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Association of the small latent transforming growth factor-β with an eight cysteine repeat of its binding protein LTBP-1

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Transforming growth factor-βs (TGF-βs) are produced by most cells in large latent complexes of TGF-β and its propeptide (LAP) associated with a binding protein. The latent TGF-β binding proteins (LTBPs-1, -2 and -3) mediate the secretion and, subsequently, the association of latent TGF-β complexes with the extracellular matrix (ECM). The association of β1–LAP with LTBP-1 was characterized at the molecular level with an expression system in mammalian cells, where TGF-β1 and various fragments of LTBP-1 were co-expressed and secreted with the aid of a signal peptide synthesized to the LTBP-1 constructs. Immunoblotting of the fusion protein complexes indicated that the third 8-Cys repeat of LTBP-1 bound covalently to the LAP region of TGF-β1. The cysteine required for the association between LTBP-1 and β1–LAP was mapped to Cys33 of β1–LAP. The N-terminal region of LTBP-1 consisting of the first 400 amino acids was found to associate covalently with the ECM. The data indicate that an 8-Cys repeat of LTBP is capable of covalent and specific protein–protein interactions. These interactions are mediated by exchanging cysteine disulfide bonds between the core 8-Cys repeat and an optionally associated protein during the secretion. This is, to our knowledge, the first demonstration of an extracellular protein module that is able to exchange cysteine disulfide bonds with heterologous ligand proteins.

Keywords: eight cysteine repeat/extracellular matrix/transforming growth factor-β

Introduction

Transforming growth factor-βs (TGF-βs) are a family of multifunctional growth-modulating polypeptides, which affect the growth, differentation and morphology of cells, the homeostasis of extracellular matrix (ECM) synthesis and proteolysis and developmental processes (reviewed in Massagué, 1990; Laiho and Keski-Oja, 1992; McCartney-Francis and Wahl, 1994). Three different TGF-βs have been found in mammals, TGF-β1, -β2 and -β3, all having similar biological effects. TGF-βs belong to a large family of proteins, comprising inhibins/activins, bone morphogenetic factors, Müllerian-inhibiting substance and the decapentaplegic of *Drosophila* (reviewed in Kingsley, 1994; Massagué *et al.*, 1994). Currently over 20 different members of the TGF-β superfamily have been identified.

TGF-βs are secreted from cells as biologically inactive homodimers. The pro-TGF-β is cleaved into N- and C-terminal parts during secretion. The C-terminal mature TGF-β is biologically latent after secretion because it remains non-covalently bound to the N-terminal latency-associated propeptide (LAP). Both LAP and TGF-β are disulfide-linked homodimers. TGF-β is activated by dissociation from the LAP, which can be accomplished *in vitro* by heat, acid or alkaline treatment, deglycosylation or proteolysis by plasmin (Lawrence *et al.*, 1985; Lyons *et al.*, 1988; Miyazono and Heldin, 1989; Brown *et al.*, 1990).

TGF-βs are generally produced as large latent complexes, where LAP is bound to another high molecular weight protein (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988; Olofsson *et al.*, 1992), although in some transformed cell lines (Olofsson *et al.*, 1992; Dallas *et al.*, 1994) and malignant prostate neoplasia (Eklöv *et al.*, 1993) the large latent TGF-β complexes cannot be detected. Best characterized of the latent TGF-β-associated proteins is a large glycoprotein, called latent TGF-β binding protein-1 (LTBP-1) (Kanzaki *et al.*, 1990; Tsuji *et al.*, 1990). TGF-β has been found to be complexed to LTBP-1 in platelets (Miyazono *et al.*, 1988; Wakefield *et al.*, 1988), fibroblasts (Kanzaki *et al.*, 1990; Taipale *et al.*, 1994), smooth muscle cells (Flaumenhaft *et al.*, 1993a), glioblastoma (Olofsson *et al.*, 1992) and osteosarcoma cells (Dallas *et al.*, 1994) and in cells of epithelial and endothelial origin (Taipale *et al.*, 1995a). In platelets, LTBP-1 has an M_r of ~130 kDa, while in fibroblasts it is ~190 kDa. The smaller form detected in platelets is probably due to proteolytic processing.

Secreted LTBP-1 binds covalently to the ECM of cultured endothelial, epithelial and fibroblastic cells (Taipale *et al.*, 1994, 1995a), resulting in the deposition of latent TGF-β in the ECM. The matrix-bound large latent TGF-β complexes can be released by treatment with certain proteases like plasmin, thrombin, leukocyte elastase and mast cell chymase (Taipale *et al.*, 1992, 1995a; Benezra *et al.*, 1993; Falcone *et al.*, 1993). These proteases release TGF-β by cleaving LTBP at specific site(s). The apparent molecular weight of released LTBP-1 is approximately the same as that of LTBP-1 found in platelets. With the exception of high concentrations of plasmin, these proteases do not activate the large latent TGF-β (Lyons *et al.*, 1988; Taipale *et al.*, 1995a).

The released large latent complex can be activated after targeting of the complex to the surface of smooth muscle cells in co-cultures with endothelial cells. Activation in this model seems to involve matrix-released large latent TGF-β complexes (Flaumenhaft *et al.*, 1993a; Sato *et al.*, 1993). LTBPs are also needed for the secretion and correct folding of TGF-β (Miyazono *et al.*, 1991). All recent data indicate that TGF-β is normally deposited in the ECM,

and suggest that release from the ECM is probably the first step in the activation of TGF-β (reviewed in Flaumenhaft *et al.*, 1993b).

LTBP-1 contains 18 epidermal growth factor (EGF)-like repeats and four novel 8-Cys repeats (also called LTBP-like repeats), which appear to be relatively rare. EGF-like domains are considered to be important in some protein–protein interactions (Appella *et al.*, 1988; Davis *et al.*, 1990), whereas the role of 8-Cys repeats has not been elucidated thus far. Recently, two other LTBPs, LTBP-2 and LTBP-3, have been cloned and found to be similar to LTBP-1 in domain structure (Morén *et al.*, 1994; Li *et al.*, 1995; Yin *et al.*, 1995). In addition to LTBPs, fibrillins, components of extracellular microfibrils, also contain several copies of 8-Cys repeats.

At least LTBPs-1 and -2 have two or more alternatively spliced forms, the larger form containing an ~350 amino acid extension at the N-terminus. This variation is likely to play a role in the matrix association, as the larger form of LTBP-1 is deposited in the ECM more efficiently than the smaller splice variant (Olofsson, 1995).

The present study was carried out to localize the sites of interaction between LTBP-1 and the small latent TGF-β complex. We found that the Cys33 of TGF-β1–LAP is required for the interaction with LTBP-1. β1–LAP associates specifically with the third 8-Cys repeat of LTBP-1, providing for the first time a biological function for this type of protein domain.

Results

Co-expression of TGF-β1 and LTBP-1 in CHO cells leads to the secretion of the large latent complex

TGF-βs are secreted as large latent complexes by most cells studied. To address the question of whether the secretion of the small latent complex observed in some cell lines results from the lack of LTBP(s), we produced CHO cell clones overexpressing both TGF-β1 and LTBP-1.

Aliquots of culture medium from normal CHO cells were analyzed by SDS–PAGE under non-reducing conditions, followed by immunoblotting for TGF-β1. As the antibodies against the human β1–LAP did not recognize endogenous hamster LAP, antibodies for TGF-β1 were used. Previously, it has been shown that a fraction of TGF-β is not properly proteolytically cleaved apart from the LAP during secretion and they remain covalently bound to each other. It was found that CHO cells secrete TGF-β1 in a complex, where TGF-β1–LAP is bound to a large protein (Figure 1A, lane 1). The small latent complex, devoid of this binding protein, was not detected. In cell clones overexpressing TGF-β1, the amount of the large latent complex was significantly increased (Figure 1A, lane 2). Further increased amounts of the large latent complex were found in cell clones overexpressing both TGF-β1 and the LTBP-1 (Figure 1A, lane 3). The complex co-migrated with the large latent TGF-β complex observed in control CHO cells. The results with overexpressed proteins were the same when anti-β1–LAP antibodies were used (data not shown). Because the levels of overexpressed LTBP-1 were quite low, we analyzed the cell-conditioned medium from CHO cell clones expressing TGF-β1 and the product from pJS-2. The expression of construct pJS-2, encoding the C-terminal part of LTBP-1, starting at the

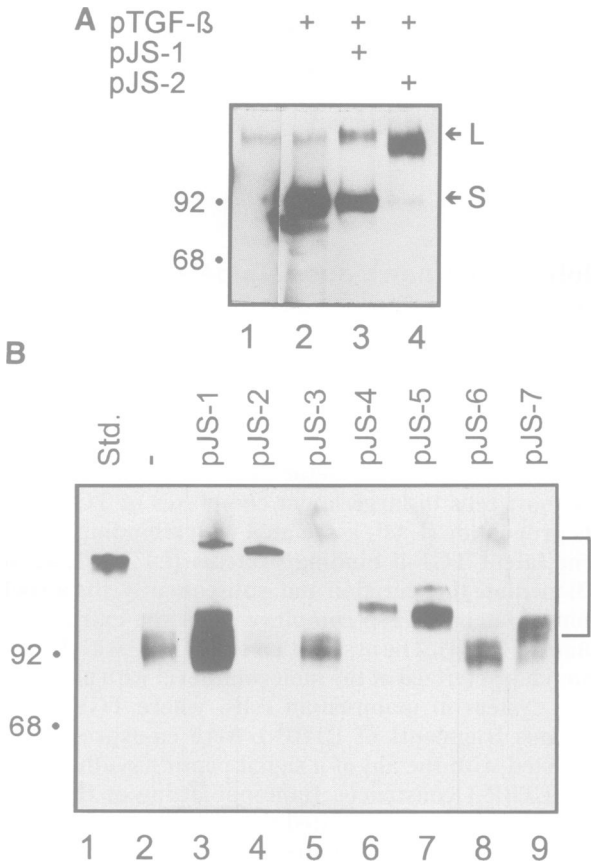

Fig. 1. Expression of the small and large latent complexes in CHO cells and identification of the domain of LTBP interacting with small latent TGF-β1. (**A**) Transfected stable CHO cell clones were grown to confluency. Cells were washed with serum-free MEM and the cell culture medium was harvested at 48 h. Secreted proteins of the conditioned medium samples were separated by 4–15% gradient SDS–PAGE under non-reducing conditions, followed by immunoblotting with antibodies for TGF-β1. Arrowheads indicate the small (S) and large (L) TGF-β1–LAP complexes. Key: (1) control CHO cells; CHO cells expressing products from (2) pTGFβ; (3) pTGFβ and pJS-1; (4) pTGFβ and pJS-2 (see Figure 2 for designations). Lane 1 is from a 10-fold longer exposure due to the low expression levels of endogenous TGF-β1. (**B**) COS-7 cells were transfected with pTGFβ and different pJS constructs (see Figure 2 for designations). After transfection, the cells were washed with serum-free MEM and the cell culture medium was harvested at 48 h. Secreted proteins of the conditioned medium were separated by 7.5% SDS–PAGE under non-reducing conditions, followed by immunoblotting with antibodies for β1–LAP. Standard (lane Std) contains 1 ng of large latent complex (see Materials and methods, M_r ~220 kDa). The bracket on the right indicates the M_r range of products where a part of LTBP-1 is bound to the small latent TGF-β1.

long domain of EGF-like repeats (see Figure 2), yielded almost totally large latent complexes and, subsequently, the secretion of the small latent complex was strongly decreased (Figure 1A, lane 4).

TGF-β1 binds to the third 8-Cys repeat of LTBP

To determine the domain(s) of LTBP responsible for the binding of the small latent TGF-β1, we prepared a series of deletion constructs of LTBP-1 (Figure 2). To allow the detection of the proteins expressed from these constructs, a sequence coding for the hemagglutinin (HA) epitope was added at the 3′ end of the constructs. In the constructs lacking the N-terminal region of LTBP-1, a partially

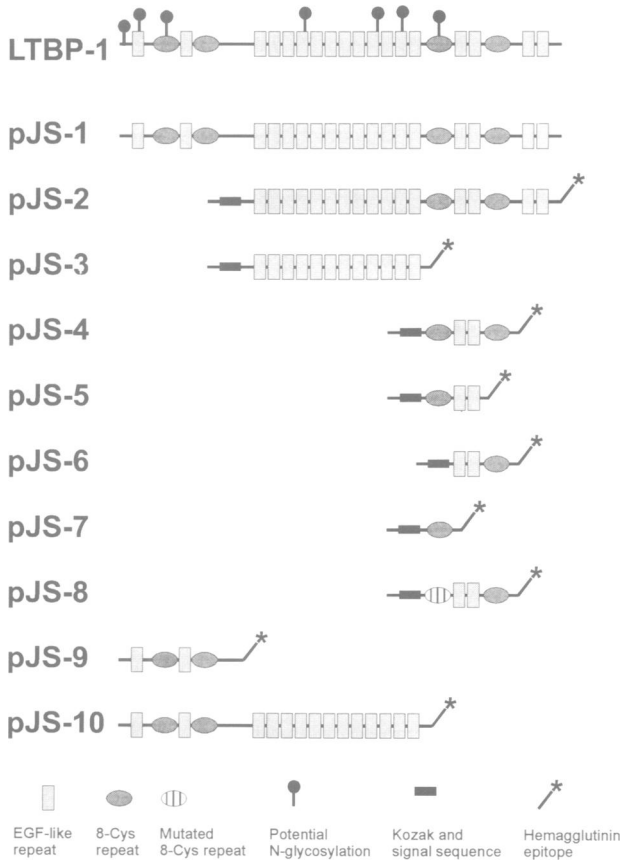

Fig. 2. Schematic representation of the cDNA constructs. The constructs were designed using different domains for the analysis of the site of LTBP-1 interacting with small latent TGF-β1 and ECM. All constructs were cloned in the polylinker of pcDNA3 eukaryotic expression vector in the sense orientation as *Hin*dIII–*Xba*I fragments. The mutated third 8-Cys repeat (Asn1039→Gln) in construct pJS-8 is indicated by shading. For clarity, the potential *N*-glycosylation sites have not been shown in the different LTBP constructs.

optimized Kozak sequence and an IgG heavy chain signal sequence were attached to the 5′ end (see Materials and methods). All constructs were sequenced. The constructs were co-expressed with TGF-β1 in COS-7 cells. In order to characterize the ability of a given LTBP-1 construct to bind the TGF-β1–LAP, cell-conditioned medium was analyzed by SDS–PAGE under non-reducing conditions, followed by immunoblotting for β1–LAP.

The protein from construct pJS-2, which encodes the C-terminal portion of LTBP-1 starting from the long domain of EGF-like repeats (amino acids 527–1394), was found to bind TGF-β1 very efficiently (Figure 1B, lane 4, see also Figure 1A, lane 4). The protein from this construct captured almost all of the small latent complex formed when overexpressed with TGF-β1, indicating a strong ability to associate with the small latent TGF-β1. In order to further localize the domain responsible for the association with TGF-β1, pJS-2 was divided into two new constructs. The protein encoded by pJS-3 consists of the long domain of EGF-like repeats (amino acids 527–1014) and pJS-4 of the third and fourth 8-Cys repeats with the two EGF-like repeats between them (amino acids 1006–1271). When these constructs were analyzed as above, only the protein encoded by pJS-4 was able to associate with TGF-β1–LAP (Figure 1B, lanes 5 and 6).

pJS-4 was further divided into two new constructs. The protein encoded by pJS-5 consists of the third 8-Cys repeat and the subsequent two EGF-like domains (amino acids 1006–1198) and pJS-6 consists of the fourth 8-Cys repeat and the two preceding EGF-like domains (amino acids 1075–1271). The protein from pJS-5 was able to associate with TGF-β1–LAP, whereas the protein encoded by pJS-6 was not (Figure 1B, lanes 7 and 8). Finally, the construct pJS-7, which encodes only the third 8-Cys repeat (amino acids 1006–1096), was made. The protein produced associated with TGF-β1–LAP (Figure 1B, lane 9), indicating that the TGF-β1 binding site of LTBP-1 is located in the third 8-Cys repeat of LTBP-1.

The expression of each LTBP-1 construct was confirmed by SDS–PAGE under non-reducing conditions, followed by immunoblotting with the monoclonal anti-HA antibody (data not shown). The proteins shown to be complexed with TGF-β1–LAP were seen both as free forms and in association with TGF-β1–LAP. The secretion of TGF-β1 was slightly lower when produced with construct pJS-6. The amount of recombinant proteins in the conditioned medium of cells transfected with constructs pJS-9 and pJS-10 was significantly lower than the amount of proteins expressed from other LTBP-1 constructs. The lack of association of TGF-β1–LAP with proteins encoded by pJS-9 (encoding amino acids 1–487) and pJS-10 (amino acids 1–1014) was confirmed after concentration of the cell culture media from COS cells co-transfected with pTGFβ and pJS-9 or pJS-10 by SDS–PAGE analysis followed by immunoblotting (data not shown).

The third 8-Cys repeat of LTBP-1 is glycosylated, but glycosylation is not required for binding of the small latent TGF-β1

The M_rs of products from constructs pJS-4, pJS-5 and pJS-6 were determined by SDS–PAGE under reducing conditions, followed by immunoblotting with the anti-HA antibody (Figure 3A). The recombinant, reduced protein encoded by pJS-7 was not detected readily by immunoblotting, possibly due to its small size and thus its inability to bind to nitrocellulose. Interestingly, the apparent M_r of the product from pJS-5 was ~8 kDa higher than from pJS-6, although the difference in calculated M_r is only 1 kDa, the product encoded by pJS-6 being larger than that encoded by pJS-5. Furthermore, the apparent M_rs of the products from constructs pJS-4, pJS-5 and pJS-6 were 8–15 kDa higher than those calculated from the amino acid sequence. Since the third 8-Cys-like repeat in all three LTBPs contains a potential *N*-glycosylation site (Asn–X–Ser/Thr), we deglycosylated the proteins from the conditioned medium of COS-7 cells, transfected with pJS-4, by *N*-glycosidase F. This treatment, which removes all *N*-linked sugar residues, slightly reduced the apparent M_r of the product of construct pJS-4. In order to detect whether glycosylation is required for the association with TGF-β1, we mutated Asn1039 in the third 8-Cys repeat in construct pJS-4 into a Gln codon. Formation of the complex between β1–LAP and the product from pJS-8 indicated that glycosylation of Asn1039 is not required for the association of the third 8-Cys repeat with small latent TGF-β1 (Figure 3B, lanes 2 and 3).

Cys33 of TGF-β1–LAP is required for covalent association with LTBP-1

The LAP of TGF-β1–LAP contains three cysteines. Cys225 and Cys227 are important for the dimerization of LAP, while Cys33 does not contribute to the stability of the LAP dimer (Brunner *et al.*, 1989). In order to characterize the function of Cys33 of TGF-β1–LAP in binding to LTBP-1, this cysteine was mutated to serine. The mutated TGF-β1 was transfected with constructs pJS-1, pJS-2, pJS-4, pJS-5 and pJS-7 to COS-7 cells as described above, and the association of products from LTBP-1 constructs with TGF-β1–LAP was analyzed. It was found that mutation of the Cys33 of TGF-β1–LAP abolished the covalent association with LTBP-1, as shown by immunoblotting (Figure 4). Mutated TGF-β1–LAP migrated as a doublet in SDS–PAGE, the higher M_r band being pro-TGF-β1, as reported by Brunner *et al.* (1989).

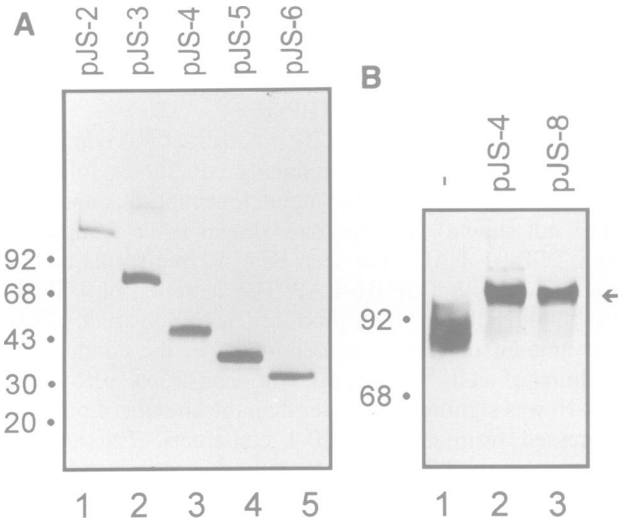

Fig. 3. Role of *N*-glycosylation of the third 8-Cys domain in the association with small latent TGF-β1. (**A**) COS-7 cells were transfected with different pJS constructs. After transfection, the cells were washed with serum-free MEM and the cell culture medium was harvested at 48 h. Secreted proteins of the conditioned medium were separated by 4–15% gradient SDS–PAGE under reducing conditions, followed by immunoblotting with the antibody against the HA epitope attached to the constructs. (**B**) COS-7 cells were transfected either with constructs pTGFβ and pJS-4 or with constructs pTGFβ and pJS-8, in which the *N*-glycosylation site of the third 8-Cys repeat is mutated to Gln (see text). Subsequently, the cells were washed with serum-free MEM and the conditioned medium was harvested at 48 h. Aliquots of medium were separated by 4–15% gradient SDS–PAGE under non-reducing conditions, followed by immunoblotting with antibodies for β1–LAP. Key: COS-7 cells expressing proteins from (1) pTGFβ only; (2) pTGFβ and pJS-4; (3) pTGFβ and pJS-8.

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

In order to characterize the region of LTBP-1 essential for the covalent association with the ECM, we produced CHO cell clones expressing both TGF-β and the proteins encoded by either pJS-1 or pJS-2. The deposition of TGF-β in the ECM was analyzed using a deoxycholate-insoluble, plasmin-digested matrix fraction (see Taipale *et al.*, 1994). In cells expressing the protein from pTGFβ only, the human TGF-β1 produced was deposited in the ECM and released by plasmin (Figure 5A, lane 2). In cell clones expressing the proteins from pTGFβ and pJS-2, which encodes the C-terminal part of LTBP-1 starting at the long domain of EGF-like repeats (see Figure 2), the level of ECM-associated, plasmin-released TGF-β1 was similar to that in cell clones expressing TGF-β1 alone (Figure 5A, lane 4). In cell clones expressing both pTGFβ and pJS-1, which encodes the whole smaller splice form of LTBP-1, the amount of matrix-bound TGF-β1 was increased ~10-fold (Figure 5A, lane 3) compared with the clones expressing TGF-β1 alone or TGF-β1 and the protein encoded by pJS-2. This is in agreement with the data from the conditioned medium, where cells expressing the protein encoded by pJS-2 produced higher amounts of the large latent complex (Figure 1A). The expression levels of TGF-β1 in all cell clones used were comparable, as detected by immunoblotting from cell culture medium (data not shown). The data shows that the smaller splice form of LTBP-1 is able to bind TGF-β1 and enhance its deposition in the ECM. Cell clones expressing proteins

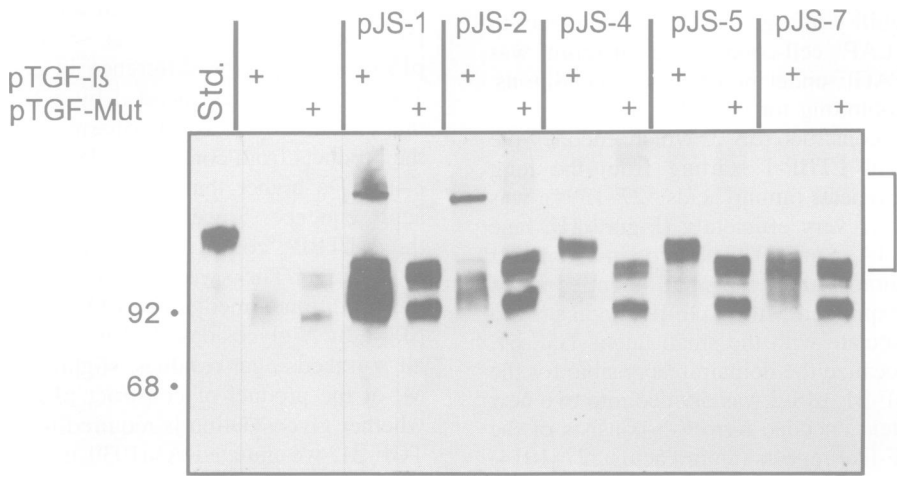

Fig. 4. Requirement for the Cys33 of TGF-β1–LAP for covalent interaction with LTBP. COS-7 cells were co-transfected with the LTBP-1 constructs which bind TGF-β1 and pTGFβ or pTGF-Mut, as indicated in the figure. Subsequently, the cells were washed with serum-free MEM. Cell-conditioned medium was harvested at 48 h and analyzed by 7.5% SDS–PAGE under non-reducing conditions, followed by immunoblotting for TGF-β1–LAP. The bracket on the right indicates the M_r range of products where a part of LTBP-1 is bound to the small latent TGF-β1. Standard (lane Std) contains 1 ng of large latent complex (see Materials and methods, M_r ~220 kDa).

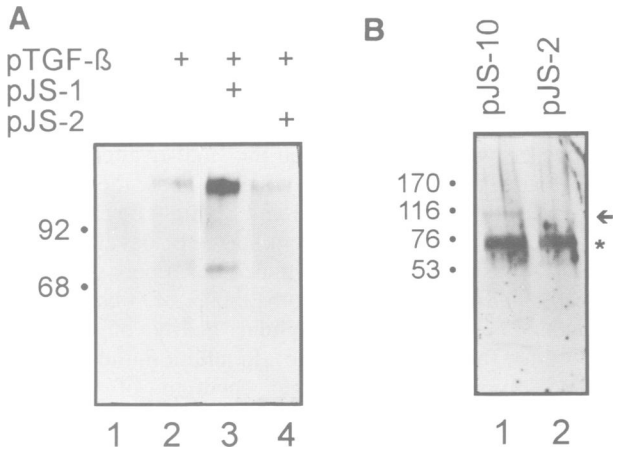

Fig. 5. ECM deposition of TGF-β1. (**A**) CHO cell clones stably transfected with pTGFβ and pJS-1 or pJS-2 (as indicated in the figure) were grown to confluency. The cells were lysed with sodium deoxycholate and washed with phosphate-buffered saline. The deoxycholate-insoluble fraction containing the ECM was partially solubilized with plasmin digestion. The samples were analyzed subsequently by 4–15% gradient SDS–PAGE under non-reducing conditions, followed by immunoblotting with β1–LAP antibodies. Key: CHO cells expressing products from: (1) non-transfected control cells; (2) pTGFβ; (3) pTGFβ and pJS-1; (4) pTGFβ and pJS-2. (**B**) The ECMs from confluent, CHO cell clones stably transfected with pJS-10 and pJS-2, were isolated as above. The plasmin-digested samples were analyzed with 4–15% gradient SDS–PAGE under reducing conditions, followed by immunoblotting for the HA epitope at the C-terminus of the protein encoded by pJS-10 and pJS-2. Key: (1) CHO cell clones expressing products from pJS-10; (2) pJS-2. Arrowhead indicates the protein released from the matrix encoded by construct pJS-10. Asterisk (*) indicates the plasmin background.

from both pJS-2 and pTGFβ did not contain enhanced levels of TGF-β1 in the matrix as compared with the cell clones expressing TGF-β1 only, indicating that the N-terminal region of LTBP-1 is essential for the accumulation of LTBP-1 in the ECM. The association was characterized further using stable CHO cell clones overexpressing proteins from constructs pJS-10 and pJS-2. The expressed proteins were detected in deoxycholate-insoluble, plasmin-digested ECM by immunoblotting for the HA epitope. Only the protein encoded by pJS-10 was found to be deposited in the ECM (Figure 5B, lanes 1 and 2).

Discussion

The present study describes the molecular basis for the association of the LTBP-1 with the small latent TGF-β1 and with the ECM. We found that CHO cells normally produce latent TGF-β1–LAP in a complex with a high molecular weight protein, which might be a hamster homolog to LTBP, as suggested by its migration in SDS–PAGE. When TGF-β1–LAP is overexpressed, the amount of secreted large latent complex is substantially increased, indicating that these cells normally produce an excess of LTBP-like proteins compared with TGF-β, or that increased expression of TGF-β induces the expression of LTBP-like protein(s). However, overexpression of TGF-β1 apparently saturates the endogenous LTBP-like proteins, and the small latent TGF-β becomes the predominant secreted form (see also Gentry *et al.*, 1987). Concomitant overexpression of LTBP-1, and especially the soluble LTBP-1 fragment capable of binding TGF-β1, decreases the secretion of small latent TGF-β1 and induces the secretion of the large latent form (see also Morén *et al.*, 1994). Thus, the observed secretion of the small latent complex in some cultured cell lines (Olofsson *et al.*, 1992; Dallas *et al.*, 1994) and intracellular accumulation of TGF-β1 without LTBP-1 in malignant prostate neoplasia cells (Eklöv *et al.*, 1993) seems to be a consequence of the lack of LTBP-like proteins. This is in accordance with previous results, where pulse-labeling experiments indicated that the small latent TGF-β1 is secreted very slowly (Miyazono *et al.*, 1991; 1992).

LTBPs-1, -2 and -3 consist mainly of EGF-like and 8-Cys repeats. There have been no suggestions for a function for the 8-Cys repeats. In the present study, the TGF-β1–LAP binding site of LTBP-1 was located to the third 8-Cys repeat by cDNA deletion analysis. The association of TGF-β1–LAP with LTBP-1 is thought to occur only inside the cells. Since the redox potential of the extracellular milieu is strongly in favor of disulfide bond formation, the TGF-β binding 8-Cys repeat seems to be able to adopt two different conformations and exchange cysteine disulfide bonds between optionally bound TGF-β and itself or other, uncharacterized protein(s).

There is a potential *N*-glycosylation site in the third 8-Cys repeats of all LTBPs. Mutation of the asparagine residue to glutamine in this site of LTBP-1 abolished *N*-glycosylation, but did not change the ability of this repeat to associate with the small latent TGF-β1. The apparent M_rs of the products from constructs encoding the third 8-Cys repeat were 10–15 kDa higher than those calculated from the sequence as detected from reduced samples in gradient SDS–PAGEs. The treatment of the protein encoded by construct pJS-4 with *N*-deglycosylating enzyme or the mutation of the conserved *N*-glycosylation consensus site reduced the observed M_r only slightly. These data suggest that there might be another unknown amino acid modification in the third 8-Cys repeat. This is further evidenced by the observed M_r of the protein coded by pJS-6, which does not display strongly anomalous migration in gradient SDS–PAGE. As the other 8-Cys repeats of LTBP-1 were not able to bind β1–LAP, this implies that the putative protein modification could be required for the association.

Another obvious difference between the 8-Cys repeats of LTBPs-1, -2 and -3 and fibrillins is the number of cysteine residues, since some of these domains have only seven cysteines. These repeats are also widely called hybrid domains between the EGF-like and 8-Cys repeats. The odd number of cysteines might indicate that one or more of the cysteines are oxidized to the sulfhydryl groups of some other proteins and that, in general, these domains are important in mediating protein–protein interactions.

As TGF-βs are a part of a large protein superfamily, the members of which all have a similar structure, a question can be raised as to whether other members of this superfamily also are deposited in the ECM by an association with proteins containing 8-Cys repeats. Fibrillins-1 and -2 are structurally very similar to the LTBPs (Sakai *et al.*, 1986; Lee *et al.*, 1991; Zhang *et al.*, 1994) and, accordingly, fibrillins also contain several 8-Cys repeats. We carried out a computer analysis (Thompson *et al.*, 1994) of the 8-Cys repeats of LTBPs and fibrillins

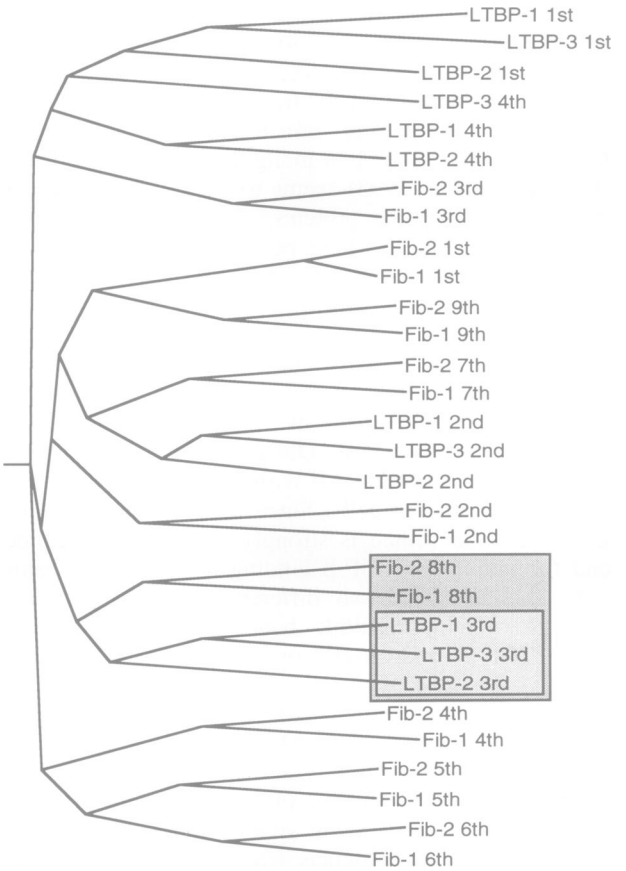

Fig. 6. The relatedness of the 8-cys repeats of LTBPs and fibrillins. The amino acid sequences coding for the 8-Cys repeats of LTBPs-1, -2 and -3, and fibrillins-1 and -2 were aligned by Clustal W (version 1.4, Thompson *et al.*, 1994). The graphics was generated by Phylip (version 3.5, Joseph Felsenstein) using pre-set values. For clarity, the hybrid domains between an EGF-like and a 8-Cys repeat are shown also as 8-Cys repeats, because of their strong sequence similarity to these repeats. It was found that the eighth 8-Cys repeat of fibrillins-1 and -2 and the 3rd 8-Cys repeat of LTBPs-2 and -3 were the most similar to the 3rd 8-Cys repeat of LTBP-1, which is responsible for the binding of TGF-β1–LAP.

(Figure 6). The eighth 8-Cys repeats of fibrillins displayed the highest similarity to the third 8-Cys repeats of LTBPs. It should be noted that the most similar 8-Cys repeats of fibrillins and other LTBPs to the third 8-Cys repeat of LTBP-1 were always the second from the C-terminus, thus making those repeats the most likely potential candidates for TGF-β binding. In addition, these proteins may mediate the association of growth factors structurally similar to TGF-βs with the ECM, targeting deposition to the microfibrillar networks.

The data from the Cys33-mutated β1–LAP suggest that the covalent bond between TGF-β1–LAP and LTBP is formed by the Cys33 of the LAP propeptide, or at least that the Cys33 is required for the covalent association. This is in accordance with previous results, where the function of the other two cysteines (Cys223 and Cys225) of LAP was found to be in the dimerization of the LAP propeptide (Brunner *et al.*, 1989).

The other function of LTBPs-1 and -2, and probably also LTBP-3, is their fate to be deposited in the ECM. The region required for covalent interaction with the ECM was mapped in the region of the first 400 amino acids of LTBP-1. The importance of the N-terminus for the ECM interaction is in accordance with Olofsson (1995), who has cloned the N-terminally extended form of human LTBP-1 and found that it interacts even more efficiently with the growth substratum than the smaller alternatively spliced form used in the present study. Thus, the alternatively spliced forms of LTBPs-1 and -2 might be expressed in different tissues or at different stages of development.

LTBP-1 associates with extracellular fibers morphologically indistinguishable from those of fibronectin–collagen fibers in the pericellular matrix of cultured fibroblasts (Taipale *et al.*, 1995b). In addition, the LTBP-1 isolated from the conditioned medium of cultured fibroblasts is able to interact with cellular fibronectin *in vitro*. This interaction was not seen with *in vitro* expressed LTBP-1. It is thus possible that LTBPs-1, -2 and -3 are secreted with some accessory protein(s) that assist in their deposition in the ECM. The absence of adequate amounts of this accessory molecule, when LTBP-1 is overexpressed, could prevent the secretion of LTBP-1. This could explain, in part, the results in this work concerning the low expression levels from the constructs containing the N-terminal parts of LTBP-1. The secretion of elastin is a known example of this kind of a mechanism (Mecham, 1991). Also, the interaction of LTBPs-1, -2 and -3 with the ECM could require some enzymes like transglutaminase to link LTBPs to the ECM. Taken together, the data from the interaction of LTBP-1 with TGF-β1–LAP and, further, the association with extracellular fibers is suggestive of a domain structure (Figure 7).

Materials and methods

Reagents

Taq thermostable DNA polymerase was from Perkin Elmer Cetus (Branchburg, NJ) and *Pfu* from Stratagene (La Jolla, CA). All other molecular biology enzymes were from Promega (Madison, WI) and New England Biolabs (Beverly, MA). pcDNA3 eukaryotic expression vector was from InVitrogen (Oxford, UK). The *N*-glycosidase F was from Boehringer Mannheim (Mannheim, Germany). Recombinant human large latent TGF-β1 LTBP-1 was a kind gift from Dr Hideya Ohashi (Kirin Brewery Co. Ltd, Gumma, Japan).

Cell culture

SV40-transformed African green monkey kidney cells (COS-7, American Type Culture Collection, Rockville, MD) were cultured in Eagle's modification of minimal essential medium (MEM) containing 10% fetal calf serum (FCS), 100 IU/ml penicillin and 50 μg/ml streptomycin. Chinese hamster ovary cells (CHO K7, ATCC) were cultured in the medium described above supplemented with 0.2% bovine serum albumin (BSA). All experiments were carried out in serum- and BSA-free conditions. For the collection of conditioned medium, cells were washed twice with serum-free medium, and the subsequently added serum-free medium was collected after 48–72 h.

Antibodies

Rabbit anti-human TGF-β1 (#627) and TGF-β1–LAP antibodies (#680) have been described previously (Taipale *et al.*, 1992, 1995a). Antibodies #680 are human specific, and they do not recognize the hamster β1–LAP. The antibodies were affinity-purified using the antigenic peptide. Mouse monoclonal antibody 12CA5 against the HA epitope was purchased from Berkeley Antibody Co. (Berkeley, CA) and used as purified IgG.

cDNA constructs

Full-length cDNAs for the smaller alternatively spliced transcript of human LTBP-1 and human TGF-β1 were obtained from Dr C.-H.Heldin

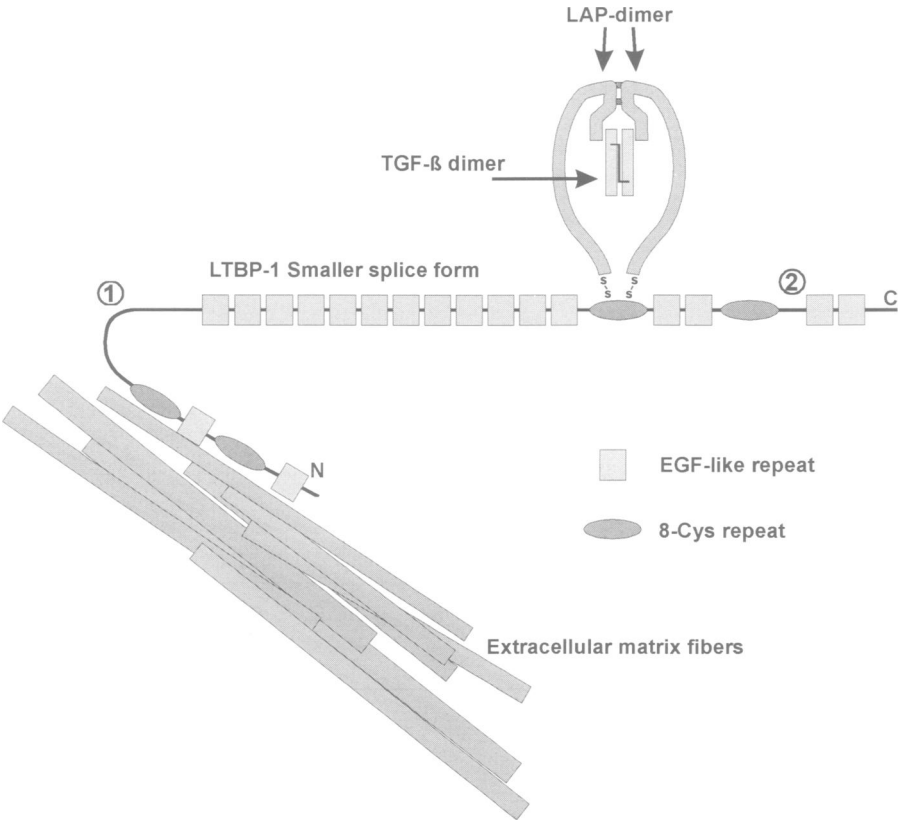

Fig. 7. The structure of the large latent TGF-β complex. Schematic representation of the ECM-associated large latent TGF-β complex. TGF-β itself is a covalent dimer held together by the disulfide bond formed by Cys77 (Daopin *et al.*, 1992; Schlunegger and Grütter, 1992). The LAP portion is dimerized by two dislfide bonds at Cys223 and Cys225 (Brunner *et al.*, 1989). The third 8-Cys repeat of LTBP covalently associates with the LAP region of TGF-β1, possibly by direct disulfide bonding to Cys33 of β1–LAP. The N-terminal region of LTBP-1 interacts with extracellular microfibrils. LTBPs have a proline and basic amino acid-rich region between the second 8-Cys repeat and the third EGF-like repeat (1); this region could be the target for proteases that release the latent complex from the ECM (Taipale *et al.*, 1994, 1995a). Also, the form of LTBP-1 found in platelets (M_r ~130 kDa) might be cleaved at this region by the endoproteinases in the regulated secretory pathway. Another possible proteinase-sensitive region is located near the C-terminus of LTBP-1, between the fourth 8-Cys repeat and the two last EGF-like repeats (dibasic RR sequence) (2). It should be noted that the dimeric TGF-β1 has an antiparallel structure but, for simplicity, this has not been taken into account here.

(Ludwig Institute for Cancer Research, Uppsala, Sweden) (Derynck *et al.*, 1985; Kanzaki *et al.*, 1990).

Construct pEpitope contained a synthetic HA epitope, generated from two partially overlapping oligonucleotides. Oligonucleotides were annealed and filled with Klenow polymerase. Subsequently, the fragment was cloned as a *Bam*HI–*Xba*I fragment to pcDNA3 eukaryotic expression vector. This epitope coded for the amino acid sequence ENSDYKDI-YDVPDYASLQI-STOP, and had a *Xho*I restriction site next to a *Bam*HI site at the 5′ end. A partially optimized Kozak sequence (CCACCATGA) (Kozak, 1986) and mouse IgG heavy chain signal sequence (Stern *et al.*, 1987) were constructed from two partially overlapping oligonucleotides, as above. This signal sequence coded for the amino acids MKCSTVIFFL-MAVVTGVNS and was cloned into pEpitope as a *Hin*dIII–*Bam*HI fragment, generating construct pSignal.

Various segments of the cDNA for human LTBP-1 were amplified by PCR with oligonucleotides containing appropriate restriction endonuclease recognition sites at the 5′ end. Amplification products were cloned as *Bam*HI or *Bgl*II–*Xho*I fragments into pSignal, generating constructs pJS-2 (nucleotides 1659–4269, human LTBP-1 has a stop codon at 4273), pJS-3 (1667–3132), pJS-4 (3104–3904), pJS-5 (3104–3681), pJS-6 (3312–3904), pJS-7 (3104–3378), except segment 75–1553 which was cloned into pEpitope, thus generating construct pJS-9. pJS-10 (nucleotides 75–3132) was generated by cloning the *Bsu*36I–*Pvu*I insert from pJS-1 into a similarly cut pJS-3 backbone. All these constructs were designed to retain the open reading frame (ORF) starting from the native or synthetic Kozak sequence, and ending at the stop codon at the 3′ end of the epitope. Construct pJS-1 coded for the whole ORF of the small splice variant of LTBP-1, which was cloned as a blunted *Dra*I–*Bsr*DI fragment into the *Eco*RV site of pcDNA3. The numbering of nucleotides refers to the sequence of LTBP-1 published by Kanzaki *et al.* (1990).

Construct pJS-8 is the same as pJS-4, except that the codon AAT coding for Asn1039 was mutated to the Gln codon CAA by PCR-mediated mutagenesis.

Construct pTGFβ contained the full-length cDNA for human TGF-β1, cloned into pcDNA3 as an *Eco*RI–*Pst*I fragment. In construct pTGF-Mut, the codon TGC coding for Cys33 was mutated to TCC to code for serine.

All PCR-generated fragments were sequenced using an ABI 373A automatic DNA sequencer. The regions of LTBP-1 coded by pJS constructs are presented schematically in Figure 2.

Transfection of cell lines

Cells were seeded in 100 mm diameter tissue culture dishes at a density of 1×10^6 cells/dish and transfected the following day with 15 μg of the plasmids indicated. Transfections were carried out using a calcium phosphate transfection kit (Gibco-BRL, Gaithersburg, MD) according to the manufacturer's instructions.

After transfection, the cells were fed with fresh medium containing 10% FCS. A day later, the cells were washed with serum-free medium, and the conditioned medium was collected after 48 h (COS-7 cells), or the selection was started with G418 (0.7 mg/ml, Gibco-BRL) in complete medium (CHO cells). All CHO cell clones were cloned by two subsequent cloning steps.

Isolation of the ECM, SDS–PAGE and immunoblotting

ECMs were prepared essentially as described by Hedman *et al.* (1979), with the modifications of Taipale *et al.* (1994), including the treatment with plasmin to solubilize the high molecular weight complexes containing LTBP-1.

SDS–PAGE was carried using a commercial 4–15% gradient or 7.5% standard Laemmli gels (Bio-Rad Co., Richmond, VA). The gels were electrophoretically transferred to nitrocellulose membranes and treated at 80°C for 2 h in order to fix the proteins.

Immunodetection of the proteins was performed as described (Taipale *et al.*, 1994), except that for 12CA5 a washing buffer containing 0.05% Tween-20, 140 mM NaCl and 25 mM Tris–HCl buffer pH 7.4 was used.

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