Thrombospondin 1 does not activate transforming growth factor β 1 in a chemically defined system or in smooth-muscle-cell cultures

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The cytokine transforming growth factor β 1 (TGF- β 1) is secreted in a latent form that has no known biological activity. The conversion of latent TGF- β 1 into its biologically active 25 kDa form is thought to be an important step in the regulation of TGF- β activity both in cell culture and *in vivo*. Thrombospondin (TSP)-1, a 360 kDa platelet α -granule and extracellular matrix protein, has been shown to participate in TGF- β 1 activation. We have used a chemically defined system to examine the mechanism of TSP-1-mediated TGF- β 1 activation. However, the addition of two different preparations of TSP-1 to recombinant small latent TGF- β 1 in the test tube resulted in only a very small increase in the proportion of the TGF- β 1 able to bind to the TGF- β type II receptor: from 0.1 % to a maximum of 0.4 %. This small effect was not specific for TSP-1: matrix metalloproteinase 2, tissue

inhibitor of matrix metalloproteinase 2 and active plasminogen activator inhibitor 1, but not transglutaminase, human serum albumin or immunoglobulin, had quantitatively similar effects on latent TGF- β 1. Furthermore, no change in the activity associated with small latent TGF- β 1 was noted in either mink lung epithelial cell or rat aortic smooth-muscle cell culture systems in the presence of TSP-1 (or TSP-1-derived peptides). We conclude that TSP-1, either alone or in the presence of cultured smooth-muscle cells (a cell type known to activate latent TGF- β *in vitro* and *in vivo*) is unable to activate latent TGF- β 1. Any TSP-mediated activation of TGF- β 1 must depend on additional factor(s) not present in our systems.

Key words: cytokine, fibroblast, platelet, wound healing.

INTRODUCTION

Transforming growth factor β 1 (TGF- β 1) has been reported to have a wide range of effects, both in cell culture systems and *in vivo* [1–3]. For example, TGF- β 1 inhibits the growth of most non-transformed mammalian cells in culture, although in certain circumstances it can also promote cell growth [3]. TGF- β 1 also modulates cell migration, extracellular matrix production and gene expression pattern *in vitro* [1]. Many of these effects of TGF- β 1 have also been reported *in vivo*, where TGF- β 1 is thought to have a dominant role in regulating the immune system [4,5]. As a result, aberrant TGF- β activity has been implicated in a range of disease processes, many of which have an important inflammatory component, including fibrosis [6], atherosclerosis [7], cancer [3] and autoimmune disorders [8].

There are three mammalian isoforms of TGF- β (β 1, β 2 and β 3) as well as a large superfamily of related cytokines. The best studied member of the superfamily is TGF- β 1, the product of the *tgfb1* gene. Translation of the mRNA product of the *tgfb1* gene yields a 390-residue polypeptide that rapidly dimerizes. After cleavage of the signal sequence, the dimer undergoes glycosylation and further proteolytic processing, resulting in cleavage between the pro-region and the active cytokine at the C-terminus [9] (Scheme 1). The pro-region dimer [termed the latency-associated peptide (LAP)] remains non-covalently associated with the mature TGF- β 1 dimer to form a small latent complex of approx. 110 kDa, which has no known biological activity. Most cell types *in vitro* secrete TGF- β 1 either in this small latent complex form or after the addition of other proteins such as

members of the latent TGF- β binding protein ('LTBP') family to form large latent complexes [10].

To exert its wide range of biological effects, the latent TGF- β 1 complexes must be activated to allow them to interact with the high-affinity TGF- β 1 receptors [11]. Because TGF- β receptors are present on most cell types *in vitro* and *in vivo*, and many cell types produce the latent TGF- β 1 complexes, it seems likely that this activation step is a major control point in the regulation of TGF- β 1 activity [11]. However, the mechanism of TGF- β activation is a subject of continued research. It is generally accepted to be a two-step process, the first stage of which is the proteolytic cleavage between LAP and the mature homodimer, which occurs before secretion of the latent complex. Recent evidence suggests that furin, a well-characterized Ca^{2+} -dependent serine protease of the mammalian subtilisin-like proprotein convertase family, might be involved in cleaving the TGF- β 1 precursor at a dibasic site [RHRR (single-letter amino acid codes)] at the N-terminus of the mature TGF- β 1 peptide [9].

It is generally assumed that the second step depends on the dissociation of the LAP dimer from the mature TGF- β 1 homodimer [9]. One candidate mediator of this dissociation step is the serine protease plasmin [12]. The proteolytic activity of plasmin, possibly cleaving the LAP, is thought to be important because inhibitors of plasmin activity [such as apolipoprotein(a) and active plasminogen activator inhibitor (PAI-1)] can decrease TGF- β activity *in vitro* [13]. Consistent with this model was our finding that transgenic overexpression of apolipoprotein(a) *in vivo* decreased TGF- β 1 activity in the blood vessel wall [14]. However, the observation that plasminogen-deficient (PLG^{-/-})

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LAP, latency-associated peptide; PAI-1, active plasminogen activator inhibitor 1; raSMC, rat aortic smooth-muscle cell; SMC, smooth-muscle cell; TCA, trichloroacetic acid; TGF- β 1, transforming growth factor β 1; TSP, thrombospondin.

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Scheme 1 Two-step model of TGF-β1 activation

Translation of the mRNA product of the *tgbf1* gene yields the TGF-β1 monomer (1) composed of the N-terminal signal sequence (zig-zag line), LAP propeptide (thin line) and the mature TGF-β1 sequence at the C-terminus (thick line). This rapidly undergoes dimerization and loss of the signal sequence (2). This dimer then undergoes the first step of the activation process, proteolytic cleavage between the LAP and mature TGF-β1 sequences, to yield the small latent TGF-β1 complex (3). The small latent complex is then exported from the cell (4a) and can become associated with additional binding proteins either before or after secretion (4b). The second step of the activation process is then required to release the mature 25 kDa TGF-β1 dimer (5) from its non-covalent association with the LAP dimer. The disulphide bond linking the two LAP monomers and the intrachain disulphide bonds have been omitted for clarity.

mice (which are also presumably plasmin-deficient) do not exhibit phenotypic characteristics in common with TGF-β1-deficient mice [15] suggests that plasmin is not the only, or possibly even a major, mediator of TGF-β activation in normal physiological circumstances.

Another candidate mediator of TGF-β activation is thrombospondin (TSP), a trimeric 360 kDa protein found at high concentrations in platelet α-granules and in extracellular matrix produced by a variety of cell types. Murphy-Ullrich and colleagues have suggested that TSP is capable of activating both small and large latent TGF-β1 complexes, using the ability of NRK-49 cells to form colonies in soft agar as an assay of TGF-β activity [16–21]. They propose this non-proteolytic activation to be dependent on the interaction between specific sequences in both TSP (K⁴¹²RFK⁴¹⁵) and LAP (L⁵⁴SKL⁵⁷), because the LSKL tetrapeptide can competitively inhibit TSP-mediated TGF-β activation and the KRFK tetrapeptide is as efficient as TSP-1 in mediating TGF-β1 activation [16,17]. More recently it has been shown that TSP-1-deficient mice display phenotypic traits in common with TGF-β1-deficient mice [21]. For example, TSP-1-deficient mice display cellular hyperplasia in a number of organ systems (bronchial smooth muscle, testes and stomach mucosa) to a similar extent to that described for TGF-β1-deficient mice.

In addition, inflammation is also seen in pancreas, lung and liver from both mouse lines, although it is generally weaker in TSP-1-deficient mice than TGF-β1-deficient mice. Interestingly, however, rampant inflammation in the heart is a generally accepted consequence of TGF-β1 deficiency, but no inflammation of the heart was seen in TSP-1-deficient mice [21]. Nevertheless, both the inflammation and the cellular hyperplasia were largely abolished by treatment of the TSP-1-deficient mice with KRFK peptide, strongly suggesting that they resulted from decreased TGF-β1 activation in the absence of TSP-1 [21].

Several questions about the mechanism of TSP-mediated TGF-β1 activation remain unanswered. In particular, much of the characterization of TSP-1-mediated TGF-β activation by Murphy-Ullrich and colleagues depended on adding both the latent TGF-β1 and the TSP-1 to a particular cell line [17–20]. It is possible that other factors provided by this cell culture system were required for TSP-1-mediated TGF-β1 activation. To assess the extent to which this model can be used as a general paradigm for TGF-β1 activation, we have quantitatively assessed the capacity of TSP to activate latent TGF-β1 both in a chemically defined system and in cultures of smooth-muscle cells (SMCs), a cell type known to produce active TGF-β both *in vivo* and *in vitro* [14,22].

MATERIALS AND METHODS

Materials

Small latent TGF-β1 and active TGF-β1 (both carrier-free) were obtained from R&D Systems. The purity and composition of this material were checked by gel electrophoresis and N-terminal protein sequencing. Partly purified platelet large latent TGF-β1 was obtained as described previously [23]. The concentration of latent TGF-β1 in each sample was determined by using the Quantikine TGF-β1 ELISA kit (R&D Systems) after complete activation of the sample with acid and urea in accordance with the manufacturer's instructions. TSP-1 (purified from human platelets) was either obtained from Biogenesis or freshly prepared by the method of Clezardin et al. [24]. In brief, washed platelets prepared from 100 ml of citrated human blood were stimulated by the addition of α-thrombin (1 unit/ml) at 22 °C for 2 min, after which hirudin (2 units/ml) was added. The platelet aggregates were allowed to settle out on ice and the supernatant was retained and centrifuged at 48000 *g* for 30 min. The supernatant was diluted 1:3 with 20 mM triethanolamine buffer, pH 7.4, and a volume containing approx. 1 mg of total protein was subjected to ion-exchange chromatography with a MonoQ column (5 ml bed volume) on an FPLC system (Amersham Pharmacia Biotech). Bound proteins were eluted with increasing concentrations of NaCl; the fractions containing TSP-1 (identified by Western blotting) were pooled and concentrated to 1 mg/ml. The purity and composition of the TSP-1 used in this study were checked by gel electrophoresis (more than 90 % purity) and immunoblotting. Peptides were synthesized with standard solid-phase chemistry by Affiniti Research Products and purified to more than 95 % purity.

Detection of active TGF-β *in vitro*

Incubations of TSP-1 and latent TGF-β1 were performed in Dulbecco's PBS solution [137 mM NaCl/2.7 mM KCl/8.1 mM Na₂HPO₄/0.9 mM CaCl₂/0.5 mM MgCl₂/1.5 mM KH₂PO₄ (pH 7.2)] unless otherwise indicated. The proportion of TGF-β1 in a sample capable of binding to the type II TGF-β receptor was then determined by incubating the sample with the extracellular

Figure 1 Effect of TSP-1 on small latent TGF- β 1 in a chemically defined system

(a) TSP-1 was incubated with latent TGF- β 1 under various conditions; the proportion of TGF- β 1 capable of binding to the extracellular domain of the type II receptor was then determined. (b) Small latent TGF- β 1 complex (1600 pM), active TGF- β 1 (400 pM) and small latent TGF- β 1 after treatment with 1 M HCl for 10 min, then neutralized (1600 pM), were assayed as shown in (a). Note that the complete activation of 1600 pmol of small latent TGF- β 1 (approx. 100 kDa) yields approx. 400 pmol of active TGF- β 1 (25 kDa). (c) Small latent TGF- β 1 (1600 pM) was preincubated for various durations (from 0 min to 24 h) with TSP-1 (31 nM) at either 4 °C or 37 °C before assay as in (a). (d) Small latent TGF- β 1 (1600 pM) was preincubated with various concentrations of TSP-1 for 2 h at 37 °C before assay as in (a). Grey bars indicate TSP-1 purchased from Biogenesis; stippled bars indicate TSP-1 purified by the method of Clezardin et al. [24]. (e) Small latent TGF- β 1 (1600 pM) was preincubated with (stippled bars) or without (grey bars) 31 nM TSP-1 for 2 h at 37 °C in PBS containing a range of Ca^{2+} ion concentrations before assay as in (a). ' + EGTA ' indicates that 2 mM EGTA was added to the buffer to decrease the free Ca^{2+} ion concentration as far as possible. 'None' indicates that no Ca^{2+} ions were added as CaCl_2 . (f) Small latent TGF- β 1 (1600 pM) was preincubated with various concentrations of highly purified human serum albumin (HSA) for 2 h at 37 °C before assay as in (a). (g) Small latent TGF- β 1 (1600 pM) was preincubated with various proteins (31 nM) for 2 h at 37 °C before assay as in (a). Abbreviations: TSP, TSP-1; MMP, matrix metalloproteinase 2; TIMP, tissue inhibitor of matrix metalloproteinase 2; PAI, PAI-1; TG, transglutaminase; apo(a), apolipoprotein(a); HSA, human serum albumin; IgG, serum gamma globulin. All proteins were of human origin. All assays were performed in triplicate. * $P < 0.05$ compared with small latent TGF- β 1 complex alone (Student's unpaired t test). In each experiment, activation by acid (with 1 M HCl) was used as a positive control.

Figure 2 Effect of TSP-1 on large latent TGF- β 1 in a chemically defined system

Partly purified large latent TGF- β 1 complex from human platelets (5000 pM) was preincubated with various concentrations of TSP-1, or with human serum albumin (HSA; 31 nM) or PAI-1 (PAI; 31 nM) for 2 h at 37 °C before assay as shown in Figure 1(a). Note that the complete activation of 5000 pmol of small latent TGF- β 1 (approx. 300 kDa) yields approx. 400 pmol of active TGF- β 1 (25 kDa). All assays were performed in triplicate. * P < 0.05 compared with large latent TGF- β 1 complex alone (Student's unpaired t test). Activation by acid (with 1 M acetic acid/5 M urea) followed by neutralization was used as a positive control.

domain of the type II TGF- β receptor coated on an ELISA plate (Quantikine kit; R&D Systems) for 2 h at room temperature with shaking. Unbound components were then washed off with three rapid washes of wash buffer (Quantikine kit). Bound TGF- β 1 was detected with affinity-purified chicken anti-(TGF- β 1) conjugated with horseradish peroxidase (Quantikine kit) in accordance with the manufacturer's instructions.

MvLu1 cell bioassay

The MvLu1 cell bioassay for active TGF- β was performed as described previously [25]. In brief, MvLu1 cells (ATCC; passage 64–70) were subcultured into 24-well plates at 10000 cells per well and grown for 24 h in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). Samples were then added in fresh DMEM/10% (v/v) FCS to triplicate wells and incubated for 23 h at 37 °C. Thereafter, 1 μ Ci/ml [3 H]thymidine (Amersham Pharmacia Biotech) was added to each well and incubated for a further 1 h at 37 °C. At the end of this incubation, cells were washed three times with ice-cold PBS and lysed with 1% (w/v) SDS; the macromolecular fraction was precipitated by the addition of an equal volume of 15% (w/v) trichloroacetic acid (TCA). The TCA cold-precipitable fraction was then filtered off over GF/C filters, which were counted for incorporated 3 H.

SMC culture and proliferation assay

Rat aortic SMCs (raSMCs) were isolated by using the enzyme dispersal technique as described previously [26]. Cells were maintained in DMEM/10% (v/v) FCS and subcultured every 4 days at 1:3 dilutions. Cells were used between passages 6 and 12. The proliferation assay was performed exactly as described previously [27]. In brief, confluent cultures of raSMCs were subcultured at 1:3 dilution and plated into 24-well plates in DMEM/10% (v/v) FCS. After 24 h, samples were added to triplicate wells in fresh DMEM/10% (v/v) FCS; the cell number at this point was determined by releasing cells from a further triplicate set of wells with trypsin/EDTA solution and counting the released cells with a haemocytometer. The number of cells in each triplicate set of wells was then determined after incubation with the samples at 37 °C for 72 h. At this time, cells in the

absence of growth inhibitors were approx. 90% confluent. Cell numbers were determined by releasing the cells with trypsin/EDTA and counting with a haemocytometer as described previously [27]. The rate of proliferation was determined by subtracting the number of cells present when the samples were added from the number of cells present at the end of the experiment.

To measure the effects of TGF- β 1 on protein synthesis, raSMCs were subcultured into 24-well plates exactly as described above for the proliferation assay. After 24 h, samples were added to triplicate wells in fresh DMEM/10% (v/v) FCS and cultured for a further 48 h. [3 H]Proline (Amersham Pharmacia Biotech) (0.8 μ Ci per well) was added and the cells were incubated for a further 24 h. The incorporation of proline into the macromolecular pool was assayed by precipitation with TCA and scintillation counting exactly as for thymidine incorporation described above. Proline incorporation was then corrected for variations in the number of cells from one triplicate to another (determined by counting replicate wells) and expressed as c.p.m./ 10^4 cells.

RESULTS

We first determined whether TSP-1 could activate latent TGF- β 1 in a chemically defined system containing only latent TGF- β 1 and TSP-1 in Dulbecco's PBS solution. Small latent TGF- β 1 was incubated with various concentrations of TSP-1 at either 4 or 37 °C for various durations. The mixture was then incubated with the extracellular domain of type II TGF- β receptor (which can bind only active TGF- β 1 [28]). Any active TGF- β 1 that was captured was then detected with a peroxidase-labelled anti-(TGF- β 1) antibody (Figure 1a). In the absence of TSP-1, approx. 0.1% of the TGF- β 1 present in the preparation of small latent TGF- β 1 was able to bind to the receptor (Figure 1b). We cannot determine whether this is because 0.1% of the TGF- β 1 was already in the active form or whether a small degree of activation occurs on incubation with the receptor. In marked contrast, approx. 100% of the TGF- β 1 in a preparation of active TGF- β 1 was detected by this method (Figure 1b).

Incubation with TSP-1 (11 μ g/ml; 31 nM) at either temperature resulted in a very small dose-dependent increase in the proportion of TGF- β 1 able to bind to the receptor. The increase in TGF- β 1 able to bind to the receptor was similar at both 4 and 37 °C and did not require preincubation of the TSP-1 with the latent TGF- β 1. No further increase (beyond 0.4%) in the fraction of added TGF- β 1 that was detectable was seen even after 24 h of preincubation at 37 °C (Figure 1c). All subsequent experiments were performed with a 2 h preincubation at 37 °C. Under these conditions we observed a dose-dependent increase in the proportion of the TGF- β 1 able to bind the receptor by using two different preparations of TSP-1 (to a maximum of 0.4% activation at concentrations of TSP-1 above 3 nM) (Figure 1d). Because Ca^{2+} ion concentration can affect TSP-1 structure [29], we next examined the effect of Ca^{2+} concentration on TSP-1-mediated TGF- β activation. TSP-1 in the presence of Ca^{2+} ion concentrations from 0 mM (in the presence of 2 mM EGTA) to 10 mM could not increase the proportion of latent TGF- β 1 converted into the active form to more than 0.4% (Figure 1e). However, in the absence of TSP-1, 10 mM CaCl_2 alone increased the proportion of latent TGF- β 1 able to bind to the receptor by approx. 4-fold. Thus 10 mM CaCl_2 and 31 nM TSP-1 were similarly effective as activators of latent TGF- β 1. In summary, TSP-1 increased the proportion of TGF- β 1 capable of binding to the receptor by approx. 4-fold, although the total amount capable

Figure 3 Effect of TSP-1 on latent TGF- β 1 in cultures of mink lung epithelial cells

(a) Various concentrations of TGF- β 1 [active TGF- β 1 (filled squares), small latent TGF- β 1 complex (grey circles) or small latent TGF- β 1 complex after treatment with 1 M HCl and neutralization (open circles)] were added to mink lung epithelial cells in DMEM/10% (v/v) FCS. DNA synthesis was assayed during a 1 h period 23 h after addition of TGF- β (see the Materials and methods section). The concentration of TGF- β 1 added is expressed in pM based on the number of moles of mature TGF- β 1 dimer present (i.e. the actual concentration of small latent TGF- β 1 complex added was 4-fold that shown because 4 g of small latent complex contains 1 g of mature TGF- β 1 dimer). (b) Various concentrations of small latent TGF- β 1 (expressed as the number of moles of mature TGF- β 1 dimer present) were added to MvLu1 cells as in (a), in the presence (filled triangles) or absence (grey circles) of TSP-1 (31 nM). (c) Small latent TGF- β 1 complex (1600 pM, equivalent to 400 pM mature TGF- β 1 dimer) was incubated with MvLu1 cells as in (a), in the presence of TSP-1 (31 nM) and the peptides KRFK, KFK and KWK (each at 1 mM). (d) Partly purified platelet large latent TGF- β 1 complex (5000 pM, equivalent to 400 pM mature TGF- β 1 dimer) was incubated with MvLu1 cells as in (a), in the presence of TSP-1 (31 nM) and the peptides KRFK, KFK and KWK (each at 1 mM). As a positive control, small latent TGF- β 1 complex was activated with 1 M HCl and large latent TGF- β 1 complex with 1 M acetic acid plus 5 M urea.

of binding the receptor was still very small (and could not be increased further by altering the concentration of TSP-1 or Ca^{2+} ions, or the time and temperature of the preincubation).

Because supraphysiological Ca^{2+} ion concentration was as effective as TSP-1, we next tested whether the apparent TSP-1-mediated activation of latent TGF- β 1 was specific for TSP-1 over other proteins. Replacing TSP-1 with highly purified serum albumin abrogated the effect: serum albumin did not increase the proportion of TGF- β 1 capable of binding to the receptor at any concentration up to 31 nM (Figure 1f). In contrast, a variety of other proteins had a similar effect to TSP-1 on the proportion of TGF- β 1 capable of binding to the receptor, including PAI-1 and apolipoprotein(a) (two proteins previously implicated as inhibitors of TGF- β activation) (Figure 1g) [12,13]. We conclude that the small degree of TGF- β 1 activation seen in the presence of 31 nM TSP-1 is not a specific effect, although not all proteins are capable of activating TGF- β 1 to this small extent.

It is possible that other TGF- β 1 protein complexes (such as platelet large latent complexes) are more susceptible to activation by TSP-1 than the small latent TGF- β 1 complex tested above. To examine this possibility we repeated the experiments with partly purified large latent TGF- β 1 from human platelets [23]. In

these experiments approx. 0.2% of the TGF- β 1 in the untreated preparation was able to bind to the TGF- β receptor; TSP-1 (31 nM) increased this to 0.6% (Figure 2). As for the small latent TGF- β 1 complex, PAI-1 increased the proportion of the TGF- β 1 capable of binding to the receptor to a similar extent to TSP-1, but serum albumin had no effect (Figure 2). We conclude that platelet large latent TGF- β 1 complex is no more susceptible to TSP-1-mediated activation *in vitro* than small latent TGF- β 1.

It has previously been suggested that TSP-1-mediated TGF- β 1 activation does not involve proteolytic processing but stimulates the release of the active TGF- β 1 dimer from its non-covalent interaction with the LAP [17,18]. We therefore examined the possibility that the small latent TGF- β 1 that we used had not been appropriately processed to permit TSP-1-mediated activation (Scheme 1). The small latent TGF- β 1 was subjected to N-terminal sequence analysis. We identified an approximately equimolar ratio of polypeptides with N-terminus ALDTNYXFSS (corresponding to the N-terminus of active TGF- β 1) and the N-terminus GRPAAGLSTXK (corresponding to the N-terminus of LAP). In addition, there was a small but significant number of sequences with an N-terminus corresponding to cleavage within the active TGF- β 1 molecule (approx.

Figure 4 Effect of TSP-1 on latent TGF-β1 in cultures of raSMCs

(a) Various concentrations of TGF-β1 [active TGF-β1 (filled squares), small latent TGF-β1 complex in the absence of TSP-1 (grey circles) or small latent TGF-β1 complex in the presence of TSP-1 (31 nM) (filled triangles)] was added to raSMCs in DMEM/10% (v/v) FCS. Cell proliferation over a 72 h period after the addition of TGF-β was measured (see the Materials and methods section). The concentration of TGF-β1 added is expressed in pM on the basis of the number of moles of mature TGF-β1 dimer present. (b) Partly purified platelet large latent TGF-β1 complex (5000 pM, equivalent to 400 pM mature TGF-β1 dimer) was incubated with raSMCs as in (a) in the presence or absence of TSP-1 (31 nM); the effect on cell proliferation was determined. The small growth stimulatory effect of the platelet preparation was abolished by co-incubation with a neutralizing antibody against platelet-derived growth factor (αPDGF; 30 μg/ml). (c) Various concentrations of TGF-β1 [active TGF-β1 (filled squares), small latent TGF-β1 complex in the absence of TSP-1 (grey circles) or small latent TGF-β1 complex in the presence of TSP-1 (31 nM) (filled triangles)] was added to raSMCs in DMEM/10% (v/v) FCS. Protein synthesis over a 24 h period at 48 h after the addition of TGF-β was measured by the incorporation of labelled proline (see the Materials and methods section). The concentration of TGF-β1 added is expressed in pM on the basis of the number of moles of mature TGF-β1 dimer present. (d) Partly purified platelet large latent TGF-β1 complex (5000 pM, equivalent to 400 pM mature TGF-β1 dimer) was incubated with raSMCs as in (c) in the presence or absence of TSP-1 (31 nM); the effect on protein synthesis was determined.

10% of the polypeptides showed evidence of additional proteolytic cleavage). No other N-terminal sequences were detected. These studies confirm that all, or almost all, of the small latent TGF-β1 molecules in our sample had been appropriately processed. Consistent with these findings was our observation that more than 40% of the small latent TGF-β1 could bind to the TGF-β receptor after treatment with 1 M HCl for 10 min (Figure 1b).

Because TSP-1 is unable to mediate the activation of more than a small percentage (less than 1%) of latent TGF-β1 in a cell-free system, it is possible that other components of the NRK-49 cell culture assay used by Shultz-Cherry et al. [17–20] were required for the TSP-1-mediated TGF-β1 activation that they described. We therefore examined TGF-β1 activation in two cell culture assays that allowed the fraction of TGF-β1 activated to be quantified. Small latent TGF-β1 was incubated with mink lung epithelial cells (MvLu1) in the presence or absence of TSP-1 (31 nM) for 23 h; DNA synthesis was then measured by thymidine incorporation into DNA during the following 1 h period. Active TGF-β1 (Figure 3a, filled squares), but not latent TGF-β1 (grey circles), inhibits DNA synthesis by MvLu1 cells in a dose-dependent fashion. At very high concentrations of latent

TGF-β1 some inhibition of DNA synthesis is observed, although we cannot determine whether this is due to the presence of a small (less than 1%) amount of active TGF-β1 in our preparation of small latent TGF-β1 or whether small latent TGF-β1 is able to inhibit DNA synthesis when present at very high concentrations (Figure 3a). Treatment of small latent TGF-β1 with 1 M HCl for 10 min, followed by neutralization, resulted in efficient conversion into active TGF-β1, with more than 90% of the TGF-β1 in the preparation of small latent TGF-β1 detectable as active TGF-β1 after treatment with acid (Figure 3a, open circles). In marked contrast, the addition of TSP-1 (31 nM) did not affect the activity associated with the small latent TGF-β1 (Figure 3b, filled triangles). Furthermore, the peptides KRFK and KFK, previously reported to activate TGF-β1 to the same extent as TSP-1 [17], did not affect the activity associated with the small latent TGF-β1 even at 1 mM (Figure 3c). We conclude that TSP-1 cannot activate small latent TGF-β1 to any significant extent even in the presence of epithelial cells. The same experiment was also performed with partly purified platelet large latent TGF-β1 complex; it demonstrated that neither TSP-1 nor the KFK-containing peptides activated this complex to any significant extent (Figure 3d).

To address the question of whether mink lung epithelial cells are specifically lacking pathways necessary for TGF- β 1 activation (in the presence or absence of TSP-1), we repeated the assays using raSMCs. SMCs were selected because this cell type is known to be able to produce active TGF- β 1 both *in vitro* and *in vivo* [14,22]. The proliferation of raSMCs in culture is inhibited by active, but not small latent, TGF- β 1 in a dose-dependent fashion (Figure 4a), which is consistent with several previous reports [13,22,27]. At very high concentrations, some inhibition of proliferation is seen with small latent TGF- β 1, which might have been due to the presence of a small fraction (less than 2%) of active TGF- β 1 in the preparation of small latent TGF- β 1. The addition of TSP-1 (31 nM) did not affect the activity associated with the small latent TGF- β 1 (Figure 4a, filled triangles). In a second series of experiments, the addition of partly purified platelet large latent TGF- β 1 did not inhibit (and actually slightly increased) the rate of proliferation of raSMCs in the presence or absence of TSP-1 (31 nM) (Figure 4b). The small increase in proliferation rate was inhibited by neutralizing antibodies against platelet-derived growth factor (Figure 4b).

As an alternative assay of TGF- β 1 activity in the raSMC cultures, we measured the rate of protein synthesis with the labelled amino acid [3 H]proline. Because TGF- β 1 stimulates the production of various collagens (which have a high proline content), this provides a sensitive assay for TGF- β 1 activity. Active TGF- β 1 (Figure 4c, filled squares) induced a 3-fold increase in proline incorporation per cell at a concentration of 400 pM, but small latent TGF- β 1 complex (grey circles) affected proline incorporation only at concentrations above 4 nM [consistent with the presence of a small fraction (less than 2%) of active TGF- β 1 in the preparation of latent TGF- β 1] (Figure 4c). The addition of TSP-1 (31 nM) (Figure 4c, filled triangles) did not affect the ability of small latent TGF- β 1 to stimulate proline incorporation. In a similar experiment, with partly purified platelet large latent TGF- β 1 complex, TSP-1 (31 nM) again had no effect on the TGF- β 1 activity present in the preparation of latent TGF- β 1 (Figure 4d).

We conclude that, in a chemically defined system, TSP-1 (or TSP-1-derived peptides) are unable to activate to any biologically significant extent either small latent TGF- β 1 or platelet large latent TGF- β 1. Furthermore, in two different cell culture systems, TSP-1 did not significantly activate either small or large latent TGF- β 1.

DISCUSSION

In marked contrast with the conclusions of Murphy-Ullrich and colleagues [16–21,30], we found no evidence for a biologically significant activation of TGF- β 1 by TSP-1 in either a chemically defined system or two different cell culture systems. Incubating two different preparations of TSP-1 (31 nM) with small latent or large latent TGF- β 1 alone resulted in a statistically significant increase in the amount of active TGF- β 1 detectable, but the effect was small and not specific for TSP-1 over a range of other proteins at a similar concentration. TSP-1 had a similar effect to both apolipoprotein(a) and PAI-1, which have previously been reported as inhibitors of TGF- β 1 activation [3,12,13]. Furthermore, no change in the activity associated with either the small or large latent TGF- β 1 complexes was noted in MvLul and raSMC cultures in the presence of 31 nM TSP-1.

Although we cannot exclude the possibility that the activation of 0.3% of the small latent TGF- β 1 complex by TSP is biologically significant, the lack of selectivity of this effect strongly suggests that we are not observing the effects of TSP-1 responsible for the phenotypic traits reported in TSP-1-deficient mice [21],

including increased inflammation and cellular hyperplasia. Thus cofactors other than TSP-1 or TGF- β 1 are required for the efficient activation of latent TGF- β 1. However, our results strongly suggest that these cofactors are not present (or are present at too low a concentration) in cultures of either mink lung epithelial cells or raSMCs.

Despite the apparently conflicting conclusions, our observations are consistent with previous reports from the laboratory of Murphy-Ullrich [16–21]. For example, Shultz-Cherry et al. [17] reported the stimulation of TGF- β activity over the same TSP-1 concentration range as used in our study [compare Scheme 1 in [17] with Figure 1(d) here]. In addition, they examined the effect of TSP-1 on small latent TGF- β 1 in a chemically defined system that was similar to ours and found that TSP-1 increased the proportion of active TGF- β 1 from 0.05% to 0.15% (Table III in [17]). Although the proportion of TGF- β 1 in an active form was slightly lower in their study than in ours, in both cases only a very small fraction (less than 0.4%) of the latent TGF- β 1 was activated even in the presence of TSP-1. We have extended these studies by examining a range of other proteins to demonstrate that the small degree of TGF- β 1 activation mediated by TSP-1 is not a specific effect, although not all proteins are able to activate TGF- β 1 to this small extent.

Because Schultz-Cherry et al. did not assess the proportion of the latent TGF- β 1 that became activated by TSP-1 in the assay with NRK-49 cells [17–20], it is possible that a greater fraction of the latent TGF- β 1 was activated by TSP-1 under these conditions. For instance, the immortal NRK-49 cell line might be atypical, producing essential cofactors that are absent from the systems we examined. If this is so, identification of these factors might provide useful new insights into both the mechanism of TGF- β 1 activation and possibly also the process by which cells become transformed. More plausibly, however, the increased growth of NRK-49 cells in soft agar reported by Schultz-Cherry et al. [18] might have resulted from the 3–4-fold increase in active TGF- β 1 (from 0.1% to 0.4% active) that we observed. Because the studies reported previously did not calibrate the assay system with recombinant small latent TGF- β 1 and purified active TGF- β 1, the proportion of the TGF- β 1 in the active form is not known. However, we can infer that the proportion of TGF- β 1 in the active form is not high (certainly less than 10%), even in the presence of TSP-1, because very high concentrations of latent TGF- β 1 were used (at least 2 nM) [17–20], yet cell growth in soft agar is highly sensitive to TGF- β 1 (in the range 1–10 pM) [31].

Our observations suggest that TSP-1 is very unlikely to be a direct activator of TGF- β 1 either *in vitro* or *in vivo*, although it might modulate TGF- β 1 activity in concert with other cofactors yet to be identified. This suggestion is supported by the observations of Bailly et al. [32], who were unable to detect any direct interaction between latent TGF- β 1 and TSP by surface plasmon resonance. However, it is possible that TSP-1 might impact indirectly on the activation of TGF- β 1 mediated by other pathways: TSP-1 might modulate plasmin activity through pathways that are at present poorly defined [33]. More plausibly, TSP-1 might affect the rate of TGF- β 1 activation by integrins [11,34] because TSP-1 can act as a ligand for certain integrins [35]. Whatever the role of TSP-1 in regulating TGF- β 1 activity, further studies are required for a definitive identification of the pathways responsible for activating most latent TGF- β 1 molecules *in vivo*.

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