Analysis of Small Latent Transforming Growth Factor-β Complex Formation and Dissociation by Surface Plasmon Resonance

ABSENCE OF DIRECT INTERACTION WITH THROMBOSPONDINS

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Transforming growth factor-β (TGFβ) is a pluripotent regulator of cell growth and differentiation. The growth factor is expressed as a latent complex that must be converted to an active form before interacting with its ubiquitous high affinity receptors. This conversion involves the release of the mature TGFβ through disruption of the noncovalent interactions with its propeptide or latency associated protein (LAP). Complex formation or dissociation between LAP and TGFβ plays a very important role in TGFβ biological activity at different steps. To further characterize the kinetic parameters of this interaction, we have employed surface plasmon resonance biosensor methodology. Using this technique, we observed real-time association of LAP with mature TGFβ1. The complex formation showed an equilibrium $K_a$ around 3–7 nM. Furthermore, we observed dissociation of the complex in the presence of extreme pH, chaotropic agents, or plasmin, confirming their effects on TGFβ1 activation. The same approach was used to examine whether latent TGFβ1 could interact with thrombospondins, previously described as activators of latent TGFβ1. Using this method, we could not detect any direct interaction of thrombospondins with either LAP alone, TGFβ1 alone, or the small latent TGFβ1 complex. This suggests that activation of latent TGFβ1 complex by thrombospondins is through an indirect mechanism.

Transforming growth factor-β1 (TGFβ1)$^1$ belongs to a family of peptides regulating cell growth and differentiation and extracellular matrix production (for reviews, see Refs. 1 and 2). Its roles in growth inhibition of many epithelial cells, potentiation of wound repair, angiogenesis, regulation of endocrine functions, and immunomodulation are among its many documented actions. TGFβ1 exerts its effects via binding to high affinity receptors that are expressed on virtually all cell types, and as TGFβ1 production is ubiquitous, there must be strict regulation of its activity.

TGFβ1 is synthesized as a 390-amino acid precursor protein, termed propro-TGFβ1, which undergoes several processing steps. These include proteolytic cleavage of the signal peptide (3), glycosylation and mannose-6-phosphorylation of the prodomain (4, 5), and cleavage at a multibasic residue site by a proconvertase, releasing mature TGFβ1 (3). Completion of posttranslational processing results in a pro-domain (amino acid residues 30–278), called latency-associated peptide (LAP), and a mature TGFβ (amino acid residues 279–390). LAP exists as a homodimer, linked by disulfide bonds, and has been shown to noncovalently bind a mature covalent TGFβ1 homodimer with a 1:1 ratio (6). This biologically inactive complex does not bind to TGFβ receptors. The resulting latent form may then be activated in different ways (for review, see Ref. 7). Dissociation can be accomplished in vitro by heat, extreme pH (above pH 9 or below pH 3), and selected chaotropic agents. In vivo, the mechanism may involve specific proteases such as plasmin (8). Thrombospondins (TSPs), extracellular matrix-associated proteins, have also been described as activators of latent TGFβ1 complex (9, 10). It has been proposed that TSP1 activates latent TGFβ via a protease- and cell-independent mechanism (9).

It is now clear that LAP is implicated at different levels in TGFβ1 biological activity. Previous studies have indicated that LAP plays important roles in secretion and proper folding of the mature growth factor molecule (5, 11, 12). Furthermore, specific binding of LAP to TGFβ may be an important mechanism in regulating the biological functions of secreted TGFβ (6, 13). Indeed, several studies suggest that LAP is a potent inhibitor of bioactive TGFβ both in vitro and in vivo (6, 14). Furthermore, formation of latent complexes increases plasma TGFβ1 half-life about 50-fold (15). Moreover, LAP interacts with the mannose 6-phosphate receptor that has been shown to be required for cellular activation of latent TGFβ1 (16); thus LAP could potentially contribute to enhanced and targeted TGFβ activity.

The first purpose of the present work was to analyze the affinity parameters of the complex formation between LAP and TGFβ1. We thought that a method of choice for determining these parameters would be surface plasmon resonance (SPR) using the BIAcore$^TM$ apparatus (Pharmacia Biotech Inc.). The principle is that one molecule involved in the interaction to be studied is covalently immobilized to a sensor chip, and the other interactant is then passed over the chip in solution. The detection system measures and records a signal proportional to the mass of the protein bound to the surface. In this way, the association phase can be visualized in real time as the ligand-containing solution flows over the surface, and the subsequent dissociation is similarly displayed after the flow switches back to buffer containing no ligand (17). Using this technique, we were able to show direct interaction between LAP and mature TGFβ1, that is, formation of the small latent complex with TGFβ1 immobilized and LAP injected as analyte or vice versa.

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$^*$ The abbreviations used are: TGFβ1, transforming growth factor-β1; LAP, latency-associated peptide; TSP, thrombospondin; SPR, surface plasmon resonance; HBS, HEPES-buffered saline; GcCI, guanidine chloride; RU, resonance unit(s); LTBP, latent TGFβ1 binding protein.
We measured the affinity parameters of this interaction.

The second goal was to use this technique to visualize the dissociation of the small latent TGFβ1 complex by known activators. We confirmed direct activation of the small latent recombinant TGFβ1 by extreme pH, chaotropic agents, or plasmin. However, we did not detect any direct interaction of thrombospondins with either LAP, TGFβ1, or the small latent TGFβ1 complex.

MATERIALS AND METHODS

Equipment and Reagents—The BIAcore™ instrument, sensor chips CM5 and SA5, surfactant P20, and the amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N-(3-dimethylaminopropyl)carbodiimide, and ethanolamine hydrochloride were acquired from Pharmacia Biosensor AB (Uppsala, Sweden). Ultrapure human TGFβ1 was from Genzyme (Cambridge, MA), recombinant human LAP (TGFβ1), and recombinant latent TGFβ1 were from R&D Systems (Abingdon, UK). Bovine plasmin was from Boehringer Mannheim (Abingdon, UK). TSP1 was purified from human platelets. Biotinylated heparin was a generous gift from Dr. Lortat-Jacob (Institut Pasteur, Lyon, France).

Immobilization of TGFβ1 or LAP on Sensor Chip—The BIAcore running buffer was 10 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 0.005% P-20 (HBS, Pharmacia), except where indicated. Equal volumes of 0.1 mM N-hydroxysuccinimide and 0.1 mM N-ethyl-N-(3-dimethylaminopropyl)carbodiimide were mixed, and 35 μl were injected over the surface of the sensor chip to activate the carboxymethylated dextran. TGFβ1 (6.66 μg/ml in 25 mM of 10 mM acetic acid, pH 4) or LAP (13.3 μg/ml in 10 mM of 10 mM acetic acid, pH 4) was injected over the activated surface, followed by 35 μl of ethanolamine to block remaining active carboxyl groups. The immobilization procedure was carried out at 25 °C and at a constant flow rate of 5 μl/min. Control immobilization was performed under the same conditions in the absence of TGFβ1 or LAP.

Kinetic Assays on the BIAcore—All experiments were carried out at 25 °C with a constant flow rate of 25 μl/min in HBS buffer. 50 μl of the analyte were injected for 2 min (association phase), followed by a 2-min period when HBS was passed (dissociation phase). Equal volumes of each protein dilution were also injected over a mock blocked surface to serve as blank sensorgrams for subtraction of bulk refractive index background and nonspecific binding of the analyte. All kinetic assays were followed by an injection of 25 μl of 6 mM guanidine chloride (GnCl) to dissociate remaining bound ligand, leaving only the immobilized interaction (regeneration phase). We worked at low immobilization level, high flow rate (25 μl/min), and concentrations of analyte suitable to limit mass transport. Further, the mass transport limitations were checked using the BIAsimulation program. All the steps described were fully automated and carried out by the BIAcore systems robotics. Association, dissociation, and regeneration phases were followed in real time as a change in signal expressed in resonance units (RU). Curves derived from these assays were used to generate kinetic constants.

Data Analysis—Sensorgrams were analyzed by nonlinear least squares curve fitting using the BIAevaluation program (Pharmacia). Kinetic constants were generated from the association and dissociation curves from the BIAcore experiments by fitting to a single-site binding model (A + B = AB). This model gave a single exponential fit with a χ2 < 0.5. Comparison fitting with more complicated models did not give a better interpretation of the data. The equation was used for the dissociation phase, where was the amount of ligand remaining bound in RU at time and was the beginning of dissociation phase. The final dissociation rate constant, k2, was calculated from the mean of the values obtained from a series of injections. To analyze the association phase, the equation was employed, where was the amount of bound ligand (in RU) at equilibrium, was the starting time of injection, and was the concentration of analyte injected over the sensor chip surface. The association rate constant, k1, was determined from the slope of a plot of versus . The apparent equilibrium dissociation constant was determined from the ratio of these two kinetic constants (k1/k2).

RESULTS AND DISCUSSION

LAP Forms a Molecular Complex with Immobilized TGFβ1—The first goal of our study was to determine whether the association between LAP and mature TGFβ1 could be visualized by SPR. Therefore, experiments were performed in two ways. Either TGFβ1 was immobilized on the chip and LAP injected as the analyte or vice versa, where LAP was immobilized and TGFβ1 was injected. Immobilization of TGFβ1 yielded approximately 600 RU, and the maximal binding capacity of the surface (Rmax) was 1326 RU for LAP binding. Association was started by injection of 50 μl of LAP followed by regeneration. Fig. 1A shows the association and dissociation curves from a representative experiment performed with five different concentrations of LAP (50–200 nM). Corrected sensorgrams are shown after subtraction of a sensorgram performed with the same concentration of LAP on the control flow cell. The association phase (0–120 s) was analyzed by nonlinear least squares curve fitting as described under “Materials and Methods” to yield values at each concentration. A plot of versus concentration of LAP produced a straight line (Fig. 1B)
Association was started by injection of 50 Kd TGF. The squares curve fitting. The dissociation rate constant, \( k_d \), was also analyzed by nonlinear least squares fitting. The dissociation constant \( K_d \) was determined from the ratio of these two rate constants, \( k_a/k_d \), was 7.5 nM. This experiment was repeated on three different chips, producing a mean \( K_d \) of 5.6 ± 1.4 nM (Table I).

**FIG. 2.** TGF1 forms a complex with immobilized LAP. LAP was immobilized (around 800 RU) and then TGF1 was injected to form small latent TGF1 complex. Sensorgrams (relative response in RU vs. time in sec) of TGF1 binding (bottom to top curve: 50, 80, and 100 nM of TGF1).

**FIG. 3.** Activation of latent TGF1 complex. TGF1 was immobilized (around 600 RU), and then LAP (50 nM) was injected to form small latent TGF1 complex. A, over this complex, 25 \( \mu \)l of 6 \( \mu \)M guanine chloride (GnCl) was injected at a flow of 5 \( \mu \)l/min, followed by another injection of LAP (50 nM) and then a second injection of 6 \( \mu \)M GnCl. B, over this complex, bottom to top curve: 50 \( \mu \)l of HBS (CTL), pH 8.6; *, plasmin (0.1 units/ml in HBS, pH 8.6); or **, heat-inactivated plasmin (0.1 units/ml in HBS, pH 8.6) was injected at a flow of 2 \( \mu \)l/min.

TGF1 Forms a Complex with Immobilized LAP —Similar experiments were performed with immobilized LAP (approximately 800 RU, yielding an \( R_{max} \) of 370 RU for TGF1 binding). Association was started by injection of 50 \( \mu \)l of TGF1 followed by regeneration. Fig. 2 shows the association and dissociation curves from a representative experiment performed with three different concentrations of TGF1 (50, 80, and 100 nM). Constants were determined as described above. The \( k_a \) value for TGF1 binding to immobilized LAP (Table I) was determined to be 31.7 ± 26.6 \( \times 10^4 \) s\(^{-1}\) mM\(^{-1}\). The \( K_d \) value for TGF1 dissociation from LAP surface was calculated as 8.5 ± 2.0 \( \times 10^{-4} \) s\(^{-1}\) (Table I). The apparent equilibrium dissociation constant \( K_d \) was 2.7 nM. This experiment was repeated on three different chips, producing a mean \( K_d \) of 3.2 ± 1.3 nM (Table I).

Whether LAP or TGF1 was immobilized, we observed a similar equilibrium constant (\( K_d \) = 3.2 or 5.6 nM, respectively). However, the respective \( k_a \) and \( k_d \) values obtained with each protocol differed by a log. LAP binding to immobilized TGF1 yielded smaller \( k_a \) and \( k_d \) values than TGF1 binding to immobilized LAP. This may reflect a different sensitivity of LAP and TGF1 to chemical cross-linking to the sensor chip. The immobilization procedure would appear to more faithfully preserve the binding sites of LAP than those of TGF1. This \( K_d \) was in agreement with that previously published by Miller et al. (18) (1.1–1.8 nM) using a covalent cross-linking method. The nanomolar range affinity between LAP and TGF1 could have major implications. Indeed, cellular receptors for TGF1 have been shown to have apparent \( K_d \) values between 20–50 pm (19–21), around 100-fold smaller than the apparent \( K_d \) values for TGF1 binding to LAP. The greater affinity of TGF1 for cell receptors than for LAP suggests that TGF1 interaction with the cell is favored. This would be in agreement with the latent TGF1 complex representing a circulating form of TGF1. In support of this idea, it has been shown that the latent complex of TGF1 has a serum half-life of 2 h as opposed to a half-life of 3 min observed for the mature active growth factor (15). Formation of latent complexes could thus switch TGF1 from an autocrine/paracrine mode of action to a more

### Table I

| Binding parameters derived from the BIAcore experiments |
|--------------------------------------------------------|
| \( k_a \) s.D. \(^a\) | \( k_d \) s.D. \(^a\) | \( K_d \) \(^b\) | Mean \( K_d \) s.D. \(^a\) |
|------------------------|------------------------|----------------|------------------------|
| Immobilized TGF1       | LAP analyte            | 1.2 ± 0.4 \( \times 10^4 \) | 0.9 ± 0.4 \( \times 10^{-4} \) | 7.5 | 5.6 ± 1.4 |
| Immobilized LAP        | TGF1 analyte           | 31.7 ± 26.6 \( \times 10^4 \) | 8.5 ± 2.0 \( \times 10^{-4} \) | 2.7 | 3.2 ± 1.3 |

\(^a\) Kinetic rate constants determined as described under "Materials and Methods" from sensorgrams as depicted in Figs. 1 and 2.

\(^b\) Similar experiments were repeated three times on different chips from which a mean \( K_d \) was determined.
endocrine mode involving target organs distant from the site of synthesis.

Real-time Activation of TGFβ — We then wondered whether SPR could permit us to visualize the dissociation of the small latent TGFβ1 complex during the activation process. As mentioned above, latent TGFβ1 can be activated by different means. Using SPR technology, we could monitor dissociation of the complex by injecting extreme pH buffers (above pH 9 or below pH 3, data not shown). Regeneration protocols confirmed that this complex can also be dissociated by chaotropic agents such as GnCl (Fig. 3A). This demonstrates that the interaction between these two peptides results at least in part from electrostatic interactions. As illustrated in Fig. 3A, a similar amount of LAP binds to TGFβ1 before and after GnCl treatment. This indicates that the binding capacity of TGFβ1 was preserved during the activation of the latent TGFβ1 complex by GnCl and validates its use of GnCl in surface regeneration for SPR analyses.

A more physiological mode of activation of TGFβ1 is through proteolytic cleavage by plasmin, which results in the removal of the amino-terminal portion of the pro-domain (22). We checked whether we could visualize this activation in real time by SPR. To do this, small latent TGFβ1 complex was formed as described previously (TGFβ1 immobilized and LAP injected afterward), and then 50 μl of plasmin (dialyzed overnight against HBS, pH 8.6, 0.1 units/ml) was injected at a flow of 2 μl/min. As shown in Fig. 3B, plasmin binds temporarily to the small latent TGFβ1 complex, dissociates from the complex, and displaces approximately 40% of the SPR signal (159 RU bound in the control versus 90 RU after injection of plasmin, Table II). This signal loss cannot be explained by the release of the proteolytic fragment of LAP alone, which constitute one-fifth of the molecule (22). It is more probable that the loss of 40% of the SPR signal corresponds to the release of 40% of LAP molecules bound to TGFβ1. This corresponds to the level of dissociation observed in vitro with plasmin (22). As a control, heat-inactivated plasmin (5 min at 100 °C) was added; in that case, inactivated plasmin still binds to the small latent TGFβ complex but does not trigger measurable dissociation of the complex. To confirm that the entire LAP molecule was released, we reinjected LAP. We were able to reconstitute an equivalent amount of complex as was present prior to plasmin treatment, as shown in Table II. This suggests that plasmin disrupted

![Table II](image)

Reversibility of the activation of small latent TGFβ1 complex by plasmin

| Amount of small latent TGFβ complex (RU) | First association | Postplasmin injection | Second association |
|----------------------------------------|------------------|----------------------|------------------|
| Immobilized TGFβ1                      | 159              | 90                   | 161              |
| LAP analyte                            |                  |                      |                  |
| Immobilized LAP                         | 125              | 41                   | 41               |
| TGFβ1 analyte                           |                  |                      |                  |

*RU after analyte injection.

*RU left after plasmin injection.

*RU after a second injection of analyte following plasmin injection and regeneration with 6 M GnCl.

*RU left after plasmin injection versus in the absence of plasmin.

![Fig. 4](image)

Absence of association of TSP1 and TSP2 with LAP, TGFβ1, or small latent TGFβ1 forms. A, TSP1 (50 nM) was injected over immobilized LAP, TGFβ1, and a control (CTL) immobilized flow cell. B, TSP2 (50 nM) was injected over immobilized LAP, TGFβ1, and a control immobilized flow cell. C, LAP was immobilized and then TGFβ1 (50 μl) was injected to form small latent TGFβ1 complex, and over this complex, TSP2 (50 nM) or TSP1 (50 nM) was injected. D, vice versa of C. TGFβ1 was immobilized and then LAP (50 nM) was injected to form small latent TGFβ1 complex, and over this complex, TSP1 (50 nM) or TSP2 (50 nM) was injected.
TSPs with the small latent TGF
known activators, we used it to investigate the interaction of
through the observation of the dissociation of this complex by
formed recombinant latent TGF
Altogether, our data indicate that plasmin can dissociate pre-
also be dissociated by addition of plasmin (data not shown).

**FIG. 5. Association of TSP1 or TSP2 to heparin.** Biotinylated heparin was immobilized onto a streptavidin chip (300 RU of fixed molecules). 35 µl of 50 nM of TSP1 (A) or TSP2 (B) was injected at a flow of 5 µl/min.

LAP binding to TGFβ1 but did not modify the immobilized TGFβ1. In the inverse experiment with the LAP-TGFβ1 com-
plex (LAP immobilized and TGFβ1 as analyte), plasmin dis-
placed approximately 70% (125 RU bound in the control versus
41 RU after injection of plasmin, Table II) of the complex.
However, as shown in Table II, when TGFβ1 was reinjected, no
LAP-TGFβ1 complex was reformed. This suggests that a frac-
tion of LAP had been irreversibly proteolyzed by plasmin.
Finally, a recombinant commercial source of preformed small
latent TGFβ1 complex immobilized to the sensor chip could also be dissociated by addition of plasmin (data not shown).

Having validated this method through the determination of the kinetic parameters of LAP-TGFβ1 complex formation and
through the observation of the dissociation of this complex by
known activators, we used it to investigate the interaction of
TSPs with the small latent TGFβ1 complex. TSPs are extra-
cellular matrix-associated proteins that have also been de-
scribed as activators of the latent TGFβ1 complex (9, 10).
Murphy-Ullrich and co-workers (23) have found that TSP1
activates latent TGFβ via a protease- and cell-independent
mechanism, whereas TSP2 does not. Their work indicates that
TSP1 activation of TGFβ could result from the binding of the tripeptide RFK, not present in TSP2, to the amino-terminal
portion of LAP, inducing a conformational change of the com-
plex and the liberation of mature TGFβ1 (23). In contrast, our
lab has reported previously that both TSP1 and TSP2
activate the large latent TGFβ1 complex (containing LTBP
(latent TGFβ binding protein)) (10). This prompted us to
investigate the possible association of TSP1 and TSP2 with LAP or
the small latent TGFβ1 complex using SPR. TSP1 (Fig. 4A) or
TSP 2 (Fig. 4B) was injected over immobilized LAP or TGFβ1
or a control flow cell in the presence of 2 mM Ca²⁺, which is
esential to TSP structure and function (24, 25). No specific
association of TSP1 or TSP2 (50 nM) with LAP or TGFβ1 could
be detected as the SPR signal returned to the same level as
before the injection. Similar results were obtained in the ab-
ence of Ca²⁺ (data not shown). The significant change in the
signal after injection of TSP2 or TSP1 was due to the bulk
refractive index of the buffer in which the TSPs are purified,
which can also be observed on the control immobilization flow
cell. We then tested if TSP1 or TSP2 could interact with the
small latent TGFβ1 complex bound to the sensor chip through
either LAP (Fig. 4C) or TGFβ1 (Fig. 4D). Again, there was no
association between TSPs and the small latent TGFβ1 com-
plexes. These experiments were performed with a wide range of
TSP concentrations and gave similar results (data not shown).

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