Latent transforming growth factor-β complex in Chinese hamster ovary cells contains the multifunctional cysteine-rich fibroblast growth factor receptor, also termed E-selectin-ligand or MG-160

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Transforming growth factor-β (TGF-β) is secreted as latent high molecular mass complexes from producer cells. The N-terminal precursor remnant, also called latency-associated peptide (LAP), forms a non-covalently linked complex with TGF-β and confers the latency to TGF-β. In human platelets and certain other cell types, latent TGF-β binding protein-1 (LTBP-1) is disulphide-linked to LAP, and forms complexes of more than 230 kDa. In addition, LTBP-2 and -3, which are structurally similar to LTBP-1, can be part of latent TGF-β complexes. In Chinese hamster ovary (CHO) cells transfected with the TGF-β1 cDNA, a major part of the latent TGF-β secreted into the medium is a 100-kDa small latent complex containing TGF-β and LAP. In addition, we found two other forms of latent TGF-β complexes, i.e. a 220-kDa complex containing LTBP-1, and a 220-kDa complex containing a 140-kDa protein. Purification of the 140-kDa component, termed latent TGF-β complexed protein-1 (LTCP-1), followed by amino acid sequencing and cDNA cloning from a CHO cell cDNA library, revealed that it is a hamster counterpart of a previously identified, multifunctional protein known as chicken cysteine-rich fibroblast growth factor (FGF) receptor, mouse E-selectin-ligand and rat MG-160 (a 160-kDa membrane sialoglycoprotein of the Golgi apparatus). Immunoprecipitation of LTCP-1 and TGF-β1 from CHO cells stably transfected with TGF-β1 precursor cDNA revealed that the expressed protein forms a complex with LAP, and that a major part of the complex is secreted. Northern blot analysis showed that mRNA for LTCP-1 was expressed in large amounts in testis, ovary and placenta, but less abundantly in other tissues. These results suggest that TGF-β, produced in certain cell types, may form a complex with LTCP-1, which may have different properties compared with other latent TGF-β complexes. It remains to be investigated whether the complex formation between LTCP-1 and TGF-β1 also occurs in other cells, whether the association between them occurs in the Golgi complex, and whether it affects the interaction of LTCP-1 with FGF or E-selectin.

INTRODUCTION

Transforming growth factor-β (TGF-β) is a family of 25-kDa dimeric proteins that regulate the growth, differentiation, adhesion and migration of various cell types (reviewed in [1]). Three different isoforms of TGF-β, i.e. TGF-β1, -β2 and -β3, with similar but distinct biological activities, have been identified in mammalian cells. TGF-β inhibits the growth of most cell types, whereas it stimulates the production of extracellular matrix proteins, and is a potent immunosuppressive factor *in vivo*.

TGF-βs are secreted from producer cells as latent, high molecular mass complexes (reviewed in [2]). Various forms of latent TGF-β complexes have been identified in different cell types. Latent TGF-β in human platelets [3,4], rat platelets [5] and certain other cells [6–8] is composed of mature TGF-β, the N-terminal remnant of the TGF-β precursor dimer (latency-associated peptide, LAP) and latent TGF-β binding protein-1 (LTBP-1). In contrast, the latent form of TGF-β secreted from cells transfected with TGF-β cDNA [9], as well as certain osteoblasts [10] and glioblastoma cells [7], is a 100-kDa complex composed of the mature TGF-β and LAP only. LAP remains in a non-covalent complex with the mature TGF-β after proteolytic processing, and confers the latency of TGF-β [9], which is the basis for its name.

LTBP is a 125–210 kDa glycoprotein that is disulphide-bonded to LAP in the latent TGF-β complex [11,12]. LTBP occurs in various sizes, which may be due to proteolytic processing as well as alternative splicing. More than 60 % of the structure of LTBP is composed of two different types of cysteine-rich repeat sequences, including 16–18 epidermal growth factor (EGF)-like repeats and 3–4 copies of a repeat containing eight cysteine residues. EGF-like repeats are found in several other proteins, and have been suggested to be of importance in protein–protein interactions [13]. The function of the eight-cysteine repeats is unknown. LTBP-1 forms part of a larger superfamily of proteins that all contain EGF-like repeats and eight-cysteine repeats, and includes LTBP-1, -2 and -3 [14,15] and fibrillin-1 and -2 [16–18].

Whereas LTBP appears not to have a direct role in TGF-β latency, it has been shown to play an important role in assembly, secretion and association of the TGF-β complex with the extracellular matrix and subsequent activation of TGF-β [6,14,19].

Abbreviations used: CFR, cysteine-rich FGF receptor; CHO, Chinese hamster ovary; EGF, epidermal growth factor; ESL-1, E-selectin-ligand; FGF, fibroblast growth factor; LAP, latency-associated peptide; LTBP, latent TGF-β binding protein; LTCP, latent TGF-β complexed protein; MG-160, 160-kDa membrane sialoglycoprotein of the Golgi apparatus; TGF-β, transforming growth factor-β; 4-VP, 4-vinylpyridine.

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During the purification of latent TGF-β1 complexes from the conditioned medium of Chinese hamster ovary (CHO) cells that were transfected with TGF-β1 cDNA, we identified a high molecular mass, latent TGF-β1 complex, which contains a molecule distinct from LTBP-1, -2 and -3, and interacts with the TGF-β1 precursor. Purification and cloning of latent TGF-β complexed protein-1 (LTCP-1) revealed that it is a hamster counterpart of a protein known as chicken cysteine-rich fibroblast growth factor (FGF) receptor (CFR) [20,21], mouse E-selectin-ligand (ESL-1) [22] and rat MG-160 (a membrane sialoglycoprotein of the Golgi apparatus) [23,24]. The characterization and possible roles of complex formation between TGF-β1 precursor and this multifunctional protein are discussed in this paper.

EXPERIMENTAL

Purification of a latent TGF-β complex from CHO cells transfected with TGF-β1 cDNA

Conditioned medium from CHO cells transfected with human TGF-β1 cDNA was given by Dr. Hideya Ohashi (Kirin Brewery, Co., Tokyo, Japan). The concentrated conditioned medium (10–20 ml) was loaded on to an S-Sepharose cation-exchange chromatography column (Pharmacia LKB) pre-equilibrated with 10 mM phosphate buffer (pH 7.4). The bound protein was eluted by a gradient from 0 to 1 M NaCl. Aliquots of fractions were tested for TGF-β activity using inhibition of [^{3}H]thymidine incorporation in mink lung cells as an assay, with or without prior acidification to activate latent TGF-β [3]. The material eluting between 0 and 0.5 M NaCl was found to contain TGF-β bioactivity after acidification, and was pooled for further purification. The fractions from S-Sepharose chromatography were freeze-dried, resuspended in 600 μl of water and loaded on to a Superose 6 gel chromatography column (Pharmacia LKB) pre-equilibrated with TBS [137 mM NaCl/3 mM KCl/25 mM Tris base (pH 7.4)]. Fractions of 0.5 ml were collected and subjected to SDS/PAGE, followed by immunoblotting or staining for protein by Coomassie Brilliant Blue or silver.

SDS/PAGE and immunoblotting

Protein samples were mixed with equal volumes of SDS sample buffer [100 mM Tris/HCl (pH 8.8)/0.01 % (w/v) Bromophenol Blue/30 % (v/v) glycerol/4 % (w/v) SDS] in the presence or absence of 20 mM dithiothreitol. Then samples were boiled for 3 min and the reduced samples were alkylated with iodoacetamide and analysed by SDS/PAGE using 5–15 % (w/v) polyacrylamide gels [25]. After electrophoresis, proteins were transferred to nitrocellulose membranes (Hybond-C super; Amersham) by electroblotting in a buffer containing 20 % (v/v) methanol, 150 mM glycine, 20 mM Tris/HCl and 0.002 % SDS at 400 mA (80 V) for 3 h. The proteins on the nitrocellulose membranes were detected by polyclonal rabbit antisera [7] using an enhanced chemiluminescence Western blotting system (Amersham). Polyclonal antisera against LTBP-1 (Ab39) [11] and a peptide corresponding to the TGF-β1 LAP (Ab96) [7] were used for immunoblotting.

Isolation of peptides and amino acid sequence analysis

Selected fractions from Superose 6 chromatography were desalted on a C4 reverse-phase column. After loading the sample, the column was washed with 0.1 % (v/v) trifluoroacetic acid and the sample was eluted by a gradient of acetonitrile. After freeze-drying, the proteins were resuspended in PBS and reduced with 50 mM dithiothreitol for 10 min at room temperature, followed by alkylation with 125 mM 4-vinylpyridine (4-VP). The use of 4-VP produces pyridyl-ethyl-cysteine residues that can be identified by their specific absorbance at 254 nm. After separation by SDS/PAGE on a 7 % acrylamide gel, proteins were visualized by a modified non-fixing Coomassie staining method [26]. After excising components of 140 kDa and 40 kDa, they were subjected to tryptic in-gel digestion according to Rosenfeld et al. [26], with the modification that the gel pieces were allowed to dry completely before absorbing the protease solution [27]. The resulting protein fragments were isolated by reverse-phase HPLC on a μRPC C2/C18 SC 2.1/10 column, operated in a SMART System (Pharmacia Biotech.). Apparently non-homogeneous fractions were rechromatographed on a different support (Sephasil C8, 5 μm, SC 2.1/10), giving a complementary retention pattern. Amino acid sequences were determined by use of an automated gas-phase sequencer (Applied Biosystems Protein Sequencer, model 470A with an on-line phenylthiohydantoin amino acid analyser, model 120A; Applied Biosystems, Foster City, CA, U.S.A.).

Isolation and characterization of cDNA clones

Based on the amino acid sequence of peptide no. 4 (Figure 2C), a probe of 32 nucleotides was chosen based on hamster sequence preferences [28], and with inosines in the most ambiguous positions. The probe was labelled at the 5′-end with [γ-^{32}P]ATP by T4 polynucleotide kinase (New England Biolab). An oligo(dT)-primed CHO-K1 cell cDNA library in a Uni-ZAP XR vector with 2×10^6 independent clones (Stratagene) was screened with the probe. Hybridization was performed in 6 × SSC (1 × SSC = 0.15 M NaCl/0.015 mM sodium citrate), 20 mM sodium phosphate, pH 7.4, 0.4 % SDS, 5 × Denhardt's solution and 100 μg/ml salmon sperm DNA at 54 °C for 24 h. The filters were washed three times in 6 × SSC/0.1 % SDS at 55 °C for 15 min each and subjected to autoradiography using Hyperfilm MP films (Amersham). A second screening was done in the same buffer as above; filters were then washed and subjected to autoradiography as before. Excision of positive clones *in vivo* was performed according to manufacturer's protocol and the inserts were recovered in pBluescript SK$^-$ vector. A clone with a 3.0 kb insert (CHO-5) was obtained. Nucleotide sequencing was performed by the dideoxy chain-termination method [29] on both strands using T7 DNA polymerase (Pharmacia) and oligonucleotide primers.

Since CHO-5 was not a full-length clone, rescreening of the cDNA library to obtain a full-length cDNA was done with an *Eco*RI/*Sph*I restriction fragment of 1.8 kb (AO22) from the 5′-part of clone CHO-5 labelled by Megaprime DNA labelling (Amersham). Hybridization was performed in 50 % (v/v) formamide, 5 × SSPE [1 × SSPE = 180 mM NaCl/10 mM sodium phosphate/25 mM EDTA (pH 7.5)], 2 × Denhardt's solution, 0.5 % SDS and 100 μg/ml salmon sperm DNA at 37 °C for 15 h. The filters were washed three times in 0.5 × SSC/0.1 % SDS at 55 °C for 20 min each and subjected to autoradiography. A second screening was done with a 5′-end-labelled oligoprimer made from the 5′-part of AO22. After the second screening, positive clones were analysed by restriction-enzyme cleaving and the longest clones were chosen for further analysis. The clones were excised and recovered in pBluescript SK$^-$ vector. A clone of 3.8 kb (CHO-31) was sequenced and further analysed.

Metabolic labelling and immunoprecipitation

The CHO-β1 stably transfected cell line was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium, supplemented with 20 % (v/v) fetal-bovine serum,

15 mM Hepes, 500 nM Methotrexate, 100 units/ml penicillin and 50 μg/ml streptomycin. Cells were grown in 25 cm^2 culture flasks (Falcon) to confluency, whereafter they where labelled in 1 ml of serum-free MCDB medium with 50 μCi/ml *in vitro* labelling mix containing [^{35}S]methionine and [^{35}S]cysteine (Amersham) for 12 h. The medium was collected and diluted with 3 ml of solubilization buffer [1 % (v/v) Triton X-100/1 % (w/v) deoxycholate/20 mM Tris/HCl (pH 7.5)/150 mM NaCl/10 mM EDTA, supplemented with 1.5 % (w/v) aprotinin and 1 mM PMSF], and subjected to precleaning with three cycles of subsequent incubation with non-immune sera and Protein A–Sepharose (Immunosorb A; EC Diagnostics), followed by centrifugation. Immunoprecipitations were then performed using 8 μl each of antisera against the TGF-β1 precursor (LT-1) [6], and LTCP-1 (ESL-1 antisera obtained from Dr. D. Vestweber, Münster, Germany), as well as non-immune sera. After 2 h of incubation at +4 °C, 60 μl of Protein A–Sepharose was added to each tube.

After an additional 45 min of incubation, the samples were spun down and the precipitated protein washed four times with RIPA buffer [1 % Triton X-100/1 % deoxycholate/0.1 % SDS/0.15 M NaCl/50 mM Tris (pH 7.5)/10 mM EDTA] and four times with high-salt buffer [1 % Triton X-100/20 mM Tris (pH 7.5)/0.5 M NaCl], followed by one wash with water. The proteins bound to the beads were eluted by the addition of 50 μl of SDS sample buffer containing 10 mM dithiothreitol and heating for 4 min at 95 °C. Samples were then analysed by SDS/PAGE using 5–15 % polyacrylamide gradient gels [25]. The gels were fixed, soaked in Amplify (Amersham) for 20 min, dried and subjected to fluorography.

Northern blot hybridization

The restriction fragment AO22 was radiolabelled by Megaprime DNA labelling system (Amersham), and used as a probe for Northern blot analysis. Filters with human mRNA from different tissues (Clontech) were hybridized with the probe in 50 % formamide, 5 × SSPE, 2 % SDS, 10 × Denhardt's solution and 0.1 mg/ml salmon sperm DNA at 42 °C for 35 h. The filters were washed three times with 2 × SSC/0.05 % SDS at 50 °C for 10 min each, and subjected to autoradiography.

RESULTS

Different latent TGF-β complexes are formed in transfected CHO cells

In the conditioned medium of CHO cells transfected with TGF-β1 cDNA, a small latent complex composed of the mature TGF-β1 and LAP was observed as a major complex ([9]; Figure 1). We have purified the latent TGF-β1 complex from the transfected CHO cells by ultrafiltration, followed by S-Sepharose cation-exchange chromatography, and Superose 6 gel chromatography. During the purification procedure, we found that latent TGF-β1 in the transfected CHO cells occurred as high molecular mass (more than 200 kDa) complexes, in addition to the small latent complex (Figure 1A). Immunoblotting analysis using an antiserum to LAP revealed that a 90-kDa LAP dimer was observed in fractions 29–34 (indicated as pool C in Figure 1A) that represents the small latent TGF-β1 complex (Figure 1B). In addition, components containing LAP were observed at a molecular mass of approx. 220 kDa in fractions 24–32 (Figure 1A, pool B). Immunoblotting using the antiserum to LTBP-1 revealed that the 220-kDa complex in fractions 24–26 (Figure 1A, pool A) contained LTBP-1, whereas that in fractions 27–32 did not react with the antiserum to LTBP-1 (Figure 1C). Antiserum to LTBP-2 did not react with these complexes either (results not shown). Immunoblotting of the fractions under reducing condition using Ab96 revealed no cross-reactivity to the new component (Figure 1D, fractions 27–32). These results suggested that the complex in fractions 27–32 contains a novel component that is different from LTBP-1 and LTBP-2. Aliquots from fractions 29 and 30 were analysed by SDS/PAGE. Under non-reducing conditions the large, latent TGF-β complex of more than 200 kDa was seen by staining with silver (Figure 2A) or Coomassie (results not shown). Analysis under reducing conditions revealed a protein of 140 kDa in these fractions, which was very weakly stained compared with LAP (results not shown). Analogously, LTBP-1 was found to be inefficiently stained by silver or Coomassie after SDS/PAGE under reducing conditions [3,5]. We termed the 140-kDa component that forms a complex with TGF-β, LTCP-1.

Amino acid sequencing of LTCP-1

Since LTCP-1 appeared to be different from LTBP-1 and LTBP-2, we purified it and determined part of its amino acid sequence. The 140-kDa LTCP-1 in fractions 29–30 was separated from the 220-kDa TGF-β complex by SDS/PAGE after reduction and alkylation by 4-VP. The part of the gel containing LTCP-1 was cut out and subjected to digestion with trypsin; tryptic peptides were then separated by reverse-phase HPLC (Figure 2B). As seen by the 254 nm trace in Figure 2(B), LTCP-1 has a high content of cysteine residues. Due to the presence of high contents of arginine and lysine residues in LTCP-1, a large number of fragments was obtained after digestion. In order to obtain homogeneous peptides, several fractions were re-run on another reverse-phase column; pure peptides were then subjected to amino acid sequencing. Fourteen sequences were obtained of 7–14 amino acid residues length (Figure 2C). At the time of this experiment, no homologous proteins were picked up during a database search using GenBank release 77, PIR release 35 and Swiss Protein release 25. In contrast, amino acid sequencing of the 40-kDa peptide in the same fractions from Superose 6 chromatography revealed that it is identical with TGF-β1–LAP (results not shown).

cDNA cloning of LTCP-1 from a CHO cDNA library

The peptide sequence no. 4 was selected to construct a probe for screening of a CHO cDNA library. A clone with a 3.0 kb insert termed CHO-5 was obtained. Nucleotide sequencing of CHO-5 revealed that it had a poly(A) tail at its 3′-end, but did not contain the complete 5′-end. In order to obtain a full-length cDNA from hamster, the CHO cDNA library was screened using the 5′-part of CHO-5 as a probe. A clone, CHO-31, with a 3.8 kb insert was obtained. In the open reading frame, we identified amino acid sequences identical with the peptides obtained by amino acid sequencing. A database search of CHO-31 revealed high amino acid sequence similarity to an FGF binding protein sequence from chicken, known as CFR [21], a ligand for mouse E-selectin (ESL-1) [22], rat MG-160, a membrane sialoglycoprotein of the Golgi apparatus [23], and a *Candida elegans* protein of unknown function (Nematode Sequencing Project; accession number, Z66522).

The predicted amino acid sequence of LTCP-1 from hamster is shown in Figure 3(A). The hamster LTCP-1 is a protein of 1160 amino acid residues, composed of an N-terminal signal sequence, followed by an extracellular domain with 1126 amino acid residues, a single transmembrane domain, and an intra-

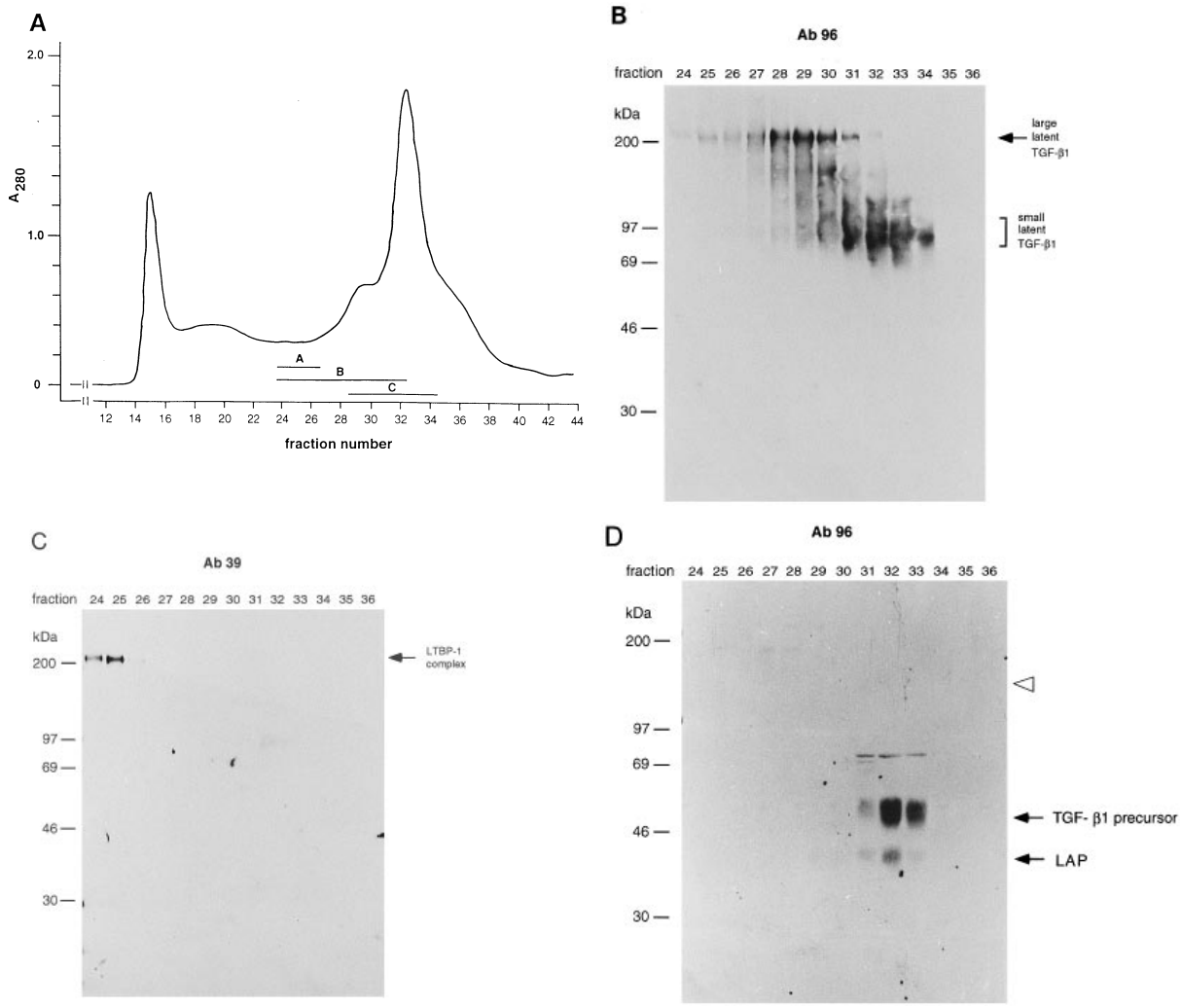

Figure 1 Identification of three different forms of latent TGF-β complexes in conditioned medium from CHO cells transfected with TGF-β1 cDNA

Chromatography of TGF-β-containing fractions from S-Sepharose chromatography on Superose 6 gel (**A**), and analysis of the fractions by immunoblotting using an antiserum to TGF-β1-LAP (Ab96) (**B**) and (**D**), and an antiserum to LTBP (Ab39) (**C**). (**B**) and (**C**) show samples run under non-reduced conditions, and (**D**) shows a sample run under reduced conditions. In (**D**), the open arrow indicates the expected position of LTCP. Molecular mass markers are indicated on the left in (**B**)–(**D**).

cellular domain with only 13 amino acid residues. The calculated molecular mass of the primary translation product without signal sequence is approx. 130 kDa. The first ATG codon encoding a methionine residue is not preceded by an in-frame stop codon. Comparison of hamster with chicken, mouse and rat homologues suggests that nine amino acid residues are lacking at the N-terminus.

The overall amino acid sequence identities between hamster and chicken, mouse, rat LTCP-1 or the related protein from *C. elegans* are 90 %, 98 %, 97 % and 23 % respectively (Figure 3B). The amino acid sequence is most divergent in the N-terminal 70-amino-acid residues; in this region the predicted chicken protein has a gap of approx. 25 amino acids when compared with the hamster, mouse and rat proteins, most probably due to alternative splicing. Five potential N-glycosylation sites were found in the extracellular domains of hamster LTCP-1, which are all conserved in chicken, mouse and rat. The hamster LTCP-1 is a cysteine-rich protein (5.9 % of the total number of residues). Sixteen copies of a conserved cysteine-rich repeat sequence composed of the motif $CX_{13-15}DX_{10}CX_{7-9}CX_nCL$ are present (Figure 3C), which is different from the EGF-like repeat, eight-cysteine repeat, or other known cysteine-rich repeat sequences. In close proximity to the transmembrane domain, two regions with potential proteolytic cleavage sites of two or three basic amino acid residues are present (Figure 3A). The intracellular domain is identical between hamster, chicken, mouse and rat.

Association of LTCP-1 with the TGF-β1 precursor in CHO cells stably transfected with cDNA for the TGF-β1 precursor

In order to confirm that LTCP-1 forms a disulphide-bonded complex with the TGF-β1 precursor, we performed immunoprecipitation of the complex from CHO-β1 conditioned medium with antisera to LTCP-1 (ESL-1 antisera) and the TGF-β1 precursor (LT-1 antisera). In support of the notion that LTCP-

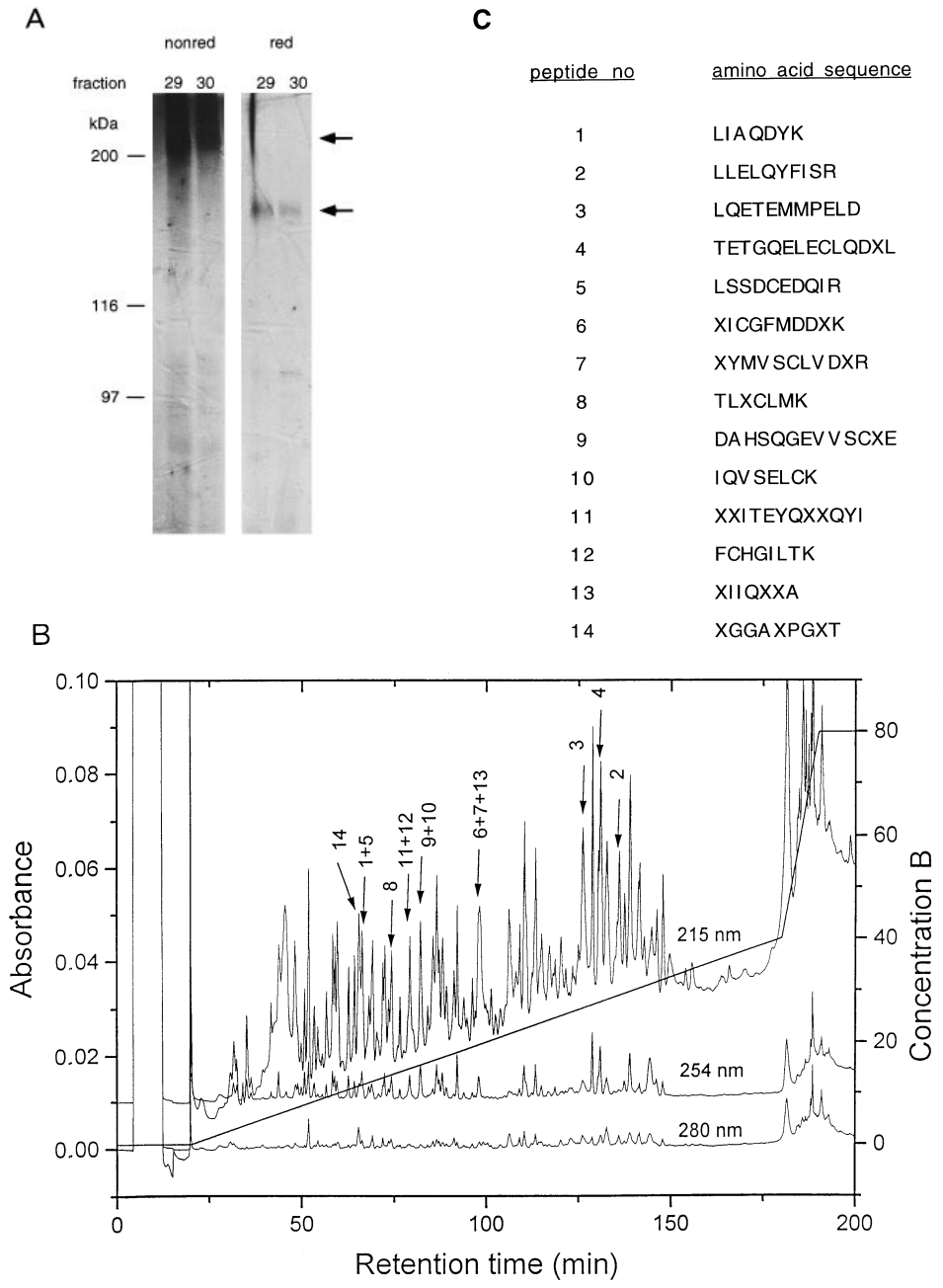

Figure 2 Amino acid sequencing of LTCP-1

Fractions 29 and 30 obtained by Superose 6 chromatography (Figure 1A) were analysed by SDS/PAGE followed by silver staining under reducing (red) or non-reducing (nonred) conditions (**A**). The 140-kDa protein (indicated by the lower arrow) was reduced and alkylated by 4-VP and separated by SDS/PAGE. The upper arrow indicates the position of the non-reduced complex containing TGF-β and the 140-kDa protein. Molecular mass markers are indicated on the left. The part of the gel containing the 140 kDa component was subjected to trypsin digestion; the peptides obtained were separated by reverse-phase HPLC on a μRPC C_2/C_{18} SC 2.1/10 column, operated in a SMART System (**B**). Solvent B is acetonitrile (%, v/v). The arrows indicate the fractions used for amino acid sequencing. Amino acid sequences were determined after individual peptides had been rechromatographed on a different support (Sephasil C_8, 5 μm, SC 2.1/10) (**C**).

1 and the TGF-β precursor forms a complex, ESL-1 antisera brought down both LTCP-1 and the TGF-β1 precursor from the conditioned media (Figure 4). However, when the LT-1 antisera were used for precipitation, a TGF-β precursor band was clearly seen, but only a faint co-precipitating LTCP-1 band was observed upon longer exposure, indicating that only a small proportion of the TGF-β1 precursor was complexed with LTCP-1 (see Figure 1D, lanes 29–33).

Expression profiles of LTCP-1 in human tissues

The expression of LTCP-1 mRNA in different human adult tissues was analysed by Northern blotting. Transcripts of 3.7, 4.6 and 8.0 kb were seen in different tissues. The 3.7 kb transcript was mainly observed in the testis, whereas the 4.6 kb transcript was seen in ovary, placenta, testis and some other tissues (Figure 5).

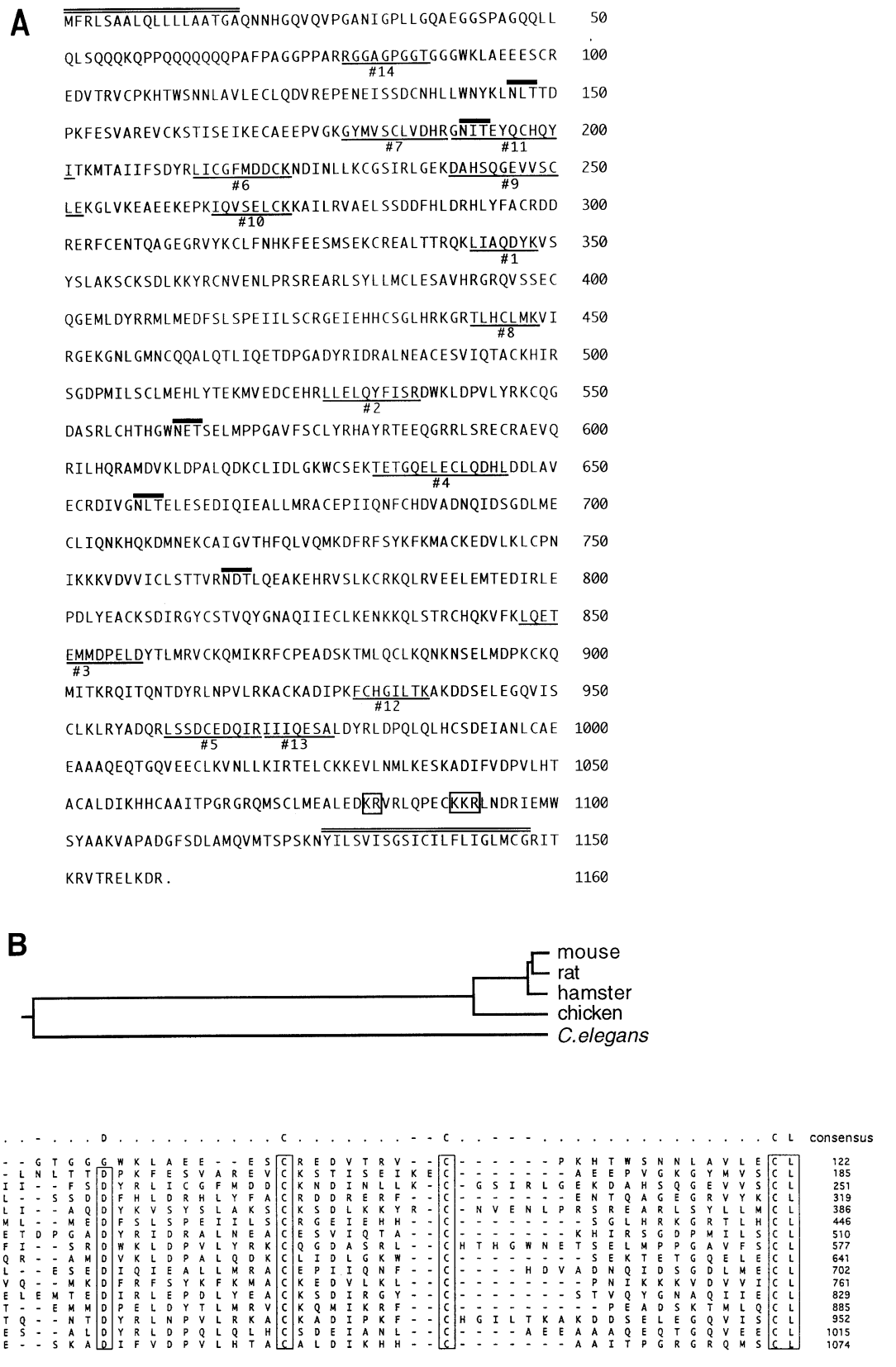

Figure 3 cDNA cloning of the 140-kDa LTCP-1 and its identification as a hamster homologue of chicken CFR, mouse ESL-1 and rat MG-160

(**A**) Deduced amino acid sequence of hamster LTCP-1 (clone CHO-31). Signal sequence and transmembrane domain are doubly overlined, peptide sequences obtained by amino acid sequencing (see Figure 2C) are underlined, N-linked glycosylation sites are overlined by a thick line, and potential proteolytic cleavage sites are indicated by boxes. The nucleotide sequence of hamster LTCP-1 has been submitted to the GenBank/EMBL Data Bank with accession number U51162. (**B**) Dendrogram of LTCP-1 from different species. (**C**) Alignment of the 16 cysteine repeats. Numbers correspond to the first and last amino acid residue in each repeated unit.

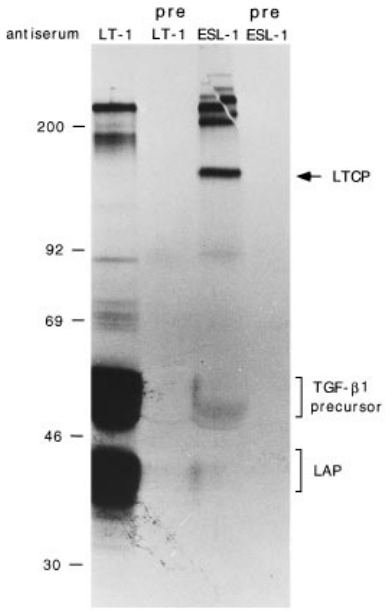

Figure 4 Interaction of LTCP-1 with TGF-β1 precursor

Conditioned media from CHO-TGF-β1 transfected cells were used for immunoprecipitation of LTCP-1 using ESL-1 antisera, and TGF-β1 using LT-1 antisera. After metabolic labelling, the media were analysed by SDS/PAGE in the presence of dithiothreitol, followed by fluorography. Molecular mass markers are indicated on the left.

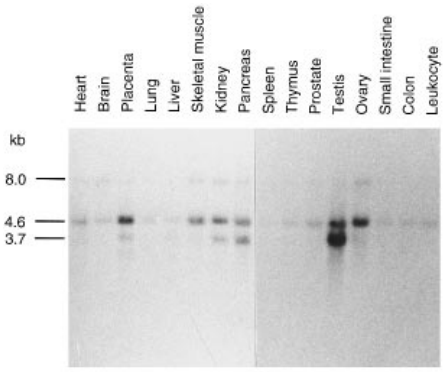

Figure 5 Northern blot analysis of LTCP-1

Human tissue blots containing various human adult tissues (Clontech) were hybridized with an *Eco*RI–*Sph*I fragment of CHO-31 and subjected to autoradiography. Size markers (kb) are indicated on the left.

DISCUSSION

In the present report, we have shown that the latent TGF-β in CHO cells transfected with TGF-β1 cDNA occurs as three different forms, i.e. a small latent TGF-β complex composed only of TGF-β1 and LAP, a high molecular mass complex containing LTBP, and a novel complex containing LTCP-1. LTCP-1 was originally identified as a transmembrane protein that binds FGFs [21], and thereafter as a ligand for E-selectin [22], and as a membrane sialoglycoprotein of the Golgi apparatus [23].

The LAP portion of the TGF-β1 precursor was found to be bound to LTCP-1 by disulphide linkage(s), similar to the complexes between LAP and LTBP-1, and between LAP and LTBP-2. Both LTBP-1 and -2 are composed of multiple copies of two different types of cysteine-rich repeat sequences, i.e. the EGF-like repeats and the eight-cysteine repeats. LTCP-1 does not contain either of these cysteine-rich repeat sequences, but has a cysteine-rich motif that has not been identified in other proteins [21]. The latent TGF-β complex containing LTCP-1 was purified from medium conditioned by transfected CHO cells, but transient transfection into COS cells revealed that a major part of LTCP-1 remains associated with the cells (results not shown). Thus, LTCP-1 in CHO cells is likely to be released into the medium by proteolytic cleavage of a membrane-associated precursor.

Latent TGF-β is observed in various forms in different cell types. In human platelets TGF-β occurs as a large, latent TGF-β complex containing LTBP-1. In other cell types, large, latent TGF-β complexes have been observed that contain LTBP-1, -2 or -3. LTBP is not needed to keep TGF-β latent, but it plays important roles during the assembly and secretion of latent TGF-β complex from certain types of cells [6]. After secretion, LTBP associates with certain extracellular matrix proteins, from which it can be released by digestion by plasmin or other proteolytic enzymes [19,30]. LTBP is also important for the efficient activation of latent TGF-β, e.g. in co-culture of endothelial cells and smooth muscle cells [31], possibly through its ability to target the complex to specific sites in the connective tissue or at the cell membrane.

The majority of latent TGF-β in CHO cells transfected with TGF-β1 was found to consist of a small, latent TGF-β complex, as reported before [9]. The small, latent TGF-β complex has also been observed in the conditioned media of certain non-transfected cells, e.g. differentiated osteoblasts and glioblastoma cells. The lack of complexed proteins in the small, latent form of TGF-β may lead to an escape from the association with the connective tissue after secretion [8,9], and TGF-β in this form may thereby reach distant target cells more efficiently.

The role of LTCP-1 in the latent TGF-β complex remains to be determined. The latent TGF-β containing LTCP-1 is associated with the producer cells, and it is most probably released from the cells after proteolytic cleavage of LTCP-1. We have thus far only seen the complex between LTCP-1 and the TGF-β1 precursor in the CHO cell line stably transfected with TGF-β1 cDNA.

We have previously reported that latent TGF-β in U-1240-MG and U-251-MGsp glioblastoma cells are observed as a small, latent complex, a large latent complex containing LTBP-1 and a 210-kDa complex containing an unidentified protein [7]. The U-251-MGsp glioblastoma cells were found to express LTCP-1 (A. Olofsson, unpublished work). Unfortunately, the cross-reactivity of the LTCP-1 antibodies with multiple proteins in the molecular mass range 200–240 kDa obscured the analysis of complex formation between LTCP-1 and TGF-β1 precursor in these cells. $SP_{2/0}$ myeloma and non-transfected CHO cells were found to express large amounts of LTCP-1, but did not express TGF-β1 (A. Olofsson, unpublished work).

Northern blot analysis showed that LTCP-1 mRNA is highly expressed in the testis, ovary and placenta, when compared with other tissues. In the chick embryo, LTCP-1 was found to be ubiquitously expressed [18,24]. TGF-β plays important roles in gonadal tissues and during development [1]; it remains to be elucidated whether TGF-β forms a complex with LTCP-1 in cell types that express both proteins.

FGF signals via tyrosine kinase receptors (reviewed in [32]). LTCP-1/CFR has been shown to bind FGF-1, FGF-2 and FGF-4 [21], but the function of LTCP-1/CFR in FGF signalling is unclear. The binding sites of FGFs and the TGF-β precursor

in LTCP-1/CFR have not been determined, and it remains to be determined whether the TGF-β precursor modulates the binding of FGFs to LTCP-1/CFR, and thereby regulates the bioactivity of FGFs.

E-Selectin is an endothelial cell adhesion molecule that mediates the binding of neutrophils. TGF-β acts as an inhibitor of inflammatory responses involving neutrophils, in part mediated by a TGF-β-induced decrease in E-selectin expression [33]. Whether the formation of a complex between TGF-β1 precursor and LTCP-1/ESL-1 inhibits the binding to E-selectin and thereby decreases the binding of neutrophils to endothelial cells, is not known. It is of interest to note that mice with inactivated TGF-β1 show a massive infiltration of leucocytes into lung and heart and die due to multifocal inflammatory disease [34,35].

The localization of LTCP-1/MG-160 to the medial cisterna of the Golgi apparatus suggests a possible involvement in the processing and secretion of TGF-β1. In the absence of LTBP, latent TGF-β was found to be retained in the Golgi complex [36]. However, whereas the intracellular TGF-β1 precursor was sensitive to endoglycosidase H, MG-160 was resistant to endoglycosidase H [37], indicating that the two proteins were present in different compartments of the Golgi apparatus.

In conclusion, we have shown that latent TGF-β occurs as high molecular mass complexes with different proteins; the different proteins in the latent TGF-β complexes may give TGF-β different functional properties *in vivo*.

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