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Ca^{2+} binding of latent transforming growth factor-β1 binding protein

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Latent transforming growth factor-β1 binding protein (LTBP) is a constituent of the latent high molecular weight complex of TGF-β1 in human platelets. In the present communication, we show that LTBP binds Ca^{2+} in its free form as well as in the latent TGF-β1 complex. The binding of Ca^{2+} induces a structural change which protects the molecule against proteolysis and changes its elution position when analyzed by anion exchange chromatography. The in vitro activation of TGF-β1 is not influenced by the presence of Ca^{2+}. The possible significance of Ca^{2+} binding of LTBP is discussed.

Transforming growth factor-β; Binding protein; EGF-like repeat; Ca^{2+} binding

1. INTRODUCTION

Transforming growth factor-β (TGF-β) is a family of regulatory proteins which influences the growth and differentiation of many different cell types (for reviews see [1]). TGF-β1 has been shown to be present in human platelets as a latent high molecular mass complex [2–4], denoted large latent TGF-β1 (LL-TGF-β1; 210 kDa). This complex is composed of the mature TGF-β1 dimer (25 kDa) non-covalently associated with the latency associated peptide (β1-LAP; 80 kDa) [5], which is linked by disulfide bonding to the latent TGF-β1 binding protein (LTBP; 125–160 kDa) [3,4]. LTBP [6] contains 16 epidermal growth factor (EGF)-like repeats and 3 copies of a new motif containing 8 cysteine residues. β-hydroxylated asparagine residues were identified in two of the EGF-like repeats by amino acid sequencing [6]. LTBP is heterogeneous and ranges in size from 125 to 205 kDa in different cell types probably because of alternative splicing of the LTBP gene and cell specific proteolysis [6].

In order to exert its activity, TGF-β needs to be released from the latent complex. Exposure to low pH or heating can activate TGF-β in vitro [7]. The in vivo activation mechanism is not fully understood but could involve the action of plasmin, glycosidases or other enzymes [8]. The β1-LAP is most important for TGF-β1 latency, whereas the function(s) of LTBP has not been fully elucidated. It has been demonstrated to be important for the proper assembly and secretion of TGF-β1 in a human erythroleukemia cell line, HEL [9]. LTBP may also be involved in targetting of the LL-TGF-β complex to the cell surface where it may undergo activation in a reaction involving the action of plasmin [10]. Since the EGF-like repeats have been shown to mediate high affinity Ca^{2+} binding in other proteins [11–13], we explored in the present communication the possibility that also LTBP binds Ca^{2+}.

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Abbreviations: TGF-β, transforming growth factor-β; LTBP, latent TGF-β1 binding protein; LL-TGF-β1, large latent TGF-β1 complex; β1-LAP, TGF-β1 latency associated peptide; EGF, epidermal growth factor.

2. MATERIALS AND METHODS

2.1. *Preparation of human LL-TGF-β1 and free LTBP*

Recombinant LL-TGF-β1 complex and recombinant small latent TGF-β1 complex were gifts from Dr. H. Ohashi (Kirin Brewery Co., Ltd., Gumma, Japan). Purification of LL-TGF-β1 was performed in the presence of 1 mM $CaCl_2$ in order to avoid proteolysis during the purification procedure (see below) Free platelet LTBP was isolated from a side fraction from the purification of LL-TGF-β1 from human platelets [6]. Conditioned medium of PC-3 human prostate carcinoma cells was used as a crude source of free LTBP.

2.2. *SDS-polyacrylamide gel electrophoresis, silver staining and immunoblotting*

Samples were analysed by SDS-gel electrophoresis (4–10% or 4–15% polyacrylamide gradients) according to Blobel and Dobberstein [14]. Silver staining was performed as described by Morrissey et al. [15]. For immunoblotting, proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membranes (Hybond-C Extra, Amersham, Buckinghamshire, England) for 6 to 8 h at 0.5 mA/cm^2 in 20 mM Tris-HCl, 150 mM glycine, pH 8.6, 0.02% SDS and 20% methanol. Membranes were then blocked by incubation in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl containing 3% bovine serum albumin for 1 h at room temperature. A rabbit antiserum raised against native human platelet LTBP (Ab39, diluted at 1:50) and [^{125}I]Protein A were used for immunological detection [6]. Autoradiography was performed at −70°C.

2.3. *$^{45}Ca^{2+}$ binding to proteins immobilized on nitrocellulose filters*

Samples were first separated by SDS-gel electrophoresis, then electrotransferred in the absence of SDS to nitrocellulose membranes, which then were washed three times in 60 mM KCl, 5 mM $MgCl_2$ and

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10 mM imidazole-HCl, pH 6.8, for 1 h as described by Maruyama et al. [16]. The membrane was incubated with $^{45}CaCl_2$ (Amersham, 10–40 mCi/mg Ca, 1 μCi/ml) in the same buffer overnight at room temperature, and then rinsed several times in distilled water, dried for 3 h at room temperature and subjected to autoradiography. A parallel nitrocellulose membrane was stained with Ponceau S (Sigma, St. Louis, MO, USA) [17] to visualize proteins.

2.4. *$^{45}Ca^{2+}$ binding to immobilized LL-TGF-β1*

Recombinant LL-TGF-β1 complex was extensively dialysed overnight at 4°C against 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA and then against the same buffer without EDTA. Five μg aliquots of LL-TGF-β1 were blotted on the surface of a nitrocellulose membrane using a 96 wells BIO-DOT apparatus (Bio-Rad Laboratories, Richmond, CA, USA). After drying at room temperature, the membrane was washed for 15 min in 60 mM KCl, 5 mM $MgCl_2$ and 10 mM imidazole-HCl, pH 6.8. It was then incubated overnight at room temperature, as described before [16], with $^{45}CaCl_2$ (1 μCi/ml) mixed with different concentrations of $CaCl_2$ (0.1 to 1,000 μM). The $^{45}Ca^{2+}$ binding was visualized and quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

2.5. *Metabolic labeling, immunoprecipitation and tryptic digestion*

PC-3 cells (obtained from S. Nilsson, University of Uppsala) were labeled with [^{35}S]cysteine (Amersham), as described [9]. The anti-LTBP antiserum (Ab39) was used for immunoprecipitation. For proteolysis, ^{35}S-labeled protein immunoprecipitated on Protein A-Sepharose beads (Pharmacia-LKB Biotechnology, Uppsala, Sweden) or recombinant LL-TGF-β1 were incubated with trypsin (Sigma) at 37°C for 2 to 40 min in 50 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA or 2 mM $CaCl_2$. Digestion was stopped by adding soybean trypsin inhibitor (Sigma) and cooling on ice. Samples were then analysed by SDS-gel electrophoresis, fluorography for the ^{35}S-labeled protein and silver staining for the recombinant LL-TGF-β1.

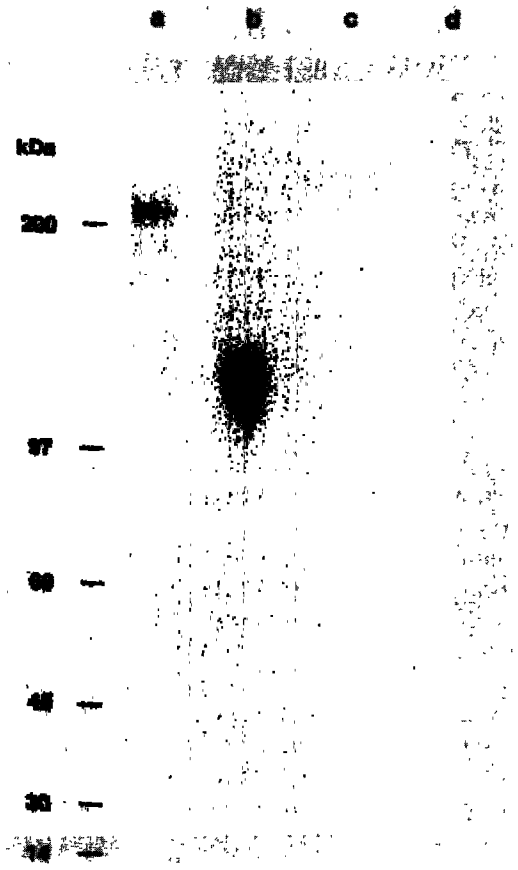

Fig. 1. $^{45}Ca^{2+}$ binding to LTBP. Recombinant LL-TGF-β1 (10 μg; lane a), human platelet LTBP (10 μg; lane b), recombinant small latent TGF-β1 complex (10 μg; lane c), and human blood (2 μl; lane d) were subjected to SDS-gel electrophoresis (4–10% polyacrylamide gradient), and then transferred to a nitrocellulose membrane. After overnight incubation with $^{45}CaCl_2$ (1 μCi/ml), the membrane was rinsed, dried and subjected to autoradiography.

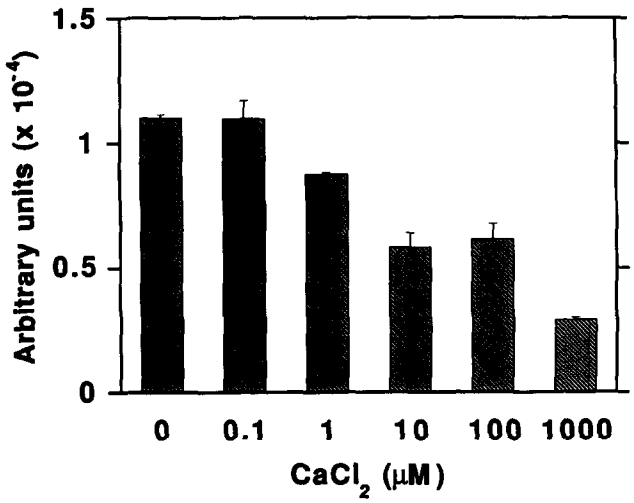

Fig. 2. Affinity of $^{45}Ca^{2+}$ binding to immobilized LL-TGF-β1. Five μg of recombinant LL-TGF-β (triplicates) were blotted on a nitrocellulose membrane, which subsequently was incubated with $^{45}Ca^{2+}$ (1 μCi/ml) mixed with different concentrations of $CaCl_2$ (0.1 to 1,000 μM). Quantification of the $^{45}Ca^{2+}$ binding was performed using a PhosphorImager.

2.6. *Anion exchange chromatography*

Conditioned medium was prepared from confluent PC-3 cells incubated for 24 h with 12.5 ml of serum-free RPMI 1640 medium per 75 cm^2 flask. Centrifuged and filtered (Millex-GV filter, 0.22 μm, Millipore, Molsheim, France) PC-3 cell conditioned medium, with or without 5 mM EDTA or 5 mM $CaCl_2$, was applied to a Q-Sepharose column (16/150, Pharmacia-LKB Biotechnology) equilibrated with 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, with or without 5 mM EDTA or 5 mM $CaCl_2$, at 150 ml/h at room temperature. Elution was performed using a 150–600 mM NaCl gradient for 45 min and at the same flow rate as during sample application. The eluate was collected in 5 ml fractions. LTBP containing fractions were identified by SDS-gel electrophoresis and immunoblotting using the Ab39 antiserum.

3. RESULTS

In order to explore the possibility that LTBP binds Ca^{2+}, free human LTBP purified from platelets and recombinant LL-TGF-β1 immobilized on nitrocellulose filters were incubated with $^{45}CaCl_2$. Both free LTBP and LL-TGF-β1 exhibited clear binding of $^{45}Ca^{2+}$ (Fig. 1). Recombinant small latent TGF-β1 and a sample of human blood used as controls did not bind $^{45}Ca^{2+}$, although Ponceau S staining revealed efficient transfer of proteins to nitrocellulose membranes (data not shown). The binding affinity of Ca^{2+} to LTBP was estimated by the ability of Ca^{2+} to compete with $^{45}Ca^{2+}$ for binding to immobilized LL-TGF-β1 (Fig. 2). Using a Scatchard

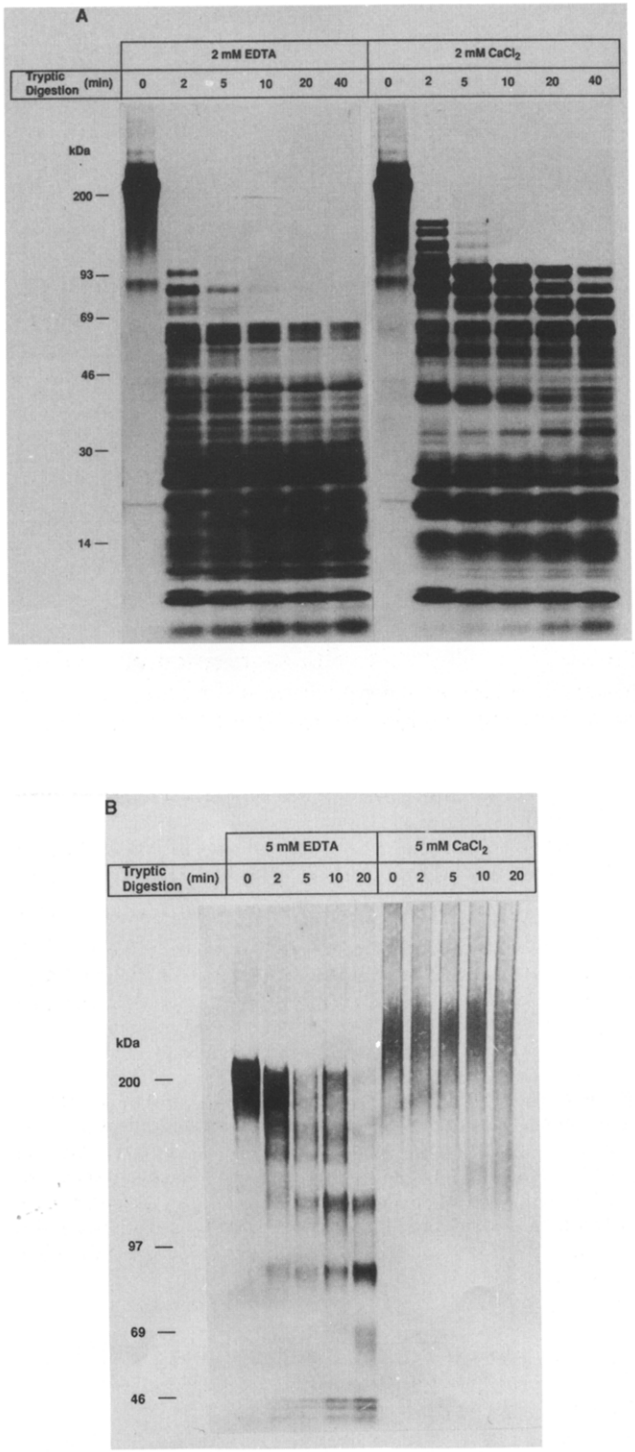

Fig. 3. Effects of Ca^{2+} on the susceptibility to tryptic digestion of the free form of LTBP and recombinant LL-TGF-β1. (A) Immunoprecipitated ^{35}S-labeled LTBP from PC-3 cells was treated with 0.5 μg of trypsin in 50 mM Tris-HCl, pH 7.4, with 2 mM EDTA or 2 mM $CaCl_2$ at 37°C for various time periods; the reactions were quenched by the addition of 2 μg of soybean trypsin inhibitor. Samples were analyzed by SDS-gel electrophoresis and fluorography. (B) One μg of recombinant LL-TGF-β1 was incubated with trypsin in 50 mM Tris-HCl, pH 7.4, with 5 mM EDTA or $CaCl_2$. Samples were then analyzed by SDS-gel electrophoresis and silver staining.

analysis program (RBinding) [18], the dissociation constant (K_d) for the binding of $^{45}Ca^{2+}$ to LL-TGF-β1 was estimated at 3 μM. Binding was not affected by other cations, i.e. Mg^{2+}, Mn^{2+} and Zn^{2+} (not shown).

In order to investigate possible effects of Ca^{2+} binding on protein structure, free LTBP produced by PC-3 cells were immunoprecipitated by Ab39 antibodies, and subjected to proteolytic digestion by trypsin for different time periods at 37°C in the presence of 2 mM $CaCl_2$ or 2 mM EDTA. A protective effect of Ca^{2+} on LTBP was observed; i.e. a delayed proteolysis compared to this obtained in the presence of EDTA (Fig. 3A). The sizes of the protease resistant fragments were similar. The same type of experiments were done using recombinant LL-TGF-β1. Tryptic digestion was performed in the presence of 5 mM $CaCl_2$ or 5 mM EDTA. Proteins were visualized by silver staining (Fig. 3B). Virtually no digestion of LL-TGF-β1 was observed in the presence of $CaCl_2$, whereas in the presence of EDTA, digestion had efficiently occurred after 20 min of incubation with trypsin.

The difference in susceptibility to tryptic digestion suggests that Ca^{2+} binding induces a conformational change in the LTBP molecule. In order to explore this possibility further, anion exchange chromatography in the absence or presence of EDTA or $CaCl_2$ was performed. Fig. 4 shows Q-Sepharose chromatograms of free LTBP from PC-3 cell conditioned medium in the presence of 5 mM EDTA or 5 mM $CaCl_2$ and analysis of individual fractions by immunoblotting using the Ab39 antiserum. When samples were chromatographed in the absence of $CaCl_2$ or EDTA, LTBP mainly eluted at around 460 mM NaCl (data not shown). In the presence of 5 mM EDTA, LTBP eluted later at about 500 mM NaCl. An earlier elution at about 420 mM NaCl was observed if Ca^{2+} was present. The lower affinity of LTBP for the anionic ion exchanger in the presence of Ca^{2+} could be due to a changed charge of LTBP after Ca^{2+} binding, or to a Ca^{2+} induced change in conformation of the molecule.

An important question was whether the Ca^{2+}-induced structural modification of LTBP influenced the activation of LL-TGF-β. Recombinant LL-TGF-β1 samples (10 ng/ml) were incubated with or without 5 mM $CaCl_2$ and activated at 37°C, 65°C or 80°C for 10 min. Then, TGF-β bioactivity was measured by the inhibition of [^{3}H]thymidine incorporation in CCL-64 cells [9]. No significant effect of the presence of Ca^{2+} was noticed on thermic activation of recombinant LL-TGF-β1 (data not shown).

4. DISCUSSION

In this communication, we have shown that LTBP binds Ca^{2+}, in the free form as well as in the LL-TGF-β1 complex, and that the binding of Ca^{2+} leads to a decreased susceptibility to proteolysis of LTBP. Similar

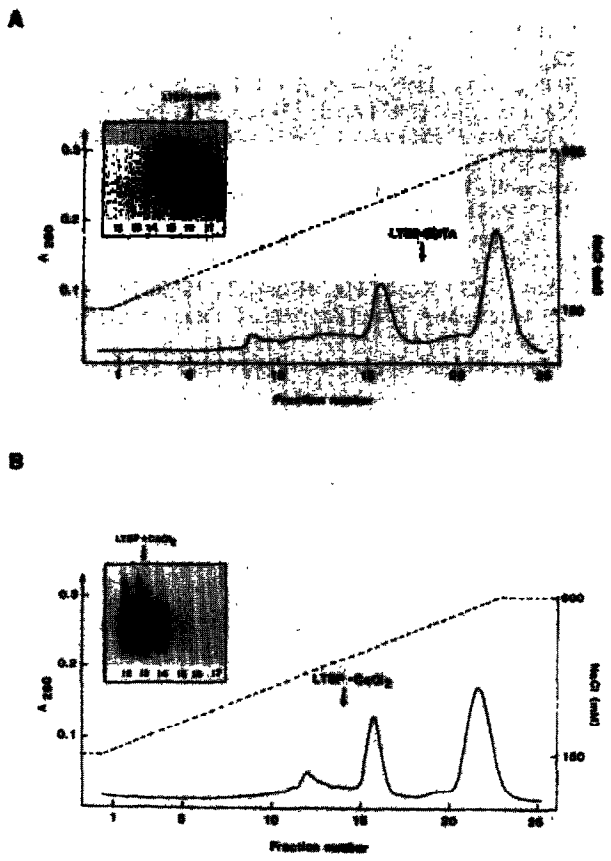

Fig. 4. Effects of Ca^{2+} on LTBP anion exchange chromatography. Aliquots of PC-3 cell conditioned medium (15 ml) were given 5 mM EDTA (A) or 5 mM $CaCl_2$ (B) and subjected to chromatography on a Q-Sepharose column equilibrated (150 ml/h) in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, with 5 mM $CaCl_2$ or EDTA, respectively. A 150–600 mM NaCl gradient (45 min) was used for elution. Absorbance at 280 nm was monitored and aliquots of 40 μl from 5 ml fractions were analyzed by SDS-gel electrophoresis and immunoblotting using the Ab39 antiserum (see insert). The arrows in (A) and (B) indicate the elution positions of LTBP in the presence of EDTA and $CaCl_2$, respectively.

protective effects of Ca^{2+} during tryptic digestion have been observed in other proteins with or without EGF-like repeats [11,19]. The majority of the binding sites was of high affinity with a dissociation constant (K_d) for the binding of Ca^{2+} to LL-TGF-β1 of 3 μM. The number of Ca^{2+} binding sites and their localization in the LTBP molecule remains to be determined. Human LTBP comprises 16 EGF-like repeats and 3 copies of eight-cysteine repeat [6]. Since several different proteins with EGF-like repeats have been shown to bind Ca^{2+}, it is possible that the EGF-like repeats in LTBP are responsible for the binding of Ca^{2+}.

Calcium ions also induced an effect on the elution position of LTBP when analyzed by anion exchange chromatography, suggesting that a conformational change and/or a modification of ionic charge of LTBP occurred. It is important to note that this effect of Ca^{2+} binding is reversible; if LTBP was chromatographed in the presence of EDTA or $CaCl_2$ and then rerun after addition of $CaCl_2$ or EDTA, a shift in elution position was observed (data not shown). The Ca^{2+}-induced conformational change of LTBP described above is not due to co-precipitation with calcium phosphate because the same results were obtained also after an extensive dialysis of the conditioned medium and the recombinant LL-TGF-β1 against Tris-buffered saline (data not shown).

The function of LTBP in vivo is not fully understood. Other proteins with many EGF-like domains have been shown to be involved in homo- or heterophilic protein–protein interactions, e.g. the specific calcium dependent aggregation of Notch with Delta or Serrate *Drosophila* proteins [20]. It is possible that the binding of Ca^{2+} to LTBP regulates its interaction with other molecules, which thus would be of importance for the targetting of LL-TGF-β to specific sites. It is also possible that a Ca^{2+}-induced conformational change of LTBP is of importance for the assembly of LL-TGF-β or for its secretion. Secretory granule biogenesis is thought to begin in the *trans* Golgi network with aggregation of secretory proteins. A decrease in pH to 6.4 and an increase in calcium ion concentration to 10 mM (i.e. conditions that mimic those in the *trans* Golgi network) are important for selective aggregation of secretory proteins, such as granins [21].

In the present study, we have shown that binding of Ca^{2+} did not influence the thermic activation of LL-TGF-β1, however this observation does not exclude a role of the Ca^{2+} binding during the in vivo activation of latent form of TGF-β. An important aim of future studies will be to explore the effect of Ca^{2+} on activation of LL-TGF-β in vivo.

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