Structural characterization of the latent complex between transforming growth factor β 1 and β 1-latency-associated peptide

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The formation of a non-covalent complex between mature transforming growth factor β 1 (TGF- β 1) and its pro region, the β 1-latency-associated peptide (β 1-LAP), is important in regulating the activity of this multipotent growth factor. We have overexpressed simian β 1-LAP in Chinese hamster ovary (CHO) cells to produce a cell line which secretes β 1-LAP into the culture medium at > 1 mg/l, thus enabling structural studies of complex formation between β 1-LAP and TGF- β 1. The simian β 1-LAP expressed in CHO cells reversed the growth inhibitory effect of exogenous TGF- β 1 on Mv1Lu (mink lung epithelial) cells and was able to form a cross-linked complex with ^{125}I -TGF- β 1. Simian β 1-LAP was purified to homogeneity by a combination

of ammonium sulphate precipitation, gel filtration, dye ligand chromatography and anion-exchange chromatography, with a yield of 15 %. The purified protein had an apparent molecular mass of 114 kDa as determined by SDS/PAGE, which is greater than that determined for the transient expression of simian β 1-LAP in COS-1 and for the simian precursor of TGF- β 1 (pro-TGF- β 1) in CHO cells, this major difference being due to more extensive glycosylation of β 1-LAP expressed by this CHO clone. Far-UV CD spectroscopy of simian β 1-LAP indicates a mostly β -sheet structure, with extensive structural rearrangements occurring upon formation of the latent complex between TGF- β 1 and β 1-LAP.

INTRODUCTION

The transforming growth factor β (TGF- β) family is composed of a number of structurally related molecules which control many aspects of cell growth and differentiation (for reviews see [1,2]). Family members include TGF- β , activins, inhibins, Müllerian inhibiting substance, bone morphogenetic proteins, *Xenopus* (Vg)-related peptides, and the *decapentaplegic* gene product from *Drosophila*. Each family member exists as a number of isoforms. Sequence conservation across the family ranges from 20 to 90 %, with the greatest sequence similarity residing in the C-terminus. All are made as larger precursors which require proteolytic processing to release the growth factor. Other important processing steps include removal of the hydrophobic leader sequence and N-linked glycosylation of the pro region. Dimerization of the growth factor is also required for biological activity. The order of these events has yet to be determined.

The prototype of the family, TGF- β 1, was initially identified by its ability to promote the transformed phenotype in untransformed fibroblasts [3]. Its most important activity is as a regulator of cell growth and differentiation. TGF- β 1 is synthesized as a 390-amino-acid precursor [4], with the active part of the molecule contained in the 112 amino acids at the C-terminus.

TGF- β 1 is secreted as a latent complex, consisting of a dimer of TGF- β 1 non-covalently associated with a dimer of its pro region. The pro region, designated the β 1-latency-associated peptide (β 1-LAP), is the peptide required for conferring latency [5,6]. The latent complex purified from human platelets [7,8]

consists of TGF- β 1, β 1-LAP and the latent TGF- β -binding protein (LTBP) which is disulphide-linked to β 1-LAP. Western blot analysis of the latent complex secreted by a number of cell types in culture showed that this three-component complex is the predominant form made by cells [7]. The LTBP is important for directing the correct processing and secretion of the latent complex [9], since the latent complex lacking the LTBP is secreted more slowly from the cell in comparison with that containing the LTBP [10]. TGF- β 1 can be released from the latent complex by extremes of pH or heat, by chaotropic agents [11], by deglycosylation [12], or by proteolysis by serine proteases such as plasmin or cathepsin D [13]. The kinetics of *in vitro* activation of TGF- β s 1, 2 and 3 by heat or by acid treatment are analogous, which suggests similar mechanisms for activation [14]. The *in vivo* mechanism of activation is not as clearly defined. Co-culture of endothelial cells and smooth-muscle cells suggests a role for plasmin in this process [15], since inclusion of plasmin inhibitors in the culture medium prevents the release of mature TGF- β 1. The fate of β 1-LAP after its release from the latent complex is unknown.

This report describes the production of a simian β 1-LAP-secreting cell line, and the purification to homogeneity of β 1-LAP from the conditioned media of these cells by ammonium sulphate precipitation, gel filtration, dye ligand chromatography and anion-exchange chromatography. Since simian [16] and human [4] β 1-LAPs differ by only two conservative amino acid replacements, CD results obtained for the simian version will be directly applicable to the human form. CD spectroscopy of simian β 1-LAP indicates that the major structural element

Abbreviations used: TGF- β , transforming growth factor β ; β 1-LAP, β 1-latency-associated peptide; pro-TGF- β 1, precursor of TGF- β 1; LTBP, latent TGF- β -binding protein; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; Mv1Lu, mink lung epithelial; M6P, mannose 6-phosphate; MTX, methotrexate; MEM, minimal essential medium.

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consists of β -sheets and random structure, and that significant structural rearrangements occur upon formation of the latent complex between β 1-LAP and TGF- β 1.

EXPERIMENTAL

Cell culture

Mv1Lu (mink lung epithelial) cells (A.T.C.C. CCL64) were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml) and streptomycin (100 μ g/ml). Chinese hamster ovary (CHO) cells, that were *dhfr* deficient, were grown in the same medium supplemented with L-proline (150 μ g/ml), adenosine (10 μ g/ml), deoxyadenosine (10 μ g/ml) and thymidine (10 μ g/ml) (Sigma Chemical Co., St. Louis, MO, U.S.A.). TGF- β -3-2000 cells were grown in DMEM containing fetal bovine serum, penicillin, streptomycin and L-proline at the above concentrations plus 20 μ M methotrexate (MTX) (Sigma Chemical Co.). Cells were passaged every 3 days using 0.05% (w/v) trypsin, containing 0.02% (w/v) EDTA in PBS. All tissue-culture reagents were from GIBCO BRL (Grand Island, NY, U.S.A.) unless otherwise indicated.

Construction of pSV2- β 1-LAP-*dhfr* vector

DNA manipulations were carried out using standard techniques [17]. A strategy similar to that used for construction of pSV2-(β TGF-*dhfr*) was employed [5]. Briefly, replicative DNA from M13mp19 containing the simian β 1-LAP cDNA was digested with *Bam*HI, the overhang filled in with DNA polymerase I (Klenow large fragment), and digested with *Hind*III. The 1 kb *Hind*III-*Bam*HI (blunt) fragment of β 1-LAP was isolated and inserted into the pSV2-*neo* (*Hind*III-*Hpa*I-digested) vector, replacing the *neo* sequence with β 1-LAP. The pSV2- β 1-LAP-*dhfr* vector was constructed by ligating the *Nde*I-*Eco*RI (blunt) fragment of pSV2- β 1-LAP into the *Nde*I-*Pvu*II fragment of pSV2-*dhfr* as previously described for the construction of pSV2-(β TGF-*dhfr*) [5] (see Figure 2a).

DNA transfections

Cells were transfected by the cationic liposome-mediated transfection procedure as recommended by the manufacturer. The *dhfr*-deficient CHO cells (0.3×10^6) were plated on to 6-cm-diam. tissue-culture dishes (Corning; Corning, NY, U.S.A.) and grown to 80% confluence. The dishes were rinsed three times with Opti MEM I reduced serum medium (GIBCO BRL). Linearized pSV2- β 1-LAP-*dhfr* (1 μ g) was added to 40 μ g of lipofectin reagent (GIBCO BRL) in 3 ml of Opti MEM I in a polystyrene tube. The mixture was added to the cells and incubated for 15 h at 37 °C in a humidified 5% CO₂ incubator. The Opti MEM I medium was then replaced with complete medium and the cells left to recover for 24 h at 37 °C. Transfected cells were then trypsinized and transferred to 15-cm-diam. tissue-culture dishes and grown in selective medium [DMEM supplemented with dialysed fetal bovine serum (10%, w/v) (GIBCO BRL), penicillin (100 units/ml), streptomycin (100 μ g/ml) and proline (150 μ g/ml)]. After 14 days visible colonies were aspirated with a Pasteur pipette and expanded. Clones were then amplified in increasing concentrations of MTX as previously described [5]. A cell line obtained in 2 μ M MTX was found to secrete the highest levels of functional simian β 1-LAP, as judged by the mink lung cell bioassay and cross-linking to ¹²⁵I-TGF- β 1. Limiting dilution clones were made and a subclone denoted CHO-2000 was selected for further studies.

Purification of simian β 1-LAP

Assay for functional β 1-LAP

Functional β 1-LAP was detected by measuring latent complex formation in an adaptation of a growth inhibitory assay using mink lung epithelial Mv1Lu cells. Serial dilutions of fractions obtained during the purification were prepared in duplicate and incubated with 80 pM TGF- β 1 for 1 h. The samples were then added to Mv1Lu cells plated on a 96-well tissue-culture plate and incubated at 37 °C for 72 h. Cell growth was measured using the tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl]tetrazolium bromide (Sigma Chemical Co.) as previously described [18]. The mitochondria of living cells convert the tetrazolium salt into a coloured product. The absorbance at 595 nm is proportional to the number of living cells. The absorbance at 595 nm was measured using a Thermo Max microplate reader (Molecular Devices Corporation, Menlo Park, CA, U.S.A.). One unit of β 1-LAP activity is defined as the amount of β 1-LAP required to produce a 50% reversal of the Mv1Lu growth inhibition by 80 pM exogenously added TGF- β 1.

Determination of protein concentration

The protein concentration of pooled fractions from each column was determined using the Micro BCA protein assay (Pierce, Rockford, IL, U.S.A.) as described by the manufacturer.

Collection of simian β 1-LAP conditioned medium

CHO-2000 cells were expanded on to 850 cm² roller bottles (Corning) and grown to confluence. Secreted proteins were collected in serum-free DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), L-proline (150 μ g/ml) and 2 μ M MTX. Collections were made every 48 h. Conditioned medium was clarified to remove cellular debris by centrifugation at 1000 g for 10 min prior to ammonium sulphate precipitation.

Ammonium sulphate precipitation

Ammonium sulphate (390 g/l; 60% satn.) (ICN Biochemicals, Cosa Mesa, CA, U.S.A.) was added slowly to 500 ml of conditioned medium at room temperature. The solution was then transferred to 4 °C and stirred overnight. The precipitate was collected by centrifugation at 4000 g for 0.5 h, dissolved in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄, pH 7.3) containing 1 mM EDTA, and dialysed overnight against the same buffer at 4 °C. The solution was clarified by centrifugation at 12000 g for 0.5 h. Protease inhibitors [PMSF (0.02 mM), pepstatin (2 μ g/ml), leupeptin (2 μ g/ml) and aprotinin (2 μ g/ml) (Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.)] were added and the solution stored at -70 °C until further use.

Gel-filtration chromatography

The ammonium sulphate-precipitated fraction was loaded on to a 2.5 cm $\times$ 115 cm column of Sephacryl S300 (Pharmacia LKB, Piscataway, NY, U.S.A.) equilibrated in PBS (pH 7.3) containing 1 mM EDTA at 4 °C, and eluted in the same buffer at a flow rate of 25 ml/h. Fractions of 4 ml were collected in siliconized tubes and assayed for simian β 1-LAP activity. The void volume (*V*₀) was determined using Dextran Blue (2000 kDa) (Pharmacia LKB), and the column calibrated with molecular-mass standards [thyroglobulin (670 kDa), bovine γ globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa) and vitamin B₁₂

(1.35 kDa) (Bio-Rad, Philadelphia, PA, U.S.A.) under the same conditions.

Dye-ligand chromatography

Simian β 1-LAP-containing fractions from the Sephacryl S300 column were pooled and dialysed against 10 mM phosphate buffer, pH 7.4, 1 mM EDTA at 4 °C overnight. The pooled sample was clarified by centrifugation at 12000 *g* for 0.5 h prior to loading on to a 1 cm $\times$ 5 cm column of Reactive Green-19 (Sigma Chemical Co.) equilibrated in 10 mM phosphate buffer, pH 7.4, 1 mM EDTA at 4 °C. The column was washed with 10 vol. of the same buffer and eluted stepwise with 10 vol. of 0.2 M, 0.4 M and 1.0 M KCl in 10 mM phosphate buffer, pH 7.4, 1 mM EDTA at 20 ml/h. Fractions of 1 ml were collected in siliconized tubes and assayed for simian β 1-LAP.

Mono Q chromatography

Simian β 1-LAP-containing fractions from the Reactive Green-19 column were pooled and dialysed overnight against 10 mM phosphate buffer, pH 7.4, 1 mM EDTA at 4 °C, clarified by centrifugation at 12000 *g* and applied to a Mono Q column (HR 5/5, 5 mm $\times$ 50 mm; Pharmacia LKB) at room temperature, at a flow rate of 0.5 ml/min. The column was washed with 5 ml of the same buffer, then eluted with a 5 ml linear gradient of 0–0.1 M KCl in 10 mM phosphate buffer, followed by a 20 ml linear gradient of 0.1–0.4 M KCl in 10 mM phosphate buffer, pH 7.4, 1 mM EDTA. Fractions of 0.5 ml were collected in siliconized microcentrifuge tubes and assayed for functional β 1-LAP.

Preparation of antibodies

The simian precursor for TGF- β 1 (pro-TGF- β 1) was partially purified as described by Gentry et al. [19]. New Zealand White rabbits were inoculated subcutaneously at multiple sites with 100 μ g of pro-TGF- β 1 emulsified in Freund's complete adjuvant, followed by boosts of 100 μ g in incomplete adjuvant at 3-week intervals. Rabbits were bled 7 days after boosting.

Electrophoresis and Western blot analysis

SDS/PAGE was performed by the method of Laemmli [20]. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes (DuPont-NEN, Boston, MA, U.S.A.) and probed for simian β 1-LAP as described previously [18]. Simian-specific β 1-LAP antibodies were affinity-purified from the rabbit pro-TGF- β 1 antiserum by the method of Smith and Fisher [21]. Proteins were also visualized by silver staining as recommended by the manufacturer (Bio-Rad). Prestained molecular-mass standards [myosin (200 kDa), phosphorylase *b* (97.4 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), β -lactoglobulin (18.4 kDa) and lysozyme (14.3 kDa) (GIBCO BRL)] were run with samples as markers.

Metabolic labelling

Parental CHO, CHO-2000 and TGF- β 3-2000 cells were plated on 3.5-cm tissue-culture dishes and grown to 80% confluence. For [32 P]P_i labelling experiments, dishes were washed in phosphate- and serum-free minimal essential medium (MEM) (GIBCO BRL) supplemented with penicillin (100 units/ml) and streptomycin (100 μ g/ml) and incubated in the same medium for 0.5 h. The medium was replaced with phosphate- and serum-free medium containing 1 mCi/ml of carrier-free [32 P]P_i (Amersham Corporation, Arlington Heights, IL, U.S.A.) and the cells

incubated for 4 h at 37 °C. For labelling proteins with [35 S]cysteine and [35 S]methionine, cells were washed as above in cysteine- and methionine-free, serum-free MEM (GIBCO BRL) and incubated in the same medium with 100 μ Ci/ml Tran 35 S-label (1.0 $\times$ 10³ Ci/mmol; ICN Biochemicals) for 6 h. Conditioned medium was collected and clarified by centrifugation at 14000 *g* for 10 min prior to immunoprecipitation.

Immunoprecipitation

To preclear the conditioned medium of proteins which would non-specifically attach to the *Staphylococcus aureus* beads, 32 P- and 35 S-labelled samples were treated with 100 μ l of 10% (w/v) Immuno-Precipitin (formalin-fixed *S. aureus* beads; GIBCO BRL) at 4 °C for 1 h. The supernatant was collected by centrifugation at 14000 *g* for 15 min. Antibody to pro-TGF- β 1 (5 μ g) was added to sample volumes equivalent to 1 $\times$ 10⁶ cells, and samples were incubated at 4 °C for 1 h. Immuno-Precipitin beads [100 μ l; 10% (w/v)] were added and incubated at 4 °C for 1 h. Immune-complexes were collected by centrifugation at 10000 *g* for 15 s and washed three times in 50 mM Tris/HCl, pH 7.5, containing 0.15 M NaCl, 1 mM EDTA, 1% Triton X-100 and 0.1% SDS. Pellets were resuspended in 1 $\times$ SDS gel buffer containing 2-mercaptoethanol. For N-glycanase digestion, pellets were resuspended in 0.05 M NaH₂PO₄, pH 7.4, containing 0.5% SDS, 50 mM 2-mercaptoethanol and 50 mM EDTA, and heated to 95 °C for 5 min. The supernatants were transferred to fresh tubes and digested as recommended by the manufacturer. Immunoprecipitated samples were analysed by electrophoresis on SDS/7.5–20%-polyacrylamide gels. Gels containing 35 S-labelled proteins were treated with Entensify (DuPont-NEN) as recommended by the manufacturer, and dried prior to autoradiography.

Purification of TGF- β 1

TGF- β 1 was purified from the TGF- β 3-2000 cell line as described previously [19,22].

Cross-linking of simian β 1-LAP to radioiodinated TGF- β 1

TGF- β 1 was labelled with Na¹²⁵I using chloramine-T [23], and cross-linking experiments were carried out as previously described [6,23].

Far-UV CD spectroscopy

Far-UV CD spectra were measured using an AVIV 62DS spectrophotometer. All measurements were made at 25 °C in a 1-cm pathlength cell. The spectral bandwidth was held constant at 1.0 nm. Spectra were recorded from 320 nm to 197 nm. Five replicates of each measurement were made, and are presented as mean residue ellipticity (deg $\cdot$ cm² $\cdot$ dmol⁻¹). The spectral curves were smoothed prior to subtraction of the blank and baseline normalization. CD spectra for simian β 1-LAP were measured either in the presence or absence of TGF- β 1. Purified simian β 1-LAP was dialysed overnight against 0.125 M KCl, 10 mM phosphate buffer, pH 7.4, 0.1 mM EDTA to decrease the absorbance of the buffer at shorter wavelengths. The protein concentration was 1.5 μ M. Purified TGF- β 1 in water was also used at 1.5 μ M. For the latent complex spectral analysis, equimolar ratios of simian β 1-LAP and TGF- β 1 were incubated at room temperature for 2 h prior to taking the spectra.

RESULTS

Amplified expression of β 1-LAP in *dhfr*-deficient CHO cells

Previous studies have indicated that simian β 1-LAP could be transiently expressed in COS-1 cells to produce a polypeptide that binds and inactivates mature TGF- β 1 [6]. We decided to produce a stable cell line expressing simian β 1-LAP in *dhfr*-deficient CHO cells. This CHO cell line, producing amplified expression of β 1-LAP, would provide enough of the polypeptide to allow far-UV CD studies to be utilized. Because the simian [16] and human [4] polypeptides are essentially identical, the designation of simian or human for β 1-LAPs will no longer be employed.

The β 1-LAP and *dhfr* cDNAs were inserted into the pSV2 vector such that both genes were placed under the control of the simian virus 40 (SV40) promoter (see Figure 1a) [5,24]. The *dhfr*-deficient CHO cells were stably transfected using cationic liposome-mediated transfection. The *dhfr* gene provided for selection of transfected clones in nucleoside-free medium, and co-amplification of β 1-LAP cDNA on addition of increasing concentrations of MTX. Western blot analysis, under non-reducing and reducing conditions, was used to determine the

levels of β 1-LAP expression upon MTX amplification (Figure 1b). The expression of β 1-LAP in the parental CHO cells was below the detection level of the antibody. The amplified clones expressed various levels of β 1-LAP immunoreactive protein. One clone, designated CHO-2000, which grew in 2 μ M MTX, secreted the highest levels of β 1-LAP and was selected for further study. Subsequent amplification of this clone in increasing concentrations of MTX did not produce any further increase in β 1-LAP expression. Under non-reducing conditions, the β 1-LAP migrated as a doublet, which may indicate some heterogeneity in the glycosylation of the protein (Figure 1b). A large proportion of β 1-LAP was secreted in the monomeric form. This polypeptide does not form the latent complex with mature TGF- β 1 [6,25]. Under non-reducing conditions, bands of 55, 40 and 22 kDa were also noted, and may be due to non-specific immunoreactivity of proteins with the antibody.

Characterization of carbohydrate moieties of β 1-LAP expressed by the CHO-2000 clone

The β 1-LAP protein expressed by the CHO-2000 clone appeared to have a higher molecular mass than that reported for the transient expression of β 1-LAP in COS-1 cells (95–105 kDa compared with 80 kDa). To examine the actual sizes of the proteins, TGF- β 3-2000 cells overproducing the TGF- β 1 precursor, CHO-2000 cells overexpressing β 1-LAP, and CHO cell control, were labelled with [35 S]cysteine and [35 S]methionine for 6 h and the labelled medium fractionated by reducing SDS/PAGE (Figure 2a). In comparison with the other lanes, the control CHO cells show minimal labelling. The TGF- β 3-2000 cells contained three monomeric TGF- β 1-related polypeptides; the pro form of TGF- β 1 (50–60 kDa), the β 1-LAP portion (38–44 kDa), and the mature TGF- β 1 molecule (12 kDa). The molecular masses of these proteins are in quite good agreement with those reported earlier [19]. CHO-2000 cells produced a β 1-LAP monomer that appeared to migrate more slowly on SDS/PAGE; β 1-LAP expressed by CHO-2000 clone migrated at $\approx$ 46 kDa whereas the β 1-LAP released by TGF- β 3-2000 cells migrated at $\approx$ 43 kDa (Figure 2a).

To determine if differences in mobility reflected differences in glycosylation of the proteins, 35 S-labelled proteins from the parental CHO cells, CHO-2000 and TGF- β 3-2000 cells were immunoprecipitated with an antibody to pro-TGF- β 1, and treated with N-glycanase (Figure 2b). No β 1-LAP-specific bands were detected in the parental CHO cells. The difference in size of the β 1-LAP expressed by the CHO-2000 clone compared with TGF- β 3-2000 cells is very apparent. N-Glycanase digestion of both CHO-2000- and TGF- β 3-2000-conditioned medium produced a deglycosylated β 1-LAP monomer which migrated identically at 27–29 kDa. This agrees with the predicted molecular mass of the core protein of β 1-LAP as determined from sequence analysis [16]. The above results in Figure 2 suggest that variations in the mobility of the protein expressed by the CHO-2000 and TGF- β 3-2000 clones reflect differences in glycosylation. The difference in glycosylation could be attributed to the different clonal origins of the parental CHO cells used to prepare the cell lines.

Secreted β 1-LAP contains mannose 6-phosphate (M6P)

Activation of the latent complex *in vivo* has been linked to binding to the M6P/insulin-like growth factor II receptor via M6P residues on β 1-LAP [26,27]. The incorporation of phosphate residues into the mannose moieties was demonstrated as pre-

Figure 1 Amplified expression of β 1-LAP in *dhfr*-deficient CHO cells

(a) Construction of the pSV2- β 1-LAP-*dhfr* vector. The β 1-LAP cDNA was inserted into the pSV2-*dhfr* vector as described in the Experimental section. Both cDNAs were placed under the control of the SV40 promoter. (b) Expression and amplification of β 1-LAP in *dhfr*-deficient CHO cells. The pSV2- β 1-LAP-*dhfr* construct was transfected into *dhfr*-deficient CHO cells and amplified in increasing concentrations of MTX. Conditioned medium was collected and analysed by SDS/PAGE on 7.5–20% gradient gels, transferred to PVDF membranes and probed with affinity-purified antibodies against β 1-LAP. Lane 1 corresponds to secreted proteins in parental CHO cells (control), while lanes 2–6 correspond to one CHO clone, amplified in 0, 400, 1000, 2000 and 4000 nM MTX respectively. The arrow indicates the migration of dimeric β 1-LAP, while the arrowhead indicates the migration of monomeric β 1-LAP. The left-hand panel shows non-reduced samples, while the right-hand panel shows reduced samples. The migration of prestained molecular-mass standards (in kDa) is as indicated.

Figure 2 Characterization of carbohydrate moieties present on β 1-LAP expressed by CHO-2000 cells

(a) Conditioned medium from parental CHO cells (control), CHO-2000 (β 1-LAP) and TGF- β 3-2000 (Pro TGF- β 1) cells was labelled with [35 S]cysteine and [35 S]methionine and analysed by SDS/PAGE on a 7.5–20% gel under reducing conditions and autoradiographed. (b) 35 S-labelled conditioned medium from the three cell lines in (a) was immunoprecipitated with an antibody directed against pro-TGF- β 1 and treated with N-glycanase as indicated prior to SDS/PAGE under reducing conditions. (c) 32 P-labelled conditioned medium from parental CHO cells (control), TGF- β 3-2000 (Pro TGF- β 1) and CHO-2000 (β 1-LAP) cells was immunoprecipitated with the pro-TGF- β 1 antibody, N-glycanase treated and analysed by SDS/PAGE under reducing conditions as in (b). The migration of molecular mass markers is indicated on the left (in kDa), while the migration of the precursor of TGF- β 1, β 1-LAP and mature TGF- β 1 are indicated as a, b, c on the right of each figure, respectively.

Figure 3 Conditioned medium from the CHO-2000 clone can form the latent complex with mature TGF- β 1

Serial dilutions of conditioned medium from CHO-2000 cells (●), and parental CHO cells (○) were incubated with 80 pM TGF- β 1 for 1 h prior to addition to the mink lung epithelial cells. The percentage cell growth relative to untreated controls was measured after 3 days as described in the Experimental section.

viously described [26]. After labelling the cells with [32 P] P_i , the β 1-LAP was immunoprecipitated, digested with N-glycanase, and run on SDS/PAGE along with the undigested [32 P] P_i -labelled proteins (see the Experimental section). If the carbohydrate contains the labelled phosphorylated sugar residues, then β 1-LAP proteins treated with N-glycanase should show no label as compared to the polypeptides from undigested [32 P] P_i -labelled cells. Parental CHO cells and TGF- β 3-2000 cells were used as controls in this experiment. Immunoprecipitation of 32 P-labelled proteins produced bands of comparable size to those observed in the 35 S-labelling experiment (compare Figure 2b with Figure 2c). No β 1-LAP-specific bands were observed after N-glycanase treatment, suggesting that the β 1-LAPs expressed by the CHO-2000 and the TGF- β 3-2000 cells contain M6P (Figure 2c). A protein of $\approx$ 14 kDa was also identified by

immunoprecipitation in all cells labelled with [32 P] P_i . This band was not present after N-glycanase digestion, implying that this smaller protein contained M6P. We assume that this protein was non-specifically immunoprecipitated by the antibody.

β 1-LAP from CHO-2000 cells is biologically active

The ability of β 1-LAP in the conditioned medium from β 1-LAP-expressing cells to form the latent complex with mature TGF- β 1 was measured using Mv1Lu cell growth as an assay for latent complex formation. Mv1Lu cells show a half-maximal growth inhibition with TGF- β 1 of 1–2 pM [22] and the addition of 80 pM TGF- β 1 to Mv1Lu cells completely inhibits their growth [6]. If exogenously added TGF- β 1 forms the latent complex with the added β 1-LAP, the inhibitory effect of TGF- β 1 is blocked. Cell growth in this assay is therefore proportional to latent complex formation. Serial dilutions of conditioned medium from parental CHO cells and CHO-2000 cells were incubated with 80 pM TGF- β 1 for 1 h prior to addition to the Mv1Lu cells. Cell growth was measured after 3 days (Figure 3). Conditioned medium from control cells was unable to reverse the inhibitory effect of TGF- β 1, while addition of CHO-2000-conditioned medium increased the growth of Mv1Lu cells in a dose-dependent manner, suggesting that the inhibitory effect of TGF- β 1 addition was reversed by increasing amounts of β 1-LAP.

To confirm that reversal of the growth inhibition of the Mv1Lu cells by added TGF- β 1 was due to latent complex formation, and not proteolysis of the protein on incubating with CHO-2000-conditioned medium, radioiodinated TGF- β 1 was incubated with CHO-2000-conditioned medium for 2 h and cross-linked with bis(sulphosuccinimidyl)suberate. SDS/PAGE and autoradiography showed a band which migrated with the complex between β 1-LAP and TGF- β 1 (Figure 4). No labelled bands of comparable molecular mass were detected in samples of parental CHO cells; however, CHO-0 cells expressing low levels of β 1-LAP could form a similar complex, when the gel was overexposed (results not shown). These results indicate that β 1-LAP secreted by the CHO-2000 cell line forms the latent complex with TGF- β 1.

Figure 4 Cross-linking of CHO-2000 conditioned medium to ^{125}I -TGF- β 1

Conditioned medium from control, CHO-0, and CHO-2000 cells was incubated with ^{125}I -TGF- β 1 for 2 h, prior to cross-linking with bis(sulphosuccinimidyl)suberate as described in the Experimental section. Samples were resolved on 7.5% SDS/polyacrylamide gels and detected by autoradiography. Molecular-mass markers (in kDa) are indicated on the left.

Purification of β 1-LAP

To obtain the purified β 1-LAP, 500 ml of conditioned medium from the CHO-2000 cell line was used as the starting material. Rapid concentration of the conditioned medium was required to minimize proteolysis of the protein at 4 °C, and to facilitate long-term storage at -70 °C. Ammonium sulphate precipitation provided an effective means of concentrating the protein; however, the purification from this step was not high (results not shown). The Mv1Lu bioassay was used to identify fractions containing β 1-LAP, and to quantify the amount of β 1-LAP during the purification. Since it was not possible to quantify the amount of β 1-LAP in the starting material, due to inhibition of the Mv1Lu cells by MTX present in the collection medium, the amount of β 1-LAP activity in the ammonium sulphate precipitation step was set at 100%.

The dialysed, clarified ammonium sulphate fraction was loaded on to a Sephacryl S300 gel-filtration column and eluted in a PBS, pH 7.3, 1 mM EDTA buffer (Figure 5a). Two major peaks of UV-absorbing material eluted from the column. The Mv1Lu bioassay showed that bioactive β 1-LAP eluted in the second broad peak with an apparent molecular mass of 133 ± 8 kDa ($n = 12$). The β 1-LAP eluted as a broad heterogeneous peak. Western blot analysis of column fractions indicated that the monomeric β 1-LAP eluted from the column with an apparent molecular mass of 53 kDa, eluting much later than the dimeric form of the protein (results not shown). This column therefore

Figure 5 Purification of β 1-LAP

(a) Gel-filtration chromatography of ammonium sulphate-concentrated conditioned medium dialysed versus PBS, pH 7.3, 1 mM EDTA and applied to the S300 column equilibrated in the same buffer. Fractions of 4 ml were collected and assayed by UV detection at 280 nm (—). Each fraction (25 μ l) was analysed for functional β 1-LAP using the mink lung cell bioassay (●). Fractions pooled for loading on to the next column are indicated by the horizontal bar. The elution position of the molecular-mass standards is indicated at the top of the Figure. (b) Reactive Green-19 chromatography from β 1-LAP containing fractions from the S300 column. These fractions were pooled and dialysed against 10 mM KH_2PO_4 , pH 7.4, 1 mM EDTA and applied to the column equilibrated in the same buffer. Proteins were eluted step-wise in 0.2, 0.4 and 1.0 M KCl in phosphate buffer as indicated by the arrows. Fractions of 1 ml were collected and assayed by UV detection at 280 nm (—). Aliquots (25 μ l) of each fraction were analysed for functional β 1-LAP as described in the Experimental section (●). Fractions pooled for loading on to the next column are indicated by the horizontal bar. (c) Mono Q anion-exchange chromatography of β 1-LAP-containing fractions from the Reactive-Green column. After these fractions were dialysed against 10 mM KH_2PO_4 , pH 7.4, 1 mM EDTA, they were loaded on a Mono Q column equilibrated in the same buffer. Proteins were eluted in a gradient of 0–0.4 M KCl (---) in the phosphate buffer and assayed by UV detection at 280 nm (—). Fractions of 0.5 ml were collected. β 1-LAP-containing fractions were identified as described. Pooled fractions are indicated by the horizontal bar.

Figure 6 PAGE of column fractions from the Mono Q column

Aliquots (5 μ l) of fractions from the Mono Q column were electrophoresed on an SDS/7.5–15% polyacrylamide gel under non-reducing conditions and silver stained. The migration of prestained molecular-mass standards are indicated on the right (in kDa), while the migration of β 1-LAP is indicated by the arrow on the left.

produced an effective separation of the functional dimeric β 1-LAP from the non-functional monomeric form of the protein.

Fractions containing β 1-LAP from the S300 column were pooled and dialysed in 10 mM phosphate buffer, pH 7.4, 1 mM EDTA prior to loading on a Reactive Green-19 dye-ligand column. The column was eluted by successive step-wise washes of KCl (Figure 5b). β 1-LAP bound tightly to this matrix, only eluting in the 1.0 M KCl wash. A 10-fold purification was achieved at this stage, with a yield of 44 %.

β 1-LAP was finally purified to homogeneity using a Mono Q anion-exchange column. A sharp peak of β 1-LAP activity eluted from the column in 0.15 M KCl followed by a much smaller peak at $\sim$ 0.3 M KCl (Figure 5c). SDS/PAGE showed that the first β 1-LAP peak contained a single band of molecular mass 114 ± 3 kDa ($n = 5$), while the later peak also contained some lower-molecular-mass contaminating proteins (Figure 6). A summary of the purification is presented in Table 1. A final yield of 85 μ g of protein was obtained from 500 ml of culture medium with an approximate 45-fold purification and a recovery of 15 %. The purified protein from three separate experiments had a specific activity of 169.7, 163.0, and 85.7 units/ μ g respectively.

CD spectral analysis

The crystal structure of TGF- β 2 [28] and the solution structure of TGF- β 1 [29] have recently been solved. Very little is known about the conformation of β 1-LAP. To obtain some information on the structural features of β 1-LAP, the far-UV CD spectrum of the protein was examined between 197 and 320 nm. Due to the

Figure 7 Far-UV CD spectral analysis of β 1-LAP

The far-UV CD spectra for the following samples from 197–250 nm are as indicated: (1) β 1-LAP (solid line), (2) TGF- β 1 (long dash line), (3) summed spectra of TGF- β 1 and β 1-LAP (dash-dot-dash line), (4) latent complex (equimolar mixture of TGF- β 1 and β 1-LAP) (short dash line) and (5) difference spectra (dotted line). The formation of the latent complex between TGF- β 1 and β 1-LAP induces a major conformational change, characterized by a decrease in the minimum at 206 nm and an increase in the shoulder at 223 nm, indicating a decrease in β -sheet content and a subsequent increase in turns and random structures.

high absorbance of the buffer below 197 nm, it was not possible to extend the spectra to shorter wavelengths. The spectra of pure β 1-LAP from 197 nm to 250 nm is shown in Figure 7. Identical spectra were obtained on different days after freeze–thawing of the sample, indicating that the purified protein is relatively stable. The minimum at 206 nm and slight shoulder at 223 nm are characteristic of a large amount of β -sheets. CD analysis of mature TGF- β 1 also suggested a high β -sheet content, in agreement with magnetic resonance spectroscopy data [29].

Table 1 Purification data for β 1-LAP

Sample	Volume (ml)	Protein concn. (μ g/ml)	Total protein concn. (μ g)	Total activity ^a (units)	Specific activity (units/ μ g)	Purification (fold)	Yield (%)
Ammonium sulphate precipitate	4.55	5565	25040	95460	3.8	—	100
Sephacryl S300 pool	42	170	7140	75720	10.6	2.8	79.3
Reactive Green-19 pool	6.5	170	1105	41990	38	10	44
Mono Q pool	1	85	85	14425	169.7	44.7	15.1

^aOne unit of activity is defined as the amount of β 1-LAP required to reverse by 50% the inhibition of 80 pM exogenously added TGF- β 1 to mink lung epithelial cells.

To determine if there are any conformational changes on latent complex formation, equimolar ratios of TGF- β 1 and β 1-LAP were incubated for 2 h at room temperature to allow latent complex formation, before recording the spectrum. This spectrum was then compared with that obtained by summation of the spectra for free TGF- β 1 and β 1-LAP, which would be indicative of the protein conformation assuming there was no significant change in secondary structure after formation of the latent complex (Figure 7). A large decrease in the minimum at 208 nm, and a more prominent shoulder at 223 nm, show that a significant conformational rearrangement occurs upon latent complex formation.

DISCUSSION

The formation of TGF- β 1 in a latent complex provides an important means of regulating the activity of this multipotent growth factor, since in this complex the mature TGF- β 1 is unable to bind to its cellular receptors and elicit its responses. The pro-domain, β 1-LAP, is the factor which confers latency on mature TGF- β 1 [5]. CHO cells overproducing TGF- β 1 make a large amount of mature TGF- β 1 and the TGF- β 1 precursor [5,19]. The TGF- β 1 precursor is a tripeptide complex representing polypeptides linked by disulphide bonds between Cys-33 of the pro form of TGF- β 1 and of β 1-LAP, with a Cys residue in mature TGF- β 1 [19]. The significance of this larger tripeptide precursor of TGF- β 1 is not understood; it may represent an expression artifact. The latent complex purified from platelets is composed of TGF- β 1, β 1-LAP and an additional protein, the LTBP [7,8], while the latent complex overexpressed in CHO cells [5] and that purified from bone [30] contain only β 1-LAP and mature TGF- β 1. That β 1-LAP confers latency on mature TGF- β 1 was further demonstrated by transient expression of β 1-LAP in isolation from mature TGF- β 1 [6]; a functional binding protein for TGF- β 1 was produced which regulated the activity of TGF- β 1 in a dose-dependent manner. To enable further studies on the mechanism by which β 1-LAP regulates the activity of TGF- β 1, we have produced a CHO cell line which secretes high levels of β 1-LAP and have purified the protein to homogeneity.

The β 1-LAP expressed by the CHO-2000 cells migrates as a doublet of 95–105 kDa under non-reducing conditions on SDS/PAGE (Figure 1B). The protein appears to be more highly glycosylated than in previous reports [5,31–33]; the difference may be due to the different clonal origin of the cells used in the expression systems. It has been proposed that the carbohydrate moieties of β 1-LAP play a role in latent complex activation, since treatment with endoglycosidase F or sialidase released mature TGF- β 1 in a dose-dependent manner [12]. This suggests that removal of part or all of the carbohydrate residues can activate the latent complex. Conversely, Wakefield et al. [33] demonstrated that an undersialylated form of β 1-LAP still forms the latent complex with TGF- β 1. Although β 1-LAP expressed by the CHO-2000 clone is glycosylated more than in other systems, this difference does not affect the ability of β 1-LAP to form a complex with TGF- β 1 (Figures 3 and 4). These results suggest that some features of the carbohydrate moieties on β 1-LAP are not critical for the activation process.

A large proportion of the β 1-LAP protein made by the CHO-2000 cells is secreted as a monomeric form. This incorrectly processed form of the protein does not form a complex with mature TGF- β 1 [6,25]. Earlier studies on the overexpression of pro-TGF- β 1 in CHO cells also demonstrated the secretion of incorrectly processed protein [5]. Western blot analysis showed the secretion of unproteolytically processed pro-TGF- β 1 and disulphide-linked aggregates between β 1-LAP and TGF- β 1.

High-molecular-mass aggregates of β 1-LAP and TGF- β 1 have also been reported in the purification of the latent complex from bone [30]. The LTBP may play a role in directing correct folding, disulphide-bond formation and secretion of the latent complex [9,10]. The LTBP does not appear to be expressed in these CHO cells or in bone, which may explain the aberrant processing of the precursor of TGF- β 1 and β 1-LAP when expressed at high levels by these cells.

The β 1-LAP protein was purified by a combination of ammonium sulphate precipitation, gel-filtration, dye-ligand chromatography and anion-exchange chromatography. Typically, 85 μ g of pure protein was obtained from 500 ml of conditioned medium. The purified protein had an apparent molecular mass of 114 kDa. The far-UV CD spectra of pure β 1-LAP, with a minima at 206 nm, is characteristic of a protein structure composed mostly of β -sheets (Figure 7) [34]. TGF- β 1 also contains primarily β structure. The high absorbance of the buffer at wavelengths below 197 nm precluded spectral measurements at shorter wavelengths. It was therefore not possible to obtain a reasonable estimation of the relative contribution of α -helices, β -sheets and random structures to the protein conformation, by comparison with known reference spectra [35]. Consistent with the CD studies, analysis of the β 1-LAP secondary structure by the Chou and Fasman [36] prediction model also suggests a high level of β -sheet with little α -helix.

Although there are limitations in using CD spectroscopy for determining the relative proportions of secondary structure, CD spectral analysis is a very sensitive technique for determining conformational rearrangements [37–40]. Studies of the extracellular domains of the epidermal growth factor receptor [37] and the insulin receptor [39] have demonstrated that both ectodomains undergo significant structural rearrangements upon binding of their respective ligands. Here, we demonstrate that β 1-LAP and TGF- β 1 undergo structural changes upon latent complex formation. Equimolar amounts of TGF- β 1 and β 1-LAP were incubated and the resulting spectra of the latent complex measured. This spectrum was compared with that calculated from summation of the individual β 1-LAP and TGF- β 1 spectra (Figure 7). For the latent complex, a decrease in intensity of the minima at 206 nm and the increase in intensity of the shoulder at 223 nm is indicative of a large conformational change upon latent complex formation. Although it is not clear as to what extent the conformational changes occur in β 1-LAP or TGF- β 1, it is likely that β 1-LAP undergoes major structural rearrangements on forming the latent complex. Enough β 1-LAP for X-ray crystallographic studies will permit a more definitive statement about such structural alterations.

Recent studies of the crystal structure of TGF- β 2 [28], and the solution structure of TGF- β 1 [29] indicate that the two proteins have a similar conformation, both having an elongated non-globular fold, known as a cystine knot motif [41], due to the arrangement of four intrachain disulphide bonds. Since there are seven conserved cysteine residues in the mature regions of the TGF- β superfamily, it is likely that all family members adopt similar conformations, resulting from the constraints placed on the structure by the position of the disulphide bonds. The LAP regions of the five TGF- β isoforms contain 30–45% sequence identity. The highest degree of sequence similarity is in the N-terminus, with residues 40–60 being identical. Site-specific deletion mutagenesis of amino acids 50–85 of β 1-LAP prevents mature TGF- β 1 from complexing with β 1-LAP [18], while proteolytic removal of the N-terminus of β 1-LAP also activates the latent complex [42]. Therefore, this region may be important for the interaction of the pro and mature regions. We have demonstrated that major structural rearrangements occur on

formation of the latent complex between β 1-LAP and TGF- β 1. Since β 1-LAP can form a latent complex with the mature regions of other TGF- β family members [23,43], it would be interesting to determine if similar rearrangements occur on complex formation with other isoforms.

Thrombospondin, an extracellular matrix protein, activates latent TGF- β 1 distinctly from other systems [44]. In this non-enzyme-catalysed system, thrombospondin binds to the latent complex and causes activation of mature TGF- β 1 [45]. Antibodies directed against the N-terminus of β 1-LAP, a site important for interacting with mature TGF- β 1 [18,42], prevent thrombospondin from binding and activating the latent complex, thus localizing the thrombospondin binding site to the N-terminus of β 1-LAP [44]. Association of the N-terminal region of latent TGF- β 1 with thrombospondin may potentially induce major structural rearrangements in the latent complex, releasing the mature growth factor. Therefore, conformational changes of TGF- β 1/ β 1-LAP may play an important functional role in *in vivo* activation of the latent complex.

In addition to conferring latency on TGF- β 1, β 1-LAP may also be useful in localizing the latent complex to the cell surface and/or the extracellular matrix. The latent complex binds to the M6P/insulin-like growth factor II receptor via the M6P residues on β 1-LAP [27]. The LTBP [46] and β 1-LAP [47] also play roles in binding the latent complex to the cell surface. The receptors by which these proteins associate with the cell surface are presently unknown. The LTBP and the latency-associated peptides of TGF- β s 1, 3, 4, and 5 all contain the Arg-Gly-Asp tripeptide recognition sequence for the integrin class of receptors [48], which may provide a means of binding the latent complex to the cell surface. The latent complex also binds to heparin-Sepharose [32], which suggests that it may associate with the extracellular matrix. These results indicate that β 1-LAP plays a pivotal role in associating the latent complex with the cell surface or the extracellular matrix. The availability of pure β 1-LAP will facilitate studies of cell-surface binding of the latent complex.

In summary, we have established a cell line which expresses β 1-LAP and have purified the protein to homogeneity. The availability of purified β 1-LAP will allow further studies into how β 1-LAP regulates the activities of mature TGF- β 1.

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