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Transforming Growth Factor-β1 Binds to Immobilized Fibronectin

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We have characterized the interaction of homodimeric porcine transforming growth factor-β1 (TGF-β1) with affinity-purified human plasma fibronectin. Using a solid-phase binding assay, we have demonstrated that TGF-β1 binds to fibronectin immobilized on Immunlon I™ microtiter plates. TGF-β1 binding increased with time, reaching a plateau after 4–6 h, and was dependent upon the concentration of both labeled TGF-β1 and immobilized fibronectin present. The binding of radiolabeled TGF-β1 to fibronectin was saturable and was reduced 75% in the presence of a 100-fold excess of unlabeled TGF-β1. TGF-β1 bound to fibronectin with an association rate constant (K_a) of 2.96×10^3 M^{-1} s^{-1} and did not readily dissociate under various conditions. The binding of TGF-β1 to fibronectin was insensitive to variations in ionic strength over a range of 0.1–1.0 M NaCl and was relatively insensitive to divalent cation concentration in the range of 0.1–10.0 mM as well. These data suggest that the binding of TGF-β1 to fibronectin may not be dependent upon the interaction of charged amino acids within these two molecules. However, the binding of TGF-β1 to fibronectin was strongly pH-dependent and binding decreased dramatically below pH 4.0 and above pH 10.0, suggesting that charged amino acids may influence TGF-β1/fibronectin interactions. The association of TGF-β1 with immobilized fibronectin or other extracellular matrix components and subsequent dissociation under acidic conditions or by an as-yet-unidentified mechanism may play a role in the distribution and/or activity of this potent growth regulator at sites of tissue injury and inflammation in vivo.

Key words: cell growth, cell differentiation, growth factor binding, extracellular matrix, fibronectin

The association of polypeptide growth factors with components of the extracellular matrix may play an important role in the regulation of the distribution and/or activity of these growth factors. The binding of several growth factors to extracellular matrix proteins has been reported [1]. Others, like basic fibroblast growth factor (bFGF) which is synthesized by endothelial cells, are reportedly incorporated into the pericellular matrix, presumably bound to heparan sulfate proteoglycans [2,3]. The binding of bFGF to extracellular matrix heparan sulfate may limit its distribution and increase its

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concentration in the matrix microenvironment. In addition, interactions with extracellular matrix heparan sulfate may enhance the mitogenic activity of FGF [4] or protect it from degradation [5]. The extent to which polypeptide growth factors other than heparin-binding growth factors interact with components of the extracellular matrix remains unclear.

Transforming growth factor-β (TGF-β), a ubiquitous 25 kDa polypeptide found in platelets and produced by a variety of normal and transformed cells [6–8], was originally characterized by the ability to transform normal cells but has now been clearly established as a bifunctional modulator of growth and differentiation [9]. TGF-β stimulates the synthesis of fibronectin and collagen by chick embryo fibroblasts and increases the incorporation of fibronectin into the pericellular matrix [10]. TGF-β increases the synthesis and cell surface expression of integrins by human fetal lung fibroblasts [11] and 3T3-L1 pre-adipocytes [12] as well as the expression of cell surface chondroitin and dermatan sulfate proteoglycans [13, 14]. TGF-β also influences matrix degradation by modulating protease and protease inhibitor production [15,16]. Many of the effects of TGF-β on cell growth and differentiation may be mediated in part by these effects on matrix biosynthesis [10]. Recent reports have shown that fibronectin can mimic the effects of TGF-β on fibroblasts grown under anchorage-independent conditions [10]. A previous report [17] suggested that the apparent growth-promoting effects of fibronectin were not inherent in fibronectin itself but were due instead to TGF-β that co-purified with fibronectin during isolation from plasma. However, this study [17] did not directly demonstrate the binding of TGF-β to fibronectin and did not define the conditions necessary for TGF-β/fibronectin binding or dissociation.

TGF-β is secreted by many normal and transformed cells in a latent form that can be activated by acidification [18]. In this latent form TGF-β is bound to one of several "masking" proteins that may alter its function [19–21]. Among these is a protein with an apparent molecular weight of 440 kDa [22]. The relationship between this 440 kDa protein and fibronectin (440 kDa) [23] has not been determined.

In this report we describe the interactions between porcine TGF-β1 and immobilized human plasma fibronectin by using a solid-phase binding assay and define the conditions necessary for TGF-β1/fibronectin binding and dissociation.

METHODS

Iodination of Transforming Growth Factor-β1

Porcine TGF-β1 was isolated from porcine platelets as described by Cheifetz et al. (1987) [24]. TGF-β1 was iodinated by using the chloramine-T method described by Ruff and Rizzino [25]. Briefly, 2.0 mCi $Na^{125}I$ (ICN, Irvine, CA) was neutralized with 20 μl of 1.2M phosphate buffer (pH 7.2) and transferred to a polypropylene tube. Carrier-free porcine TGF-β1 (2.5–5.0 μg in 20 μl of 30% acetonitrile/0.1% trifluoroacetic acid) was then added. Chloramine-T (5 μl of a 150 μg/ml solution in Tris-HCl, pH 7.2) was added to initiate the reaction and two 5.0 μl additions were made after 1.5 min and 3.0 min. The iodination was terminated after an additional 2.0 min by adding 200 μl of a saturated tyrosine solution, 200 μl 4.0M urea (ultra-pure urea in 1.0 M HCl), and 20 μl potassium iodide (60 mM in phosphate buffer, pH 7.2). Labeled TGF-β1 was separated from free iodine on a Sephadex G-25 column pre-equilibrated with 4 mM HCl, 75 mM NaCl, and 0.2% bovine serum albumin and eluted with this same buffer.

The specific activity of [^{125}I]-TGF-β1 labeled in this manner was generally 75–100 μCi/μg and 90–95% of the radioactivity could be precipitated with 10% trichloroacetic acid (TCA). Radiolabeled TGF-β1 was also capable of reversible, specific binding to cellular TGF-β1 receptors on mink lung epithelial cells (Mv1Lu) (data not shown). The inhibitory effect of unlabeled TGF-β1 on MvLu DNA synthesis was measured over a broad concentration range (0.1–100 ng/ml) (data not shown). The quantity of labeled TGF-β1 recovered following iodination was estimated by comparing the inhibitory activity in serial dilutions of labeled TGF-β1 with the dose-response curve for unlabeled TGF-β1. Using this method we determined that our recovery was approximately 50%.

Preparation of Fibronectin

Fibronectin was isolated by previously described methods [26]. In brief, a fibronectin- and fibrinogen-rich by-product of human Factor VIII production was obtained from Dr. Micheal Chopek (University of Minnesota). Approximately 25.0 g of this lyophilized material was resuspended in 600 ml deionized water. Fibrinogen was removed by heat precipitation at 56°C for 30 min followed by filtration on several layers of surgical gauze. The fibrinogen-free supernatant was dialyzed overnight against 0.01 M Tris/0.075M NaCl (pH 7.4), clarified by centrifugation at 7,500 rpm for 15 min, and applied to a DEAE-Trisacryl ion exchange column. A linear salt gradient (0.075–0.3M NaCl in 0.1M Tris, pH 7.4) was applied; the fibronectin peak was collected, dialyzed overnight against 1.0 mM EDTA in phosphate-buffered saline (PBS), pH 7.4, and applied to a gelatin affinity column. Fibronectin bound to the affinity column was eluted with 0.05M sodium acetate/0.5M sodium bromide at pH 5.0, dialyzed extensively against phosphate-buffered saline, pH 7.4 (12–16 liters), and stored frozen at −70°C. Fibronectin purified in this manner migrated as a closely spaced doublet of 230 kDa and 240 kDa on reducing sodium dodecyl sulfate polyacrylamide electrophoretic gels (SDS-PAGE) and was found to be greater than 98% pure by Coomassie Blue staining. The ability of affinity-purified fibronectin to stimulate NRK-49F cell anchorage-independent growth was measured to check for the presence of platelet-derived TGF-β1. NRK-49F cell anchorage-independent growth was not stimulated by fibronectin at concentrations in the range of 0.1 to 100 μg/ml.

Solid Phase Binding Assay

The binding of [^{125}I]-TGF-β1 to immobilized fibronectin or bovine serum albumin (BSA, fatty acid free, ICN Immunobiologicals, Irvine, CA) was measured by using a solid-phase binding assay [27]. Fibronectin or BSA at various equimolar concentrations (0.125–50 pmole/well) in phosphate-buffered saline (PBS), pH 7.4, was added to Immulon I™ plates (Dynatech Laboratories, Inc., Alexandria, VA) (50 μl/well) and allowed to dry overnight at 29°C (see figure legends). Approximately 80% of the fibronectin immobilized in this manner remained bound to the Immulon I™ plastic as measured by using [^{3}H]-fibronectin. Any residual protein-binding sites on the coated plates were then blocked with 200 μl of binding buffer (10 mM phosphate buffer containing 0.1M NaCl, 68 M $CaCl_2$, 0.1% BSA, pH 7.4) for 90 min at 37°C. Following blocking, 50 μl of [^{125}I]-TGF-β1 was added at various concentrations (65–2,700 pM) in binding buffer for periods ranging from 15 min to 24 h. Unbound [^{125}I]-TGF-β1 was removed by washing three times with binding buffer containing 0.05% Triton X-100. Fibronectin-bound [^{125}I]-TGF-β1 was solubilized with 200 μl of 0.5M NaOH and 1%

SDS for 30 min at 60°C and the radioactivity was measured in a Beckman gamma counter. In each experiment, the specific binding of [^{125}I]-TGF-β1 to fibronectin was calculated by subtracting the nonspecific binding of [^{125}I]-TGF-β1 to BSA-coated plates from the total [^{125}I]-TGF-β1 bound to fibronectin.

RESULTS

TGF-β1 Binding to Increasing Concentrations of Immobilized Fibronectin

Experiments were performed to determine the extent of radiolabeled TGF-β1 binding to immobilized fibronectin. Immulon I™ plates were coated with fibronectin in the concentration range of 0.125 to 50 pmol/well and the amount of [^{125}I]-TGF-β1 that bound to fibronectin increased as a function of immobilized fibronectin concentration, reaching a plateau at fibronectin concentrations above 5 pmol/well (Fig. 1A). The amount of [^{125}I]-TGF-β1 bound to fibronectin also increased as a function of the amount of labeled TGF-β1 added (Fig. 1B) and saturation was observed at higher [^{125}I]-TGFβ1 concentrations. The binding of radiolabeled TGF-β1 was reduced 75% in the presence of 100-fold excess unlabeled TGF-β1 (Fig. 1C). The nonspecific binding of [^{125}I]-TGF-β1 to BSA-coated plates under similar conditions was 10% or less of the binding to immobilized fibronectin.

TGF-β1 Binding to Fibronectin: Kinetics of Association and Dissociation

The kinetics of TGF-β1 binding to immobilized fibronectin were determined. The binding of [^{125}I]-TGF-β1 to fibronectin increased with time and reached a plateau after 3–4 h (Fig. 2A). In additional experiments, [^{125}I]-TGF-β1 was allowed to bind to immobilized fibronectin for 24 h. Unbound labeled TGF-β1 was then removed by washing the plates three times with binding buffer and the kinetics of dissociation were measured. As seen in Figure 2b, 80% or more of the radiolabeled TGF-β1 remains tightly bound to immobilized fibronectin after an additional 24 h. The dissociation of bound [^{125}I]-TGFβ1 was not enhanced in the presence of 100-fold excess unlabeled TGF-β1 (data not shown). The association rate constant (K_a) for the binding of [^{125}I]-TGF-β1 to fibronectin was calculated by using methods described by Rodbard [28] and was approximately $2.96 \times 10^3\ M^{-1}\ s^{-1}$.

Influence of pH, Ionic Strength, Divalent Cations, and Heparin on TGF-β1 Binding to Fibronectin

The binding of [^{125}I]-TGF-β1 to fibronectin over a wide pH range was studied. The binding of labeled TGF-β1 to fibronectin was unaffected by pH in the range of 4.0 to 10.0 (Fig. 3A). At extremes of pH (less than pH 4.0 or greater than pH 10.0) binding was markedly reduced.

The binding of [^{125}I]-TGF-β1 to immobilized fibronectin was insensitive to ionic strength over a wide range (.01–1.0M NaCl) (Fig. 3B). In addition, the binding of labeled TGF-β1 to fibronectin was found to be largely independent of divalent cation concentration. At Ca^{+2} concentrations below 5.0 mM, no effect on [^{125}I]-TGF-β1 binding to fibronectin was observed (Fig. 3C). At Ca^{+2} concentrations in the range of 10.0–50.0 mM, TGF-β1 binding to fibronectin increased approximately twofold. Heparin (Sigma Chemical Co.), which binds to fibronectin [29], did not alter the binding of

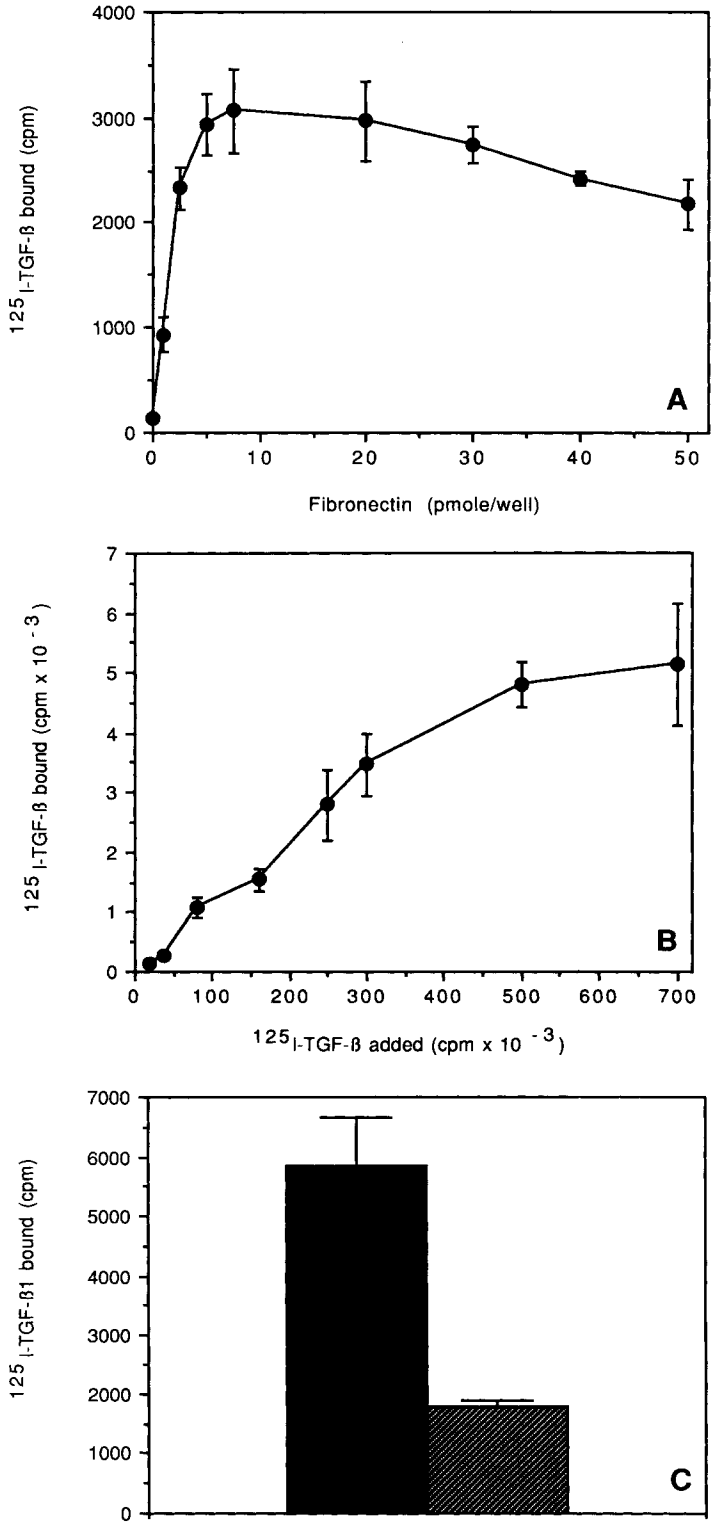
4000
3000
2000
1000
0
125I-TGF-ß bound (cpm)
A
0
10
20
30
40
50
Fibronectin (pmole/well)
7
6
5
4
3
2
1
0
125I-TGF-ß bound (cpm x 10 -3)
B
0
100
200
300
400
500
600
700
125I-TGF-ß added (cpm x 10 -3)
7000
6000
5000
4000
3000
2000
1000
0
125I-TGF-ß1 bound (cpm)
C

Figure 1.

labeled TGF-β1 to fibronectin when tested over a concentration range of 0.01 to 100 μg/ml (Fig. 3D).

pH-Dependent Dissociation of [^{125}I]-TGF-β1 From Immobilized Fibronectin

Since [^{125}I]-TGF-β1 did not associate with fibronectin at low pH (see Fig. 3A), experiments were performed to determine whether [^{125}I]-TGF-β1 could also be selectively removed from immobilized fibronectin at low pH. [^{125}I]-TGF-β1 was allowed to associate with immobilized fibronectin for 1 h, and following the removal of unbound labeled TGF-β1 it was observed that labeled TGF-β1 dissociated from fibronectin-coated plates under acidic conditions (below pH 4.0) (Fig. 4A). Under identical conditions, immobilized [^{3}H]-fibronectin could not be removed from Immulon I™ plates at low pH (Fig. 4B). These results demonstrated that the reduction in the amount of bound TGF-β1 was due to the dissociation of TGF-β1 from immobilized fibronectin and not due to the dissociation of TGF-β1/fibronectin complexes from the surface of the Immulon I™ plates.

DISCUSSION

The binding of polypeptide growth factors to components of the extracellular matrix may be an important step in the regulation of the activity and/or distribution of these growth regulators. Endothelial cells produce bFGF and incorporate it into their pericellular matrix, restricting its distribution to the local microenvironment where it may act as an autocrine regulator of endothelial cell growth [2]. The binding of bFGF by heparin (and presumably heparan sulfate proteoglycans in the extracellular matrix) enhances the mitogenic activity of this growth factor [4] and also protects it against proteolytic degradation [5].

The extent to which other families of polypeptide growth factors may interact with matrix components remains unclear. The evidence that the ubiquitous polypeptide TGF-β may be one such growth factor comes from several sources. TGF-β has profound effects upon extracellular matrix synthesis which include stimulation of fibronectin synthesis and its incorporation into the pericellular matrix [10]. Fibronectin can mimic the ability of TGF-β to stimulate anchorage-independent growth by normal fibroblasts [10]. These observations support the view that TGF-β promotes anchorage-independent growth by stimulating fibronectin synthesis, thus providing a pericellular matrix that supports cell growth. Alternatively, the ability of fibronectin to stimulate anchorage-independent growth may not be intrinsic to fibronectin itself but may be due to the association of TGF-β with fibronectin during purification from plasma [17]. A TGF-β-like polypeptide immunologically related to TGF-β can be separated from some preparations of plasma fibronectin by acidification [17].

Fig. 1. Binding of TGF-β1 to immobilized fibronectin. **A:** The binding of [^{125}I]-TGF-β1 (12×10^3 cpm) to Immulon™ I plates coated with increasing concentrations of fibronectin (0.1–50 pmol/well) was measured as described in Materials and Methods. **B:** Immulon™ I plates were coated with 0.125 pmol/well fibronectin and the binding of [^{125}I]-TGF-β1 (65–2,700 pM) was measured as described in Materials and Methods. **C:** [^{125}I]-TGF-β1 (4×10^4 cpm) binding to 0.125 pmol/well immobilized fibronectin in the absence (solid bar) or presence (cross-hatched bar) of 100-fold excess unlabeled TGF-β1. Nonspecific binding to equimolar concentrations of immobilized BSA has been subtracted and data are expressed as mean ± 1 standard deviation of triplicate determinations.

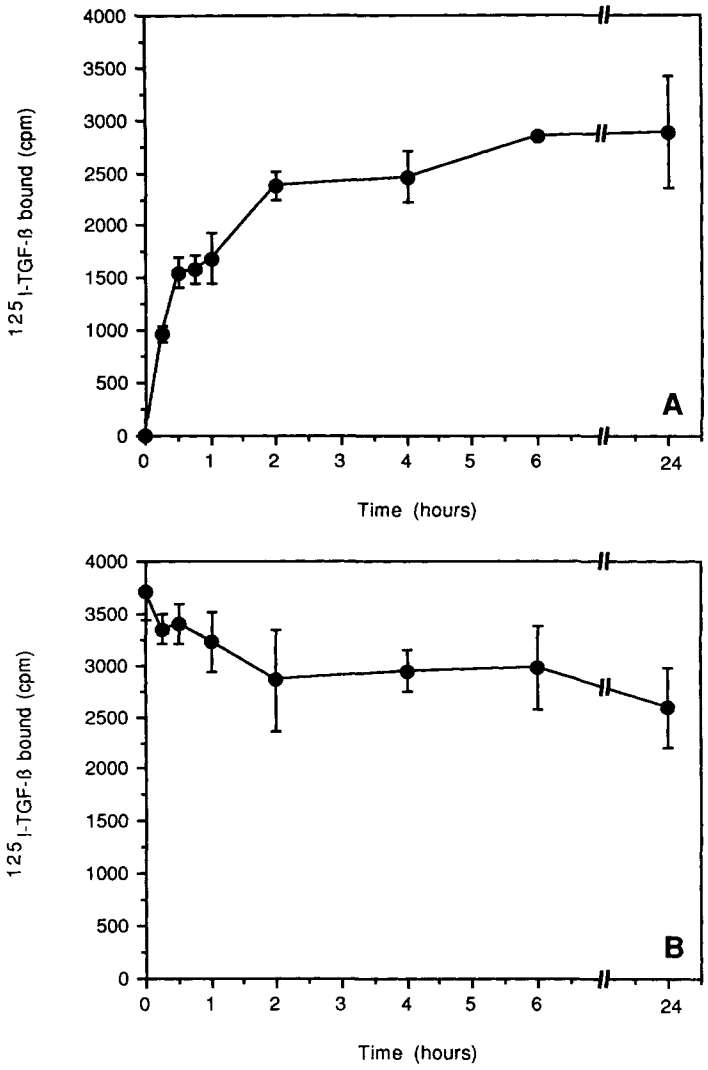

Fig. 2. Kinetics of TGF-β1 binding to immobilized fibronectin. **A:** [^{125}I]-TGF-β1 (12×10^3 cpm) was incubated with immobilized fibronectin or BSA (0.2 pmol/well) and the amount of bound TGF-β1 was determined over a 24 h period. **B:** [^{125}I]-TGF-β1 (12×10^3 cpm) was incubated with immobilized fibronectin or BSA (0.2 pmol/well) for 24 h. Buffer containing unbound [^{125}I]-TGF-β1 was then removed and replaced with fresh binding buffer. Binding was determined as a function of time following removal of labeled TGF-β1. Nonspecific binding to equimolar concentrations of BSA has been subtracted. Data are expressed as the mean ± 1 standard deviation of three determinations.

We have demonstrated that [^{125}I]-TGF-β1 binds to immobilized human plasma fibronectin using a solid-phase binding assay. This binding increases with time, reaching a plateau after 3–4 h. Binding is dependent upon the concentration of labeled TGF-β1 and upon the concentration of immobilized fibronectin. [^{125}I]-TGF-β1 binds to fibronectin with high affinity and only 10–20% of bound TGF-β1 dissociated over a 24 h period following the removal of unbound labeled TGF-β1. The dissociation of TGF-β1

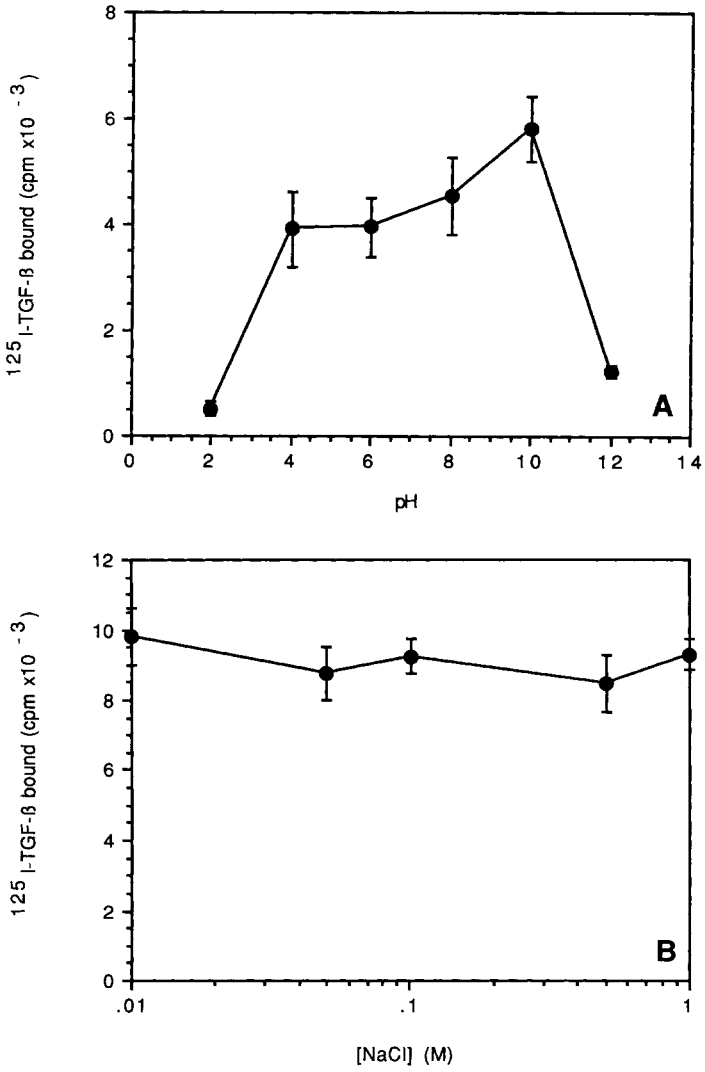

Fig. 3 Effect of pH, ionic strength, divalent cations, and heparin on the binding of TGF-β1 to fibronectin. [^{125}I]-TGF-β1 (35×10^3 cpm) was incubated with 0.2 pmol/well immobilized fibronectin and binding was measured (**A**) as a function of pH in the range of pH 2 to pH 10, (**B**) as a function of ionic strength at NaCl concentrations of 0.01 to 1.0 M, (**C**) as a function of Ca^{+2} concentration in the range of 0.1 to 100 mM, and (**D**) in the presence of heparin at concentrations of 0.01 to 100 μg/ml. Nonspecific binding to equimolar BSA has been subtracted and the data are expressed as the mean ± 1 standard deviation of three determinations.

from fibronectin was not enhanced in the presence of 100-fold excess unlabeled TGF-β1. TGF-β1 binding to fibronectin was saturable and unlabeled TGF-β1 could compete with [^{125}I]-TGF-β1 for binding. To determine the nature of the interactions between fibronectin and TGF-β1 the effects of ionic strength, divalent cation concentration, and pH on [^{125}I]-TGF-β1 binding were examined. The relative insensitivity of binding to changes in ionic strength and divalent cation concentration suggest that the binding of TGF-β1 to fibronectin may not require the participation of charged amino acids. This

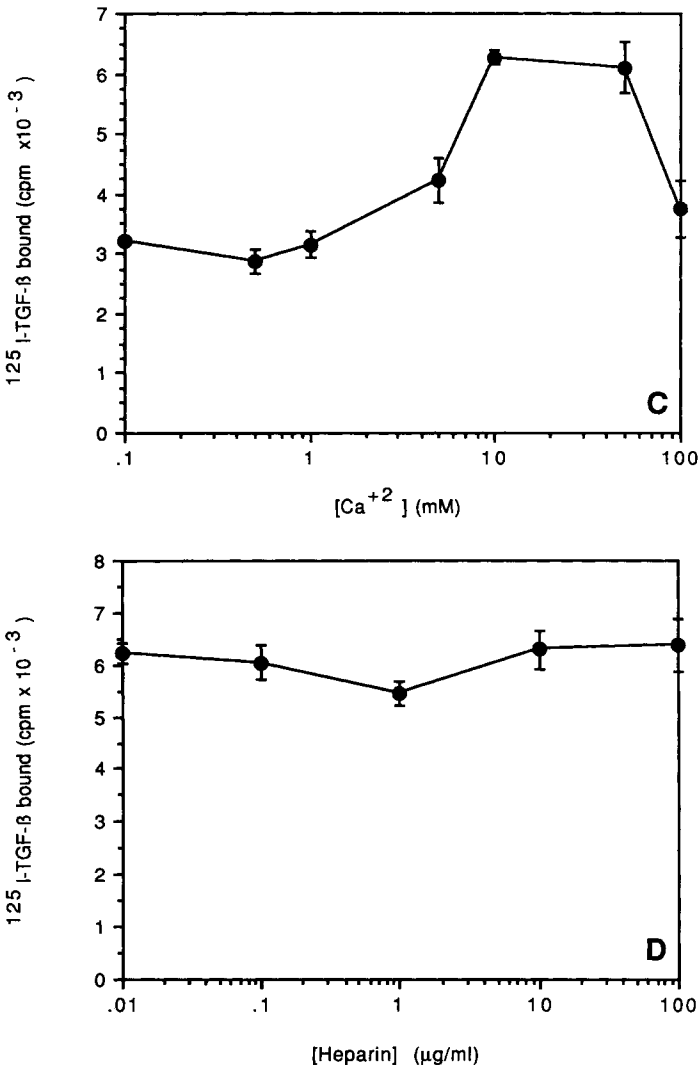

Figure 3 continued

view is supported by the observation that heparin, an anionic glycosaminoglycan which binds to fibronectin [29], does not interfere with TGF-β1/fibronectin interactions. This datum also provides indirect evidence that TGF-β1 does not interact with fibronectin via known heparin-binding domains.

TGF-β is a hydrophobic molecule that binds readily to plastic and glass surfaces [30] and the binding of TGF-β1 to fibronectin may involve hydrophobic interactions. However, the pH dependence of TGF-β1 binding to fibronectin suggests that charged amino acids may play a role in the binding of TGF-β1 to fibronectin, perhaps by influencing the conformation of TGF-β1 and/or fibronectin. Further studies will be necessary to determine the critical events in TGF-β1/fibronectin interactions.

In addition, several TGF-β-binding proteins have been described, including α_2-

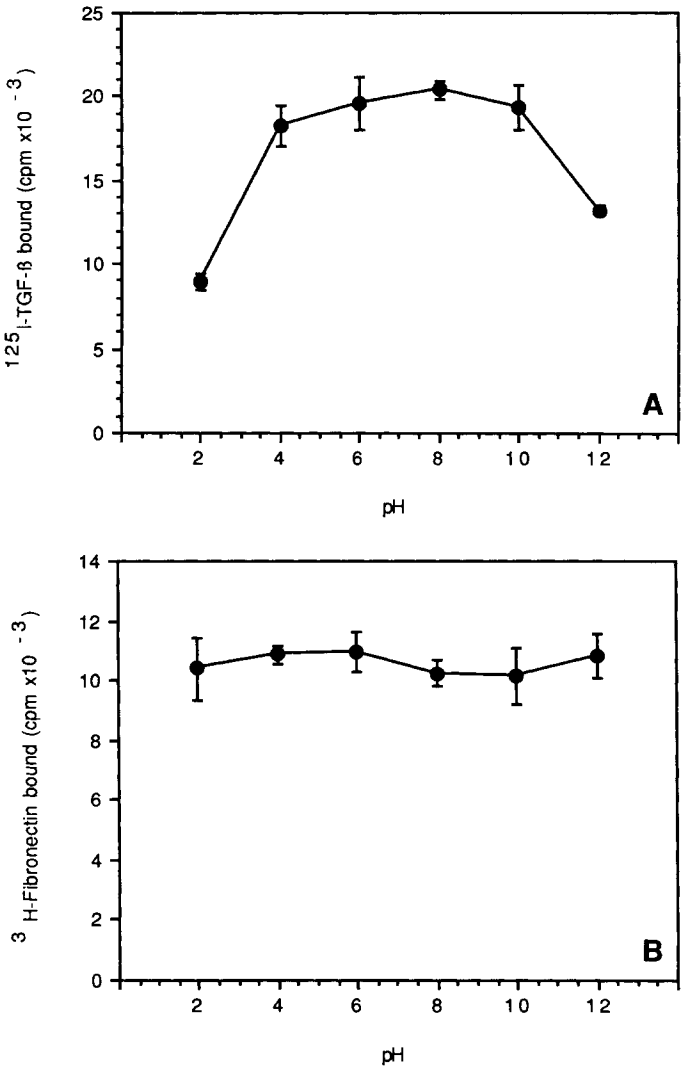

Fig. 4. **A:** pH-dependent dissociation of [^{125}I]-TGF-β1 from immobilized fibronectin. [^{125}I]-TGF-β1 (16×10^3 cpm) was incubated with 0.2 pmol/well fibronectin for 2 h and treated with PBS at pH 2, 4, 6, 8, 10, or 12, and specific binding was determined 2 h later as described in Materials and Methods. Data are expressed as the mean ± 1 standard deviation of three determinations. **B:** Effect of pH on [^{3}H]-fibronectin binding to Immulon I™ plastic. Immulon I™ plates were coated with [^{3}H]-FN (48×10^3 cpm, 0.2 pmol/well) and PBS at pH 2, 4, 6, 8, 10, or 12 was added for 2 h. Bound counts were recovered as described in Materials and Methods. Nonspecific binding to BSA has been subtracted and the data are expressed as the mean ± 1 standard deviation of three determinations.

macroglobulin [18–21]. In our experiments, TGF-β1 also bound to BSA, though its binding to BSA was five- to tenfold lower than its binding to equimolar concentrations of fibronectin. We have also demonstrated that TGF-β1 binds to immobilized laminin and does so to a greater extent than fibronectin (data not shown). It is likely that TGF-β1 may interact with a variety of extracellular matrix proteins, and it may be sequestered by extracellular matrices as a result.

Acidification (pH 2.0 for 1 h) has been shown to activate latent TGF-β, a process which involves cleavage of the TGF-β precursor and its dissociation from a "masking" protein [18–21]. One such "masking" protein has been described by Nakamura et al. [22] and is a protein with a molecular weight of approximately 440 kDa. Our results suggest that this protein may be fibronectin (440 kDa) [23].

In this report we have demonstrated that similar treatment with acid 1) inhibits the binding of [^{125}I]-TGF-β1 to fibronectin and 2) also causes the dissociation of previously bound [^{125}I]-TGF-β1 from fibronectin. Under identical conditions, immobilized [^{3}H]-fibronectin is not released from the plastic surface. These data confirm that acidification results in the dissociation of [^{125}I]-TGF-β1 from immobilized fibronectin and not merely the removal of immobilized TGF-β1/fibronectin complexes from the plastic surface.

The physiological significance of acidification in the distribution of fibronectin-bound TGF-β is unclear. A drop in pH in vivo to 2.0—under any circumstances—is highly unlikely. However, it has been reported that milder acidification (pH 4.0–5.0 for 1 h) can activate 20–30% of latent TGF-β [31]. A pH in this range—while still unlikely in vivo—is possible [31] and if sustained may lead to a similar dissociation of TGF-β from fibronectin. Our data suggest a slight reduction in the binding of TGF-β1 to fibronectin at pH 4.0 (see Fig. 3A). We do not know whether longer incubations at milder pH would result in greater dissociation of TGF-β1 from fibronectin in solid-phase binding assays. In any case, the activation of TGF-β by limited proteolysis is likely to be of greater physiologic importance than acidification [31] and experiments to investigate this possibility are now underway.

Finally, TGF-β is known to induce fibrosis and angiogenesis and to stimulate wound healing in vivo [32–34]. TGF-β1 localized at sites of vascular injury could play an important role in the normal and/or pathological growth of cells following vascular injury [35–37]. The principal source of TGF-β1 in vivo is the platelet [38], and the release of TGF-β1 by platelets which accumulate at the sight of tissue injury and the subsequent association of TGF-β1 with exposed extracellular matrix fibronectin may play an important role in normal and abnormal wound healing. In addition, inflammation and the resulting changes in tissue pH and release of proteolytic enzymes may lead to the dissociation of TGF-β1 from extracellular matrix fibronectin or other extracellular matrix components. These processes may therefore alter the distribution and/or biological activity of this potent growth regulator at sites of tissue injury and repair.

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