympus fluorescence microscope equipped with an ISIS digital image analysis system (Metasystems, Altlussheim, Germany). The chromosome identity was verified by painting with a chromosome specific probe according to manufacturer's instructions (Cambio, Cambridge, UK).

7.0.12 Primer extension

The oligonucleotide used in primer extension experiments was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The oligonucleotide was allowed to anneal with denatured total RNA and extended with SuperScript II RNase H-reverse transcriptase. Extension products were separated in denaturing urea polyacrylamide gel.

7.0.13 RNase protection

cDNA fragments to be used in RNase protection assays were cloned into pGEM-7Zf vector (Promega) and linearized. Antisense RNA probes were synthesized with T7 RNA-polymerase in the presence of [α-^{32}P]UTP. RNA probes were annealed to total RNA and single stranded RNA was degraded by RNase A/RNase T1 mix (Ambion Inc., Austin, TX, USA). Protected fragments were separated in denaturing urea polyacrylamide gel.

7.0.14 Luciferase reporter assays

Fragments of LTBP-1S and -1L promoter to be used in luciferase reported assays were cloned into pGL3 Basic vector (Promega). These constructs were transfected with pRL-TK control plasmid. The cells were washed twice with PBS 48 hours after transfection, lysed with Passive Lysis Buffer (Dual Luciferase Kit, Promega) and lysates were subjected for luciferase activity measurements.

8 RESULTS

8.1 *Identification of the ECM and TGF-β binding regions of LTBP-1 (I)*

8.1.1 N-terminal region of LTBP-1 is required for ECM-binding

The large latent TGF-β complex had previously been found to consist of TGF-β1LAP covalently bound to LTBP-1 (Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990, Miyazono *et al.*, 1991). This complex was subsequently observed to be deposited into the sodium deoxycholate insoluble ECM, from where it could be released by proteolysis (Taipale *et al.*, 1992, Taipale *et al.*, 1994). Plasmin digestion resulted thus in generation of LTBP-1 with a molecular mass approximately the same as the truncated LTBP-1 found in platelets (Miyazono *et al.*, 1988, Wakefield *et al.*, 1988, Kanzaki *et al.*, 1990). In order to identify the regions of LTBP-1 mediating the association with the ECM, expression vectors coding for different parts of the LTBP-1 cDNA were cloned and stably expressed in CHO cells. Analysis of the deposition of these LTBP-1 proteins into ECM showed that the protein encoded by LTBP-1 construct lacking the N-terminal region (first 400 amino acids) was unable to get incorporated into the deoxycholate insoluble ECM fraction, whereas both full length LTBP-1S and LTBP-1 construct containing the N-terminal region were assembled to the ECM. Thus, the first 400 N-terminal amino acids were found to contain the ECM binding region of LTBP-1.

8.1.2 Co-expression of TGF-β1 and LTBP-1 results in the formation of covalent large latent TGF-β complexes

Most studied cell types had been known to secrete TGF-β in the large latent complex, covalently bound to LTBPs. However, in certain cell lines had also been found to secrete small latent TGF-β. In order to answer to the question, whether the rarely observed secretion of small latent TGF-β is due to the lack of endogenous expression of LTBPs, CHO cells were co-transfected with TGF-β1 and LTBP-1 cDNAs. Endogenous TGF-β1 of CHO cells was observed in the large latent complex. Upon overexpression of TGF-β1 alone, the amount of endogenous large latent TGF-β1 complex increased, but the majority of TGF-β1 was seen in the small latent complex. The secretion of the small latent complex upon overexpression is most likely due to the saturation of endogenous LTBPs. However, when the cells were co-transfected with cDNAs for both TGF-β1 and LTBP-1, the majority of TGF-β1 was in the large latent complex, indicating that when both TGF-β and LTBP are available, TGF-β is secreted in the large latent complex. These results were in accordance with earlier results, indicating that in the absence of LTBP-1, the small latent TGF-β is secreted slowly and may contain anomalous disulphide bridges (Miyazono *et al.*, 1991). Furthermore, the rarely observed secretion of the small latent TGF-β in certain cell models (Bonewald *et al.*, 1991, Eklöv *et al.*, 1993, Mizoi *et al.*, 1993, Dallas *et al.*, 1994, Grainger *et al.*, 1995) is thus most likely due to the lack of LTBPs.

8.1.3 Identification of the 3rd 8-Cys repeat of LTBP-1 as the TGF-β binding domain

LTBP-1 had been found to bind covalently to the LAP part of the small latent TGF-β1. This binding is dependent on oxidized cysteine residues, since reduction of the complex breaks its association (Miyazono *et al.*, 1988, Miyazono *et al.*, 1991). However, the actual protein domain(s) of LTBP-1 involved in this association were not known. To analyze the region of LTBP-1 responsible for the association with LAP, a series of deletion constructs of LTBP-1 cDNA were created and co-expressed in COS cells together with TGF-β1 cDNA. The secreted proteins were analyzed in SDS-PAGEs under non-reducing conditions for the presence of β1LAP complexed with LTBP-1 fragments. By gradually excluding different regions of LTBP-1, the 3rd 8-Cys repeat was found to be solely responsible for the association with TGF-β1. The other 8-Cys repeats were verified not to be able to covalently associate with β1LAP.

8.1.4 Cysteine 33 of TGF-β1LAP is required for the covalent association with LTBP-1

The LAP-part of TGF-β1LAP contains three cysteines. Cys-223 and Cys-225 were previously found to be required for the dimerization of the LAP (Brunner *et al.*, 1988), whereas the function for Cys-33 was not known. Since the interaction between the 3rd 8-Cys repeat of LTBP-1 and β1LAP was found to be mediated by covalent disulphide bonds, the requirement of β1LAP Cys-33 for the interaction with 8-Cys repeat was studied. The codon for Cys-33 of β1LAP was mutated to code for serine and this mutated TGF-β1 was transfected with β1LAP binding constructs to COS-cells. The mutation of Cys-33 to serine resulted in a complete loss of the covalent complexes with the proteins encoded by all β1LAP binding LTBP-1 constructs.

8.2 Molecular analysis of the 8-Cys repeat interaction with TGF-β (II)

8.2.1 The 3rd 8-Cys repeats of LTBPs -1, -3 and -4, but not of LTBP-2, are capable of forming covalent association with TGF-βs

Fibrillins and LTBPs contain a total of 34 different 8-Cys repeats. Previously, the TGF-β1LAP binding function had been assigned to the 3rd 8-Cys repeat of LTBP-1 (**I**). However, no solid data existed of the ability of the 8-Cys repeats to associate with β1LAP. In addition, it was not known whether the two other βLAP isoforms (-2 and -3) were able to associate with the 8-Cys repeats. Therefore, an analysis of the abilities of LTBPs and fibrillins to associate with TGF-βs was carried out.

In these studies, β1LAP was found to co-immunoprecipitate with LTBP-1 protein secreted by fibroblasts. However, while present in the conditioned medium, neither LTBP-2 nor fibrillin-1 was co-precipitated with β1LAP. When fibroblasts were transfected with TGF-β1 cDNA, endogenous LTBP-1 was found to become complexed with β1LAP, whereas the overexpression of TGF-β1 did not yield any complexes with LTBP-2 or fibrillin-1. The

inability of LTBP-2 to covalently associate with β1LAP was confirmed by transient co-expression of full length LTBP-2 with TGF-β1. These results indicated that LTBP-2, unlike LTBP-1, was unable to complex with β1LAP. In addition, some of the 8-Cys repeats of fibrillin-1 were co-expressed with TGF-β1LAP and found out to be incapable in binding to β1LAP. These constructs included the 8th 8-Cys repeats of fibrillins -1 and -2, which are the most similar ones to the TGF-β binding 3rd 8-Cys repeat of LTBPs.

Next, LTBPs 1-4 were analyzed for their abilities to associate with the three mammalian isoforms -1, -2 and -3 of βLAPs. LTBP constructs coding for the 3rd and 4th 8-Cys repeats and the two intervening EGF-like repeats were co-expressed with TGF-βs 1-3. Proteins coded by LTBP -1 and -3 constructs were found to efficiently form complexes with all βLAP isoforms. On the contrary, LTBP-4 (**III**) had much weaker complex forming ability as compared to LTBPs -1 and -3, and it was detected to form a complex with β1LAP isoform only. The protein encoded by the LTBP-2 construct was not able to form covalent complexes with any of the βLAP isoforms.

In addition to the 3rd 8-Cys repeats of LTBPs -1, -3 and -4, also other 8-Cys repeats have been suggested to be capable of associating with β1LAP in co-precipitation assays (Moren *et al.*, 1994, Yin *et al.*, 1998a). In both of those reports, TGF-β1 and either full length LTBP-2 or fragments of LTBPs -2 and -3 were overexpressed in COS-cells and co-precipitation of β1LAP with LTBP-2 or fragments of LTBPs -2 or -3 was detected. In contrast with those results, other 8-Cys repeats of LTBP-1 than the 3rd one, were found to have no covalent β1LAP binding ability in different cell types, including COS-, 293T- and insect Sf9-cells (**I**, **II**, Gleizes *et al.*, 1996). In addition, no LTBP-2 - β1LAP complexes could be detected with bovine LTBP-2 (Gibson *et al.*, 1995, Robert Mecham, personal communication). Furthermore, no LTBP-2 - β1LAP complexes were detected in the conditioned medium of stable CHO-cell clones, or transiently transfected COS or 293T-cells overexpressing LTBP-2 and TGF-β1 (**II**, Hyytiäinen *et al.*, 1998). In addition, endogenous LTBP-2 did not co-precipitate with β1LAP from TGF-β1 transfected primary fibroblasts (**II**). Thus, the reported interaction of LTBP-2 as well as the other 8-Cys repeats than the 3rd ones of LTBPs -1, -3 and -4 with β1LAP most likely represents non-covalent co-precipitation under mild conditions than used in (**II**). The biological significance of non-covalent interactions between LTBPs and β1LAP is unknown and may just represent the used overexpression system, since in non-transfected cells, only covalent interactions between LTBP-1 and β1LAP have been found (Taipale *et al.*, 1994, Taipale *et al.*, 1995). In addition, the documented stoichiometry of the complex between TGF-β binding LTBPs and βLAP argues against the possibility of TGF-β1LAP interacting with more than one 8-Cys repeat of LTBPs, as suggested by Yin *et al.*, (Yin *et al.*, 1998a).

8.2.2 Identification of the TGF-β binding motif in 8-Cys repeats

The 8-Cys repeats are about 55 amino acids long protein domains, which fold into a globular structure (Yuan *et al.*, 1997, Yuan *et al.*, 1998). When the TGF-βLAP binding functions were found to be limited to a very small subset of the 8-Cys repeats, the obvious question was, what determines their βLAP binding ability. For this purpose, chimeric cDNA

constructs were generated. The backbone of these constructs was the 3rd 8-Cys repeat of LTBP-1, and all the residues between successive cysteines were exchanged to those of the 3rd 8-Cys repeat of LTBP-2. The constructs were then transfected together with TGF-β1 cDNA, and the formed complexes were analyzed from the conditioned medium. The proteins encoded by all chimeric constructs, except construct L1ΔL2-4, were able to bind β1LAP in a covalent manner, like the wild type construct. In construct L1ΔL2-4, the region between the 6th and 7th cysteine residues was exchanged (**C**EIFP**C** in LTBP-1, **C**EI**C** in LTBP-2).

To verify these results, two new chimeric constructs were made and analyzed in a similar manner. Construct L1ΔL4-4 coded for a similar chimera as L1ΔL4-2 between LTBP-1 (backbone) and LTBP-4. Protein coded by L1ΔL4-4 retained the ability to covalently associate with β1LAP, like the protein coded by wild type LTBP-1 construct. Construct LTBP-2GAIN coded for an analogous chimera between LTBP-2 (backbone) and LTBP-1. The protein expressed from LTBP-2GAIN gained the TGF-β1LAP binding ability. Thus, the change of the short specific region between the 6th and 7th cysteine residues in the 3rd 8-Cys repeat of LTBP-2 to that of LTBP-1 was enough to provide this protein domain with the β1LAP binding ability. This small sequence motif yielding latent TGF-β binding ability was named briefly as the TGF-β binding motif in 8-Cys repeats. Sequence analysis of all the 8-Cys repeats of LTBPs and fibrillins indicated that this motif is present only in three of the known 34 different 8-Cys repeats, namely in the 3rd 8-Cys repeats of TGF-β binding LTBPs, -1, -3 and -4.

8.2.3 Molecular models for TGF-β binding and non-binding type 8-Cys repeats

In order to analyze the actual consequences of the TGF-β binding motif in the 8-Cys repeats, molecular modeling was used. Several 8-Cys repeats were modeled, including both TGF-β binding and non-binding types. The previously determined structure of the 8th 8-Cys repeat of fibrillin-1 was used as a template for the modeling (Yuan *et al.*, 1997). When the backbone of the structure of the 8th 8-Cys repeat of fibrillin was aligned with the models for the 3rd 8-Cys repeat of LTBPs -1 and -2, the largest difference in the backbone alignment was in the TGF-β binding determinant region between the 6th and 7th cysteine residues of the model for the 3rd 8-Cys repeat of LTBP-1.

This resulted in the loss of altogether three hydrogen bonds that were present both in the structure of the 8th 8-Cys repeat of fibrillin and in the model for the 3rd 8-Cys repeat of LTBP-2. The lack of these hydrogen bonds suggests increased flexibility of the 3rd 8-Cys repeat of LTBP-1. The sulfhydryl groups of the cysteine residues were not consistently more exposed in the models for TGF-β binding type 8-Cys repeats. However, the surface hydrophobicity was increased considerably in all the models for the TGF-β binding type 8-Cys repeats. The increased surface hydrophobicity may have a role in creating favorable conditions for the complex formation between the TGF-βLAP and LTBPs in the secretory pathway.

8.2.4 Direct disulphide bridges mediate the binding between the 8-Cys repeat and the Cys-33 of β1LAP

Previously the covalent interaction between the 3rd 8-Cys repeat of LTBP-1 and β1LAP had been found to be dependent on the Cys-33 of β1LAP (**I**). It was also known that the cysteine residues in the 8-Cys repeat are all in oxidized form (Gleizes *et al.*, 1996, Reinhardt *et al.*, 1996a). However, the character of this interaction was still unspecified. Namely, it was not known whether the cysteine residues required for 8-Cys - β1LAP complexes are involved in inter- or intramolecular disulphide bridges. In the intermolecular disulphide bridge model, one or both of the Cys-33s of the β1LAP dimer are forming a disulphide bridge with unknown cysteine(s) of the 8-Cys repeat. In the intramolecular cysteine disulphide bridge model, all the cysteines of the TGF-β binding 8-Cys repeat and β1LAP are involved in intramolecular disulphide bridges. However, the molecules would be folded in a way, where they are kept together like two engaged circles. The complex would thus resist the non-reducing sample denaturation in SDS-PAGEs. The dimerization of β1LAP was prevented by mutating the cysteines 223 and 225 to serines, and this construct was co-expressed in COS-cells with an LTBP-1 fragment, capable of covalent interaction with the β1LAP via its 3rd 8-Cys repeat. In the conditioned medium of the transfected cells, the mutated β1LAP was found to retain the ability of the wild type β1LAP to form covalent LTBP-1 complexes. This interaction recruited both copies of the monomeric β1LAP, as indicated by both the observed mobility of the complex and by its detection in the immunoblot, since the used β1LAP antibodies did not detect the single chain β1LAP. These results suggest that the interaction between β1LAP and LTBP-1 is mediated by two direct cysteine disulphide bridges between the molecules.

8.3 Cloning of a novel latent TGF-β binding protein, LTBP-4 (III)

8.3.1 Cloning of human LTBP-4 and its alternatively spliced forms

New members to the LTBP-fibrillin gene family had been cloned based on the sequence homology (Moren *et al.*, 1994, Gibson *et al.*, 1995, Yin *et al.*, 1995a). Simultaneously, a large number of the expressed sequence tags (ESTs) sequences became available. In order to identify novel proteins containing an 8-Cys repeat, the EST databank was searched using the 3rd 8-Cys repeat of LTBP-1 as a probe (Altschul *et al.*, 1990). Several ESTs were obtained, coding for unknown 8-Cys repeat containing proteins. Using the ESTs as probes, a new cDNA of 4944 bp was obtained and named as LTBP-4S. LTBP-4S contained all the unknown 8-Cys repeats found in different EST clones. The overall structure of LTBP-4S is very similar to those of the previously identified LTBPs -1, -2 and -3. Alternatively spliced forms of LTBP-4 cDNA emerged during the cloning, which had different numbers of EGF-like repeats in the central core of successive EGF-like repeats. These alternatively spliced forms were named as LTBP-4-ΔE and LTBP-4-Δ2E. In addition, a part of LTBP-4L cDNA, coding for an alternative N-terminal end was cloned. The open reading frame of LTBP-4L cDNA continues upstream and the full-length sequence of LTBP-4L is not yet known. During this work, an LTBP-4 sequence, with yet another 5' end was described (Giltay *et al.*, 1997).

LTBP-4 cDNA was used to isolate a genomic LTBP-4 PAC clone, which was further used to analyze the chromosomal localization of the LTBP-4 gene. Human metaphase leukocyte chromosomes were hybridized with biotinylated LTBP-4 PAC probe and LTBP-4 gene was localized to chromosome 19, at the region of 19q13.1 – 19q13.2.

8.3.2 Analysis of LTBP-4 expression in different tissues

In multitissue Northern blots, a single LTBP-4 mRNA form was detected. The size of this mRNA was approximately 5.1 kb. Tissue specific expression of LTBP-4 was analyzed by using multitissue Northern blots as well as a dot-blot, containing mRNA from 50 different tissues. LTBP-4 was found to be quite ubiquitously expressed, and the highest levels of LTBP-4 expression were in the heart, aorta, uterus, small intestine, ovary and adrenal gland. Notably, the expression of LTBP-4 in most fetal tissues was significantly lower than in adult tissues suggesting that LTBP-4 expression emerges later in the development.

Two antibodies were raised against peptides derived from the 3rd and 4th 8-Cys repeats of LTBP-4 to study the protein level expression of LTBP-4. LTBP-4 was found to be present in fibroblast conditioned medium. A fraction of LTBP-4 appeared to be associated with some as yet unidentified protein(s) via its 3rd 8-Cys repeat, since the antibodies against the 4th 8-Cys repeat detected other, higher molecular mass forms of LTBP-4 in non-reduced samples. From the respective reduced samples, both antibodies detected only a single form of LTBP-4. Overexpression of full length LTBP-4 in mammalian cells turned out to be very inefficient, only very low levels of LTBP-4 were secreted. Co-expression of LTBP-4 with TGF-β1 resulted in relatively inefficient complex formation, indicating that LTBP-4 is a true TGF-β1 binding protein, but less active than LTBP-1.

8.3.3 Identification of LTBP-4 as a protease sensitive ECM component

LTBPs were known to be deposited to the ECM structures in such a way that they are not extractable without breaking covalent interactions (Taipale *et al.*, 1992, Taipale *et al.*, 1994). Using the antibodies raised against LTBP-4, also LTBP-4 was found to be assembled to ECM. Plasmin, which can release LTBP-1 from the ECM, resulted also in the release of LTBP-4 in a truncated form from fibroblast ECM, with a cleavage in its N-terminal region. LTBP-4 complexed with unidentified protein(s) via its 3rd 8-Cys repeat was detected also in the ECM extracted samples.

LTBP-1 had earlier been found to be susceptible for proteolytic release by plasmin, elastases and mast cell chymase (Taipale *et al.*, 1995). These proteases cleaved LTBP-1 at its hinge region, releasing LTBP-1 and possibly complexed TGF-βLAP as a truncated large latent complex. LTBP-4 was analyzed here for its susceptibility to proteolytic cleavage, using several proteases. Of the proteases tested plasmin, leukocyte and pancreatic elastases as well as mast cell chymase were able to process LTBP-4 to large fragments of slightly lower molecular weight than intact LTBP-4.

8.4 Identification of two independent promoter regions that regulate the transcription of LTBP-1S and LTBP-1L (IV)

8.4.1 LTBP-1S and LTBP-1L are transcribed from their independent promoters

LTBP-1 was previous found to exist in two, N-terminally different forms, that were differentially expressed in different tissues (Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990). In addition, LTBP-1 expression was shown to be reduced in malignant prostate as well as digestive tract tumors (Eklöv *et al.*, 1993, Mizoi *et al.*, 1993). In order to study the mechanism generating the different LTBP-1 forms as well as reducing their expression in certain conditions, the upstream regions of LTBP-1S and -1L in the human genome were cloned. These were subsequently inserted into a luciferase reporter vector to analyze for the presence of promoter activity. The 5' upstream regions (hereby called as "promoters") of both LTBP-1S and -1L strongly induced luciferase expression, thus indicating that both forms of LTBP-1 have their own, independent promoter regions. No TATA boxes were present in either of the promoter regions. However, a number of other potential transcription factor binding sites were predicted by the sequence, including a TGF-β inhibitory element (TIE) and SMAD binding element (SBE) in LTBP-1S promoter.

The sequence of the junction point between the LTBP-1S and -1L also revealed the utilization of a rare intraexonic splice acceptor site (**IV**, Öklu *et al.*, 1998a). This splice site is within one of the exons for LTBP-1S, whereas a part of the exon is used as a splice acceptor site in the LTBP-1L transcript.

8.4.2 Localization of the regulatory elements in the 5' upstream regions of LTBP-1S and -1L

Several 5' end deletion constructs were made from the promoters for both forms of LTBP-1. Extending of the used promoter length of LTBP-1S increased the observed luciferase activities. When the same constructs were transfected to human amniotic epithelial cells, no such correlation was observed. This is in accordance with the previous results of Northern blots suggesting negatively regulated transcription of LTBP-1S in placenta.

Transcriptional activities of similar LTBP-1L promoter deletion constructs were quite the opposite. Their activities were significantly increased upon shortening of the used promoter regions, down to the size of about 450 bp.

8.4.3 Cell type-specific transcription of LTBP-1S and LTBP-1L

The LTBP-1S and -1L promoters were found to be differently regulated in different cell types. Generally, the transcriptional activity from the LTBP-1S promoter was higher than from the LTBP-1L promoter. In the used fibroblast model, the activity of LTBP-1S promoter was more than ten fold higher than that of the LTBP-1L promoter. In primary human amnion epithelial cells, the activities of both promoters were much lower, and the LTBP-1L promoter was more active than the LTBP-1S promoter in this cell model. This indicates that the LTBP-1

promoters had independent, cell type specific activities. Earlier the LTBP-1 isoforms were found to be differentially expressed in different tissue types, as detected by mRNA Northern blotting (Olofsson *et al.*, 1995).

The SV-40 virus transformed VA-13 subline of WI-38 fibroblasts was used as a model for cells with transformed phenotype. As compared to the transcriptional activity of LTBP-1S and -L promoters in WI-38 wild type fibroblasts, promoter activities for both LTBP-1 promoter regions were very low in VA-13 cells. Similar results were obtained from HT-1080 fibrosarcoma cell line.

9 DISCUSSION

Growth factors are extracellular signal mediator molecules, that function as auto / paracrine ways. Unlike endocrine hormones, that can circulate and act all over the body, growth factors usually show a more restricted spatial pattern of their action regarding to the place of the synthesis. Good examples are many members of the TGF-β superfamily, which take part in guiding the various stages of the development, where controlled function of the growth factor induced effects both in time and space is crucial.

TGF-β and insulin like growth factor (IGF) are examples of growth factors that are bound to their respective binding proteins in extracellular space. Dissociation of the binding proteins is required for binding to the growth factor signaling receptors. Fibroblast growth factor (FGF), platelet derived growth factor (PDGF), granylocyte-macrophage colony stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), tumor necrosis factor-alpha (TNF-α) and vascular endothelial growth factor (VEGF) are all associated with ionic interactions with ECM molecules and are hence also localized in space. Another type of regulation of growth factor availability is a required proteolytic cleavage of the pre-form of the growth factor in order to gain biological activity, like in the case of bone morphogenetic proteins (BMPs) and hepatocyte growth factor (HGF).

This work has concentrated on the molecular mechanisms by which TGF-β is secreted from the cells in a complex with its binding proteins, LTBPs and subsequently, how these latent TGF-β complexes are deposited to the ECM and how the expression of LTBPs is regulated.

9.1 Extracellular matrix deposition of TGF-β via LTBP-proteins

The overall domain structure of the LTBP proteins is well conserved and can be divided to four parts, the N-terminal region, the following hinge domain, the central cluster of EGF-like repeats and the C-terminal TGF-β binding region (**Fig. 11**). The main differences between LTBPs are in the number of EGF-like repeats in the central part and in the non-homologous N-terminal region. The observed alternative N-terminal ends provide even more variability to the N-terminal regions of LTBPs.

9.1.1 Amino-termini of LTBPs are required for the ECM deposition

The N-termini of all LTBPs contain two to three copies of EGF-like repeats, including one cbEGF-like repeat, and two 8-Cys repeats, the latter being a hybrid domain type 8-Cys repeat. The region of LTBP-1 required for covalent interaction with the ECM was mapped to the N-terminus of LTBP-1 (**I**). This result was in accordance with other observations for LTBPs. The soluble form of LTBP-1 from platelets lacks the N-terminal part (Kanzaki *et al.*, 1990, Miyazono *et al.*, 1991), like the LTBP-1 released from the matrix by proteolysis (Taipale *et al.*, 1994). In addition, the N-terminally extended LTBP-1L isoform has enhanced ECM binding ability (Olofsson *et al.*, 1995; see below section *9.2 Structural variability of LTBPs*). Subsequently, the ECM association of LTBP-1 was found to involve transglutaminase-mediated cross linking (Nunes *et al.*, 1997, Verderio *et al.*, 1999). In addition, recombinant LTBP-2 is incorporated into the ECM of cultured fibroblasts in a

covalent manner, possibly also by a transglutaminase catalyzed reaction (Hyytiäinen *et al.*, 1998). Recently a putative second ECM binging region has been proposed to be located in the very C-terminal part of LTBP-1, including the 4th 8-Cys and the two last EGF-like repeats (Unsöld *et al.*, manuscript submitted 2000; see **Fig. 11**).

Large portions of the N-termini of LTBPs contain no known protein domains and include cysteine residues that are not part of any EGF-like or 8-Cys repeats. Some of the "lonely" cysteine residues may be involved in covalent disulphide linkages with the ECM structures to which LTBPs are deposited. In addition, the N-termini of LTBPs contain so-called 4-Cys repeats, in which overall sequence conservation and patterning of the cysteine residues is quite limited, as compared to the EGF-like or 8-Cys repeats. No biological functions have been identified for the 4-Cys repeats. A 4-Cys repeat is found also in the N-termini of both fibrillins -1 and -2. The hybrid type 8-Cys repeats in the N-termini of LTBPs have an odd number of

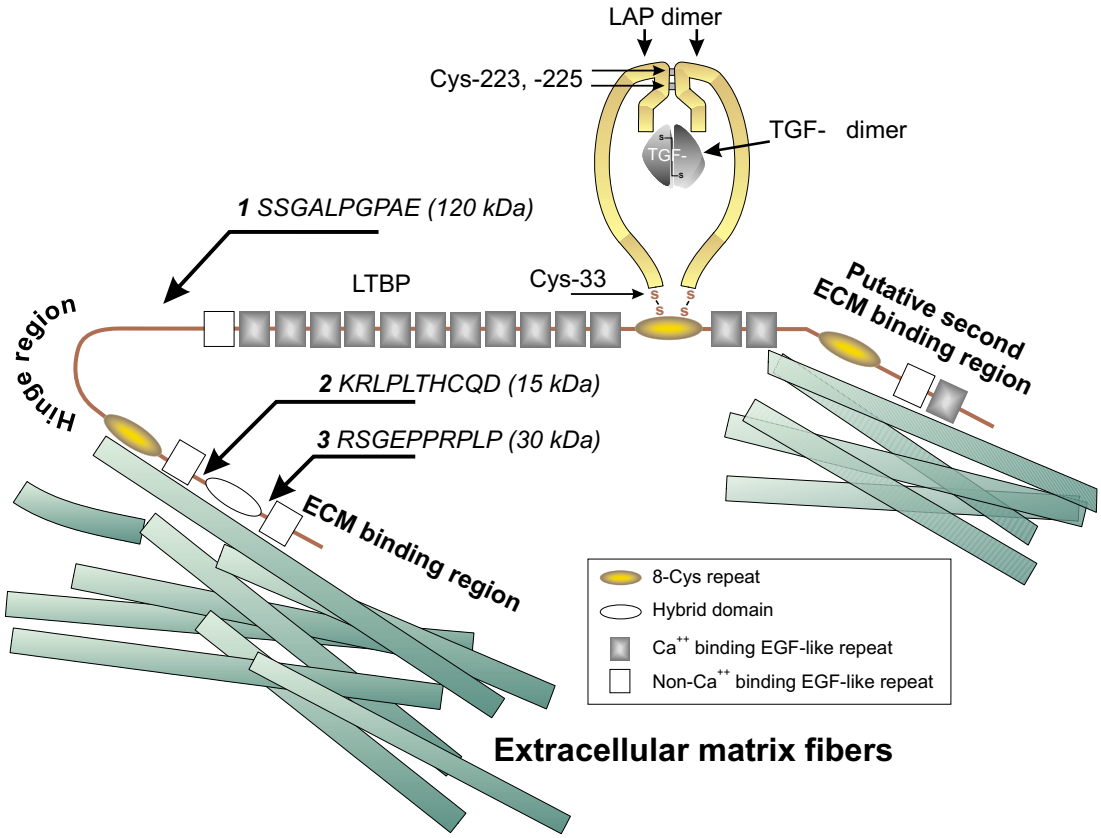

Figure 11. A schematic structure of the large latent TGF-β complex
LTBP-proteins are covalently associated with the ECM via their N- and possibly also C-termini (**I**, Taipale *et al.*, 1994, Unsöld *et al.*, manuscript submitted 2000). The 3rd 8-Cys repeat of TGF-β binding LTBPs can be covalently associated with the LAP part of small latent TGF-β by direct disulphide bonds involving Cys-33 residues of LAP-dimer (**I**, **II**, Gleizes *et al.*, 1996). The other cysteine residues of LAP, Cys-223 and Cys-225 are required for the dimerization of the LAP (Gentry *et al.*, 1988). Large latent complex can be released from the ECM by proteolysis at specific sites (**I**, **III**, Taipale *et al.*, 1994, Taipale *et al.*, 1995), three of the sequenced sites in LTBP-2 are indicated by arrows (numbers 1-3) in addition to the sequence after the cleavage site (Hyytiäinen *et al.*, 1998). Cysteine residue numbering refers to the TGF-β1 isoform.

cysteine residues. In fibrillin-1, one of the cysteines in the first hybrid type 8-Cys repeat is found to occur as a free thiol, thus being able to form inter-protein disulphide bridges (Reinhardt *et al.*, 2000).

The dimerization of the fibrillin monomers is suggested to be an early step leading to the construction of the microfibril. The N-terminal region of fibrillin-1 has been shown to direct its dimerization (Trask *et al.*, 1999). Similar results have been obtained with LTBP-1, suggesting that the 8-Cys repeats in the N-terminus may be required for disulphide-mediated dimerization (Unsöld *et al.*, manuscript submitted 2000).

9.1.2 Proteolysis at the hinge domain between the amino-terminal ECM binding region and central core of EGF-like repeats releases large latent TGF-β complex from the ECM

LTBP, possibly complexed with latent TGF-β, can be released from the ECM by proteolytic cleavage at a so-called hinge region, located after the ECM-binding N-terminal part (**I**, **III**, Taipale *et al.*, 1994, Hyytiäinen *et al.*, 1998). The release of the large latent TGF-β complex may well be a critical requirement for the activation of TGF-β. The length of the hinge region is between 90-150 amino acids in different LTBPs. It does not contain any known protein domains, but is rich in proline and basic amino acid residues. The region seems to be susceptible at least to plasmin, elastases, thrombin and mast cell chymase (**III**, Taipale *et al.*, 1992, Taipale *et al.*, 1996, Hyytiäinen *et al.*, 1998). Also the anti-adhesive functions of LTBPs -1 and -2 were localized to their proline-rich hinge regions (Hyytiäinen *et al.*, manuscript 2000; see section below *9.1.5 Other biological functions for LTBPs*). In addition to hinge region, there are other potential protease sensitive sites in the N-terminal region of LTBPs. Some of the proteolytic cleavage sites have been confirmed by amino acid sequencing from LTBP-2 (Hyytiäinen *et al.*, 1998; See **Fig. 11**).

In fibrillin-1 a proline-rich putative protease sensitive site is located in the N-terminus, between the 1st 8-Cys repeat and the successive non-Ca^{++} binding type EGF-like repeat. Fibrillin-2 does not have a proline-rich region but instead the corresponding area has high concentration of glycine residues. The glycine and proline-rich regions of fibrillins have been suggested to be involved in the initial dimerization of fibrillins (Ashworth et al., 1999a). Fibrillins also contain multiple proteinase sensitive cleavage sites, and various proteases are able to degrade fibrillins (Kielty et al., 1994, Ashworth et al., 1999b, Hindson et al., 1999).

9.1.3 Central core of LTBPs is composed of multiple consecutive EGF-like repeats

The central parts of all LTBPs are composed of a long stretch of EGF-like repeats. This part consists of 9-14 repeats, and is about a third of the total protein size. All the EGF-like repeats, except the first one, in this region are of calcium binding type. This region is resistant to proteolysis (**II**, Taipale *et al.*, 1995, Hyytiäinen *et al.*, 1998). However, the biological functions for this region in LTBPs are not well known. It has been suggested that this region would form a helical-rod like structure like the similar regions in fibrillins containing cbEGF-like repeats (Downing *et al.*, 1996).

9.1.4 Association of LTBPs with TGF-βLAP is mediated by a specific 8-Cys repeat in the carboxy-terminal part of TGF-β binding LTBPs

The localization of the covalent TGF-β1LAP interacting domain to the 3rd 8-Cys repeat of LTBP-1, near to its C-terminus, was the first observed biological function for 8-Cys repeat protein domains, which are found only in the LTBP and fibrillin proteins (**I**). These results were later verified using an insect cell expression system (Gleizes *et al.*, 1996). In the C-termini of all LTBPs, a typical structure of 8-Cys—EGF— EGF—8-Cys is found, but the covalent TGF-β binding function is present only in the first 8-Cys repeat of this region. Even though there are altogether 34 8-Cys repeats in the LTBP-fibrillin family, with quite conserved sequences, only a minor subset of those is able to associate with βLAP (**II**). The observed weak association of LTBP-4 with TGF-β1LAP may well be overcome *in vivo* by the simultaneous expression of LTBPs -1 or -3 and their efficient interaction with βLAPs. Thus, LTBP-4 seems to have a less important role in depositing TGF-β to extracellular matrix than LTBPs -1 and -3. Further, the results encourage search for other functions for the majority of the abundant 8-Cys repeats of LTBPs and fibrillins (**II**). Interestingly, the weak TGF-β binding ability of LTBP-4, together with the observed high proportion of LTBP-4 complexed with unknown protein(s) via its 3rd 8-Cys repeat (**II**), strengthens the possibility of other proteins to be covalently deposited into the ECM via an interaction with the 8-Cys repeats. The further identification of the protein(s) associating with the 3rd 8-Cys repeat of LTBP-4, and possibly with other members of the LTBP-fibrillin family is of great interest.

The interaction of the 3rd 8-Cys repeat of LTBP-1 with β1LAP occurs via two direct disulphide bonds involving both Cys-33s of TGF-β1LAP and yet unknown cysteines in the 8-Cys repeat (**I**, **II**). Since all of the eight cysteine residues in 8-Cys repeats are involved in intradomain disulphide bonding in a 1-3, 2-6, 4-7, 5-8 pattern (Gleizes *et al.*, 1996, Reinhardt *et al.*, 1996a, Yuan *et al.*, 1997), the interaction with TGF-βLAP appears to result in rearrangement of at least one disulphide bridge in the 8-Cys repeat (**II**). Previously the function of the other two cysteines (Cys-223 and -225) of β1LAP has been found to be in the dimerization of the LAP-propeptide (Brunner *et al.*, 1988). Since all TGF-β isoforms contain cysteines at analogous positions, one might expect that all βLAP - LTBP interactions are mechanistically similar to the studied β1LAP - LTBP-1 interaction. The possibility of other TGF-β superfamily members being covalently associated with the 8-Cys repeats seems thus to include the requirement of a cysteine residue in their propeptide parts analogous to the Cys-33 of β1LAP.

The TGF-β binding function was found to correlate with the insertion of two amino acids between the 6th and 7th cysteine residues of the 8-Cys repeats (**II**). In LTBPs -1 and -3, which bind TGF-β very efficiently, the area between the 6th and 7th Cys residue is coded by EIFP and EIYP, respectively, whereas in the weak TGF-β binding protein, LTBP-4, it is coded by a more diverse RIQQ sequence. All the 8-Cys repeats in LTBP-2 and in fibrillins are of the non-TGF-β binding type. Replacement of the TGF-β binding motif from the 3rd 8-Cys repeat of LTBP-1 with the analogous region of LTBP-2 results in the loss of TGF-βLAP binding ability (**II**). When the TGF-β binding motif of LTBP-1 is inserted in the non-TGF-β binding 3rd 8-Cys

repeat of LTBP-2, the modified 8-Cys repeat gains the ability to associate covalently with TGF-βLAP (**II**). The 8-Cys repeats can hence be classified to TGF-β binding and non-binding types (**II**) by their primary sequence. However, the small TGF-β binding motif is most likely not enough to provide TGF-β binding ability for all of the 8-Cys repeats, but more likely, also other replacements for the more divergent 8-Cys repeats would be required.

During these studies, the structure of the 8th 8-Cys repeat from fibrillin-1 was determined by NMR (Yuan *et al.*, 1997). This protein domain folds into a globular structure, containing six beta-strands and two alpha-helices. Cysteines 3-5, which are next to each other in the sequence, are located in the hydrophobic core and form disulphide bridges with cysteines closer to the surface of the domain. The homology models of the 8-Cys repeats of LTBPs revealed a clear difference between the TGF-β binding and non-binding type 8-Cys repeats (**II,** Saharinen, unpublished data; see **Fig. 12**). An increased hydrophobic surface, extending from the vicinity of the TGF-β binding motif was present in the TGF-β binding type 8-Cys repeats, while the non-TGF-β binding type 8-Cys repeats were resembling the structure of the fibrillin 8-Cys repeat in this aspect (**II**; see also Yuan *et al.*, 1997, Rudd *et al.*, 2000). This suggests that the association of βLAP with the TGF-β binding type 8-Cys repeats in the secretory pathway involves hydrophobic interactions. Although the interaction with TGF-β is formed by disulphide bridges, the availability of the SH-groups in the models did not correlate with the TGF-β binding ability. In the model for the 3rd 8-Cys repeat of LTBP-1 (**II**, Yuan *et al.*, 1997), some of the disulphide bridges were more accessible, and were suggested to have a plausible role required for interaction with βLAP (Yuan *et al.*, 1997). However, in the other models of the TGF-β binding type 8-Cys repeats, these disulphide bridges were not more accessible than in the non-TGF-β binding type 8-Cys repeat models (**II**). These results suggest that the surface accessibility of the disulphide bridges of an 8-Cys repeat is not critical for association with βLAP and the fold of an 8-Cys repeat undergoes structural changes upon interacting with βLAP. Nevertheless, in the absence of experimental structural data the results from the molecular modeling studies may not give the correct structural information of the TGF-β binding type 8-Cys repeats.

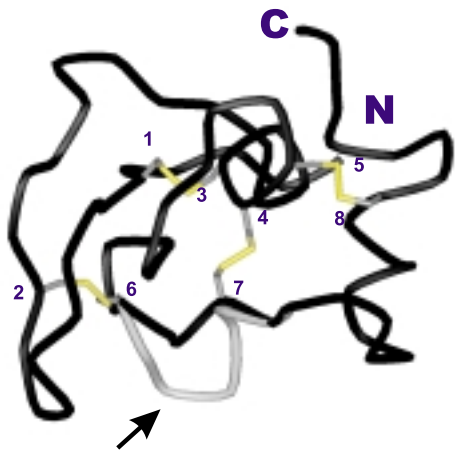

Figure 12. Structure of an 8-Cys repeat
The backbone of the NMR-derived 8th 8-Cys repeat of fibrillin-1 is shown in black. The cysteine residues are numbered and their sidechains are shown. The area between the 6th and 7th Cys-residues of the TGF-β binding type 3rd 8-Cys repeat of LTBP-1 is superimposed, shown in grey and indicated by an arrow. Modified from **II** and Yuan *et al.*, 1997.

9.1.5 Other biological functions for LTBPs

The RGD sequence motifs, possibly mediating cell adhesive functions via integrins (Ruoslahti, 1996), exist in human isoform of LTBP-1, LTBP-2 and in one LTBP-4 isoform as well as in fibrillins -1 and -2. In fibrillins, the

RGD-sequences are located on the 8-Cys repeats, unlike in LTBPs. Purified fibrillin microfibrils have been found to mediate cell adhesion and platelet aggregation (Kielty *et al.*, 1992, Pfaff *et al.*, 1996, Sakamoto *et al.*, 1996 see also Ross *et al.*, 1998). Cell attachment to fibrillin-1 is mediated by $\alpha_V\beta_3$ integrin (Pfaff *et al.*, 1996, Sakamoto *et al.*, 1996, D'Arrigo *et al.*, 1998) and to fibrillin-2 by $\alpha_V\beta_1$ and $\alpha_3\beta_1$ integrins (D'Arrigo *et al.*, 1998). The fibrillin containing microfibrils are also known to support platelet adhesion (Ross *et al.*, 1998). Also the microfibrillar protein, MAGP-2, contains the RGD sequence motif, and MAGP-2 protein is suggested to provide a link to connect microfibrils to the cell surface (Gibson *et al.*, 1998). The plausible cell adhesive capability of the RGD motifs in LTBPs has yet to be demonstrated. The RGD sequences present in TGF-β binding LTBPs may be involved in the targeting of the latent TGF-β complexes to activation at cell surface after release from ECM via the integrin-mediated TGF-β activation system.

LTBPs -1 and -2 have been found to be anti-adhesive for certain cell types (Hyytiäinen *et al.*, manuscript 2000). The anti-adhesive functions were mapped to the proline-rich hinge region of both LTBP-1 and -2. Recombinant LTBP-1 and -2 were found to prevent totally cell adhesion to fibronectin, when fibronectin and LTBP were coated on cell culture plates before the seeding of the cells. Interestingly, LTBP-1 or -2 had no effect on cell adhesion, when cells were seeded onto fibronectin-coated cell culture plates together with soluble recombinant LTBP. LTBP-1 has been reported to play a role also in vascular remodeling (Kanzaki *et al.*, 1998). After mechanical injury to rat arteries, LTBP-1 was located in the intimal layers of the arteries, and found to have strong chemotactic functions towards rat smooth muscle cells in vascular remodeling (Kanzaki *et al.*, 1998).

9.2 Structural variability of LTBPs

Structural variation has been found in all LTBPs (**Fig. 13**). Two different variants of LTBP-1 are known, the longer LTBP-1L having a 346 amino acid N-terminal extension. The fusion site is immediately after the putative signal sequence of LTBP-1S (Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990, Olofsson *et al.*, 1995; *LTBP-1L* and *LTBP-1S* in **Fig. 13**). The longer form has been reported to interact more efficiently with the ECM than LTBP-1S (Olofsson *et al.*, 1995), emphasizing the importance of the N-termini of the LTBPs for the ECM association. These isoforms have their own, independent promoter regions. (**IV**). These promoters were found to regulate transcription differentially and in cell line specific manner (**IV**). This is likely to be the reason for the tissue type specific expression of LTBP-1 isoforms (Olofsson *et al.*, 1995). The use of multiple promoter regions, regulating the composition of the N-termini of the translated proteins is found also in other ECM proteins, including different collagens (Nishimura *et al.*, 1989, Saitta and Chu, 1994, Sugimoto *et al.*, 1994, Thomas *et al.*, 1995, Pallante *et al.*, 1996, Rehn *et al.*, 1996, Zhang *et al.*, 1997) and laminin α3 (Ferrigno *et al.*, 1997). The utilization of independent promoters may allow a more precise control of expression in different tissue types and different stages of development. Similar alternative N-terminal variability as found in LTBP-1 may also occur in LTBP-2, since in Northern blots, LTBP-2 appears as two mRNA species (Moren *et al.*, 1994). Whether the cloned LTBP-2 cDNA represents the longer or smaller species detected in the LTBP-2 Northern blots is not

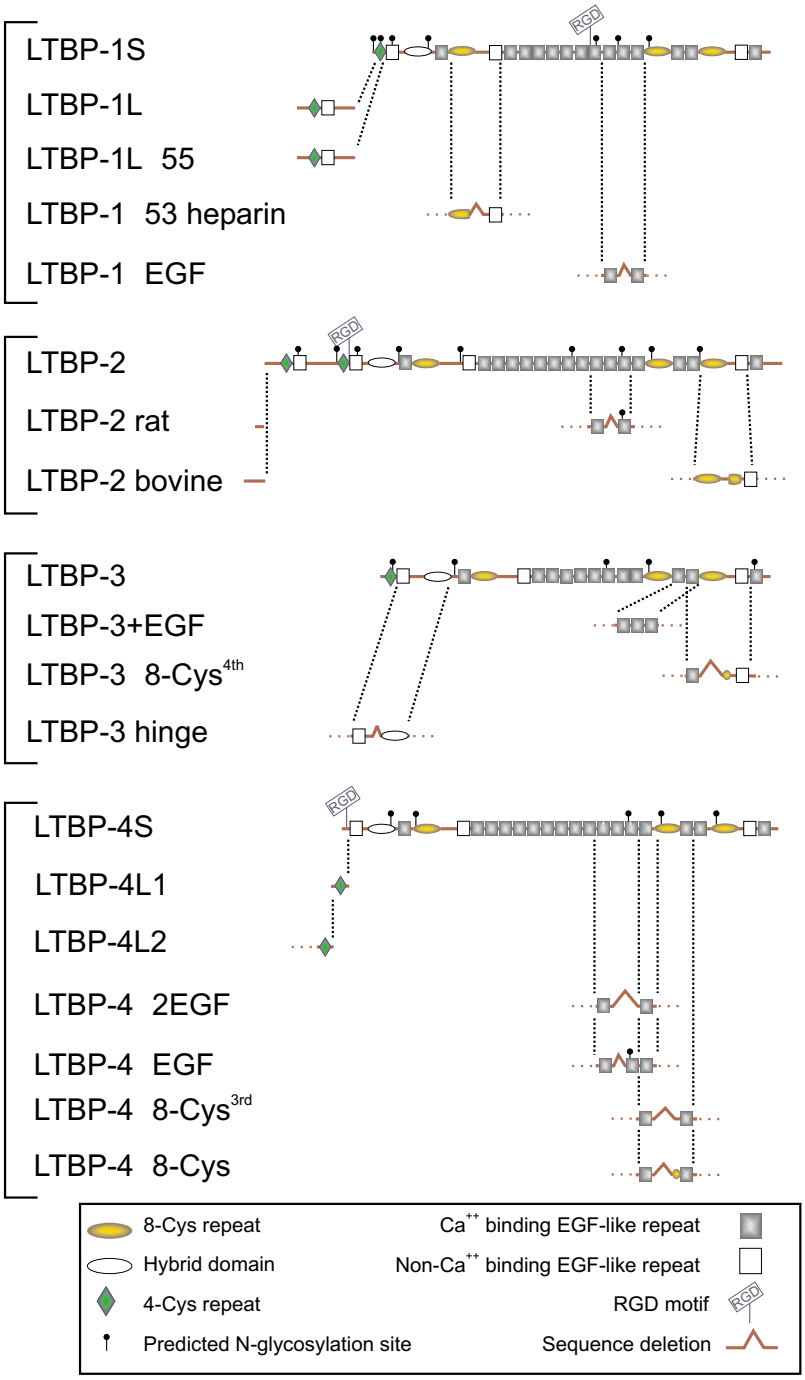

Figure 13. Structural variability of LTBPs (modified from Saharinen *et al.*, 1999)
LTBPs display an extensive structural variability, generated both by alternative splicing as well as by the use of different promoter regions. The small and large (S and L) forms of LTBPs, differing in their N-termini, are generated most likely by use of independent promoters for the different forms. Alternative splicing is known to generate variability in the number of both EGF-like and 8-Cys repeats. Deletions of one or two EGF-like repeats have been observed in LTBPs -2, -3 and -4, as well as an insertion of an additional EGF-like repeat in human LTBP-3. In mouse LTBP-3, a splice variants lacking first or both exons coding for the 4th 8-Cys repeat have been characterized. In human LTBP-4, a splice variant lacking the TGF-β binding 3rd 8-Cys repeat has been found. In LTBPs -1 and -3, splice variants lacking parts of the protease sensitive hinge regions have been identified.

known, since the size of the cloned LTBP-2 cDNA is smaller than both of the LTBP-2 mRNA forms detected in Northern blots. In LTBP-4 at least three different 5'-ends have been identified at the cDNA level (**III**, Giltay *et al.*, 1997; *LTBP-4S*, *LTBP-4L1* and *LTBP-4L2* in **Fig. 13**). Interestingly, one of these isoforms codes for an RGD sequence, suggesting a cell adhesive role for this LTBP-4 isoform. The sequence of fibrillin-1 gene suggests that there could be at least three different 5'-regions (Corson *et al.*, 1993), resulting in different N-terminally alternative forms. However, observations on the sequence conservation between the protein coding areas of human and pig genomic fibrillin-1 sequences argue against the N-terminal variability (Biery *et al.*, 1999**).**

In addition to N-terminal variability due to independent promoter regions, also several variable forms of LTBPs generated via alternative splicing have been found. These alterations scatter all around the LTBP-proteins. However, the biological functions for these variable forms are generally unknown. Alternative splicing generated variability has been observed in the N-terminal extension of LTBP-1L (Öklu *et al.*, 1998a; *LTBP-1L 55* in **Fig. 13**), creating a LTBP-1L variant lacking 55 amino acids, including two potential N-glycosylation sites.

The composition of the protease sensitive hinge region of LTBPs is also controlled by alternative splicing. An alternatively spliced form of LTBP-1 lacks 53 amino acids that include a consensus heparin binding site, and has been suggested to be less protease sensitive (Michel *et al.*, 1998, Öklu *et al.*, 1998b; *LTBP-1 53 heparin* in **Fig. 13**). Also LTBP-3 cDNA lacking parts of the hinge region has been reported, but the specific function for this splice variant is unknown (Yin *et al.*, 1998b; *LTBP-3 hinge* in **Fig. 13**).

In the central cluster of successive EGF-like repeats, there is alternatively splicing generated variation in at least LTBPs -1and -4, probably also in LTBP-2 (**III**, Saharinen *et al.*, manuscript 2000, Öklu *et al.*, 1998a; *LTBP-1 EGF*, *LTBP-2 rat*, *LTBP-4 EGF* and *LTBP-4 EGF* in **Fig. 13**). The exact biological functions of any of these splice variants are not known. In addition to affecting the length of the predicted rod like structure generated by the long stretches of EGF-like repeats (Downing *et al.*, 1996), the variability in the number of the EGF-like repeats can be involved in controlling possible protein-protein interactions, as is known for the certain EGF-like repeats of Notch and Delta (Rebay *et al.*, 1991).

Alternative splicing has also been reported to generate a partial or complete deletion of an 8-Cys repeat of mouse LTBP-3 and human LTBP-4 (Koli *et al.*, manuscript submitted 2000, Saharinen *et al.*, manuscript 2000, Saharinen *et al.*, 1996, Yin *et al.*, 1998b). Unlike the EGF-like repeats, which are encoded by a single exon, the 8-Cys repeats are encoded by two exons. This generates the observed partial deletion of 8-Cys repeats, in which the first exon coding for the 8-Cys repeat is missing and thus the region covering the first seven cysteine residues is lost. Alternative splicing resulting in the loss of the 3rd 8-Cys repeat of LTBP-4 is biologically interesting (Koli *et al.*, manuscript submitted 2000), because it affects the TGF-β binding function of LTBP-4 (**II**, **III**). In bovine LTBP-2, a repeat containing four cysteines, resembling a part of an 8-Cys repeat is located at the C-terminus, after the last 8-Cys repeat (Gibson *et al.*, 1995). Interestingly, this repeat is against the exon boundaries in the 8-Cys repeats. As soon as additional biological functions for the 8-Cys repeats are discovered, there will be more biological insights into the alternative splicing regulating the number of 8-Cys repeats.

9.3 Regulation of expression of LTBPs

The existence of four members of LTBPs and their structural similarities raise questions of the possible functional differences between these proteins. The expression patterns of different LTBPs in different tissues are partially overlapping. LTBP-1 is mainly expressed in the heart, placenta, lung, spleen, kidney, and stomach (Kanzaki *et al.*, 1990, Tsuji *et al.*, 1990, Moren *et al.*, 1994) and human LTBP-2 in lung, skeletal muscle, liver and placenta (Moren *et al.*, 1994). Strikingly, the expression pattern of mouse LTBP-2 is very limited, being expressed only in cartilage perichondrium and blood vessels (Fang *et al.*, 1997). In the developing mouse, LTBP-3 is expressed widely in mesenchymal cells (Yin *et al.*, 1995a). Significant expression was observed by *in situ* analysis in the developing osteoblasts, central nervous system, respiratory epithelial cells and connective tissue cells in the pulmonary interstitium, somites and the cardiovascular system. In Northern blotting, the expression levels of human LTBP-3 and LTBP-4 are quite similar. They both are predominantly expressed in the aorta, heart, small intestine and ovaries (**III**, Saharinen *et al.*, manuscript 2000, Giltay *et al.*, 1997). The expression levels of LTBP-3 and LTBP-4 were significantly lower in most fetal tissues than in adult tissues (**III**, Saharinen *et al.*, manuscript 2000). This might indicate that neither LTBP-3 nor LTBP-4 is required for the initial formation of microfibrillar structures, but they can provide a way to store latent TGF-β or related molecules in the extracellular fibrils. In contrast, LTBP-2 is expressed at early stages of the development, as exemplified by the drastic death of the LTBP-2 deficient mice between D3.5-6.5 (Shipley *et al.*, 2000). “Switching” of isoform expression during development may be typical of the LTBP-fibrillin family. For example, fibrillin-2 is expressed earlier and in a more transient manner in the mammalian development than fibrillin-1, which is expressed at later stages of development (Zhang *et al.*, 1995, Rongish *et al.*, 1998). Since there are at least four members in the LTBP-family, it will be of interest to learn more of the expression of different LTBPs in different stages of development, and thus to provide possible explanations and biological functions for the existence of multiple LTBPs.

The promoters regulating the gene expression are known only for LTBP-1 (**IV**). The LTBP-1 gene codes for two independent promoters, regulating the transcription of N-terminally different LTBP-1 isoforms. Treatment of fibroblasts with TGF-β either had no effect (LTBP-1L) or decreased (LTBP-1S) the activity of the promoters. However, the mRNA levels of both LTBP-1 isoforms in Northern-blots were up regulated by TGF-β, indicating post transcriptional mechanisms in the regulation of the LTBP-1 expression. LTBP-1 promoters were found to have cell type specific regulation of transcription. Interestingly, the activities of both of the promoters were reduced in the used SV-40 virus transformed cell model. This is in accordance with the previous results, indicating reduced expression of LTBP-1 in transformed cells, including cells isolated from malignant prostate and digestive tract tumors (Eklöv *et al.*, 1993, Mizoi *et al.*, 1993, Dallas *et al.*, 1994, Taipale *et al.*, 1996). Tumor cells are known to produce very little ECM components, whereas they often have increased extracellular proteolysis (Vaheri and Ruoslahti, 1975, Mignatti and Rifkin, 1993). This phenotype is required for e.g. the invasive processes of malignant tumors. TGF-β strongly regulates the

homeostasis of the ECM production and degradation, increasing production of multiple ECM components and reducing extracellular proteolysis (see above section *5.2.1 Effects of TGF- on extracellular matrix and skeletal system*). Thus, the downregulation of LTBP production may be beneficial for tumors.

10 PERSPECTIVE

The results of this study revealed a specific mechanism by which LTBPs associate with latent TGF-β to deposit it in the ECM (**I**, **II**). However, it was also found that only a minority of expressed LTBP molecules is associated with latent TGF-β and not all the LTBPs even have the latent TGF-β binding ability (**I**, **II**) .Therefore, other biological functions of LTBPs, including their role in the fibrillin-microfibrils and in other ECM structures are to be found. Interaction of latent TGF-β with LTBPs targets the localization of the growth factor and via TGF-β activation, provides a rapid mechanism for availability of a growth modulating factor, without the need of gene expression. The localization of various members of the TGF-β superfamily is strictly controlled during developmental processes. However, it is not known, whether other members of TGF-β superfamily than TGF-βs themselves associate with LTBPs. Cloning and subsequent characterization of LTBP-4 suggested that LTBP-4 might be associated with a heterologous protein other than TGF-β (**III**). A number of structurally different forms of LTBPs, generated by alternative splicing as well by the use of different promoter regions, are known (**III**, **IV**). However, the specific functions of most of these protein variants are to be found. In addition, the role of LTBPs in the TGF-β activation is an issue requiring further investigation

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