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Activation Of Latent Transforming Growth Factor-Beta By The Extracellular Matrix Protein Thrombospondin.

Stacey Lynne Schultz-Cherry
University of Alabama at Birmingham

ACTIVATION OF LATENT TRANSFORMING GROWTH FACTOR-ß BY THE EXTRACELLULAR MATRIX PROTEIN THROMBOSPONDIN

by

STACEY L. SCHULTZ-CHERRY

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Transforming growth factor-ß (TGF-ß) is a multifunctional growth regulatory protein, that is secreted by virtually all cells in vitro in a biologically latent form. Latent TGF-ß is unable to bind to cellular receptors. Receptor expression in most cell types is constitutive; therefore, the primary means of regulating TGF-ß activity involves the conversion of the latent molecule to the biologically active form. The paradigm for activation of latent TGF-ß in a cellular system requires interactions with cell surface components and activation by proteases. We now show that thrombospondin (TSP), an extracellular matrix and platelet α-granule glycoprotein activates latent TGF-ß secreted by bovine aortic endothelial cells, and in a chemically-defined system via a novel mechanism that does not involve proteases or interactions with the cell surface. Activation of latent TGF-ß by TSP is mediated via a two-step binding mechanism. The GGWSXW motif within the type 1 repeats of TSP binds to the active domain of latent TGF-ß. This initial interaction facilitates binding of the RFK sequence (amino acids 413 - 415), unique to TSP1, to the latency associated peptide (LAP) at the amino terminus of the latent TGF-ß complex, resulting in activation of TGF-ß. Activation of latent TGF-ß is unique to TSP1. These studies

are important in that they describe a new mechanism for the activation of latent TGF-ß by the extracellular matrix protein thrombospondin. TSP may be important in the activation of TGF-ß in many pathophysiological situations such as wound healing. TSP agonists and antagonists may have important therapeutic potential.

Abstract Approved by: Committee Chairman Jeanne G. Murphy-Ullrich

Program Director Thomas Linnoila

Date 6/29/95 Dean of Graduate School Joan R. Rodell

DEDICATION

I dedicate my thesis to my husband Kyle, my children, and my parents and family. Without Kyle's support and love I never would have had the self-confidence to make it through. He kept me going on those days when nothing would work, even attempting molecular biology one long Saturday afternoon. Thank you Kyle. My children Sean and Nicholas (both of whom were born while I was in graduate school) helped me keep my priorities straight and know what is important in life.

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INTRODUCTION

Extracellular Matrix

The development and normal functioning of all cell types depends on the interactions of the cell with molecules in their environment. The major classes of molecules that regulate cellular development and biological function include growth and differentiation factors, cell adhesion molecules, and components of the extracellular matrix (ECM). The ECM is composed of numerous components, including glycoproteins, collagens, proteoglycans, and growth factors. Originally, the ECM was only thought to contribute to the mechanical scaffolding and integrity of cells and organs such as skin. Although this is a very important function, we now know that the extracellular matrix is a dynamic environment that provides the cells with much more than physical support. One function of the extracellular matrix is to bind and sequester growth factors. Growth factors bound to the ECM often have altered potency and increased stability and are concentrated in the vicinity of the cell. One of the best described growth factor-matrix interactions involves the binding of fibroblast growth factors (FGF) to heparan sulfate proteoglycans (97,132). Basic FGF may require heparan sulfate proteoglycan in order to bind to its cellular receptor (175). Additionally, the binding of heparin and heparan sulfate proteoglycans to acidic and basic FGF may increase the stability of the receptor-ligand complex.

Heparin protects the FGFs from inactivation by heat, proteases, and extremes of pH (47,131) and may increase their half-lives in vitro (23).

FGF is not the only growth factor sequestered in the ECM; transforming growth factor-ß (TGF-ß) is also associated with extracellular matrix molecules. TGF-ß induces the expression of the proteoglycans decorin and biglycan (115) which then bind and inhibit the activity of TGF-ß (174). TGF-ß also binds to the matrix molecules fibronectin (96) and collagen type IV (117), although the significance of these interactions is unknown.

The interaction of growth factors with the ECM may be important in the control of growth factor activity and ECM function. The study of this interaction may lead to the understanding of many pathophysiologic systems including wound repair, cancer progression, and bone remodeling. The focus of my work is the ECM and serum glycoprotein thrombospondin (TSP) and its regulation of TGF-ß activity.

Thrombospondin Genes

The TSPs are a family of large glycoproteins (420,000 daltons) derived from separate genes (reviewed in 1,13,36,37,70,71,99). Five different isoforms of TSP have been described (1). TSP1 was described first and serves as the prototypical TSP. TSP was first identified as a secretory product from the α-granules of thrombin-stimulated platelets (13,36,37,70,71,99) and is primarily found as a homotrimer. However, in a fibroblast system where TSP1 and TSP2 have been cotransfected, heterotrimers are formed (111). TSP heterotrimers have not, thus far, been identified in non-transfected cells. Hence, heterotrimer formation may be an artifact of transfection.

Most cell types examined in vitro synthesize TSP and incorporate it into their extracellular matrices: these cells include keratinocytes, endothelial cells, smooth muscle cells, glial cells, fibroblasts, chondrocytes, and normal and malignant breast epithelial cells (4,60,90,98,122,124). However, TSP is not a constitutively expressed protein, and its mRNA synthesis is highly regulated. Immunolocalization studies have shown that TSP is primarily localized at sites of active cell migration and proliferation. TSP is also an early, but transient, component of clots (102) and is more widely distributed in the matrices of wounded or developing tissue (69,102,111). TSP is considered an early response gene similar to *myc* and *fos* (74) and is rapidly upregulated in response to serum components. The TSP1 promoter contains a serum response element and a proximal element that serves as a binding site for the CCAAT-box binding factor, NF-Y (37). These sites coordinately mediate the serum response of TSP1. In addition, TSP1 mRNA levels are induced by a number of growth factors, including platelet-derived growth factor (PDGF), TGF-ß, bFGF (116), and the tumor suppressor gene p53 (22). Synthesis can be superinduced by PDGF in the presence of cycloheximide, suggesting that TSP1 mRNA expression is de novo (87).

In contrast to TSP1, TSP2 is not a serum-responsive gene (12,72,141). There is no serum response element or NF-Y binding site within the TSP2 promoter. However, the TSP2 promoter contains two AP-1 sites and a *ras*-responsive factor 1 (RRF-1), suggesting that TSP2 synthesis may be induced by phorbol esters and the ras signalling pathway.

In spite of the differences in the promoter regions of TSP1 and TSP2, the proteins share sequence and structural homology. TSP1 and 2 share ~47% sequence homology at the amino terminus (~32% protein homology) with the homology increasing towards the carboxy terminus. Both TSP1 and TSP2 contain the type 1, 2, and 3 repeats, and share ~80% protein homology at the carboxy terminal. TSP sequence is also very highly conserved throughout different species (xenopus through mammalian).

In contrast to TSP1 and TSP2, TSPs 3-5 are not well described. TSP3 and TSP4, like TSP2, are not serum-responsive genes (163). The protein and sequence homology of TSP3-5 is greatest at the carboxy terminus (~60% as compared to TSP1). The most striking difference is that TSP3-5 do not contain the type 1 or 2 repeats and may form pentamers or oligomers rather than trimers (1,13). This fact will prove to be very important in later discussions.

The diversity of the TSP family also arises from preliminary evidence suggesting that TSP1 mRNA can be alternatively spliced. The alternatively spliced form of TSP1 exists in hamster cells and inhibits angiogenesis (37,45). However, the relevance of the alternative splicing and the function of the protein is unknown at this time.

TSP1 Structure

Similar to other extracellular matrix proteins, TSP has multiple domains that interact with various macromolecules. TSP1 is the best characterized of the TSP family; however, the other isoforms contain some similarities. This introduction will describe the structure of TSP1.

The extreme amino terminus of TSP1 is composed of a small globular heparin binding domain (HBD). This region is involved in TSP binding to cell surface heparan sulfate proteoglycans (103,104), sulfatides (128), and soluble heparin (25,77,78,146). The heparin binding domain also stimulates focal adhesion disassembly in endothelial cells (107), through a cGMP-dependent kinase pathway (Murphy-Ullrich et al., submitted). Immediately following the heparin binding domain, there are two cysteine residues, Cys-252 and Cys-256, that are responsible for the interchain disulfide-bonds of trimeric TSP1 (72,149). Following the interchain bond, there is a stalk region composed of multiple repeat motifs that share homology with other proteins. The first region is ~90 residues long, contains 10 cysteines, and shares homology with the amino terminal propeptide of human type I collagen (164). This region may be responsible for TSP binding to collagen. In addition, a synthetic peptide corresponding to the sequence NGVQYRN inhibits neovascularization in vivo, perhaps by disrupting collagen synthesis (158).

The next segments of TSP are termed the type 1, 2, and 3 repeats. The three type 1 repeats are ~60 residues in length and share homology with the complement component properdin (48) and the malaria circumsporozoite protein (130). The tertiary structure includes ß-turns and a possible ß-pleated sheet (148). There are two conserved motifs within the type 1 repeats. The first, CSVTCG, serves as an attachment factor for melanoma cells and endothelial cells (121,160,161) and has also been shown to be important in TSP's in vivo antiangiogenic activity. The second motif is WSHW. This sequence is a second heparin and sulfatide binding site (49,50) within TSP1 and TSP2, and also binds to the gelatin-binding domain of fibronectin

(145). Vogel et al. also showed that the sequence KRFKQDGGWSHWSPWSS (amino acids 412-428) located within the second type 1 repeat inhibits the proliferation of endothelial cells (162).

The three type 2 repeats are also ~60 residues in length and share homology with epidermal growth factor (EGF). This region binds to soluble molecules, including fibrinogen (80) and plasminogen (27,143,144). The second type 2 repeat conforms to the ß-hydroxylation consensus sequence consisting of an aspartic acid at position 25 and a tyrosine or phenylalanine at position 34 (21,152). These repeats also contain highly-charged amino acids that could serve as high-affinity calcium-binding sites (21).

The eight type 3 repeats form the primary calcium binding region of TSP and are responsible for the tertiary structure of TSP in the presence of calcium (30,75,92). The calcium binding region is made up of highly conserved repeats of asparagine, glutamine, and aspartic acid residues. This is functionally related to the E/F hand in calmodulin (76). In addition, the last type 3 repeat contains an RGDA sequence that can serve as a ligand for the $\alpha_v\beta_3$ integrin (77).

The globular carboxy terminus interacts with platelets, melanoma cells, and keratinocytes (13). Sequences from the carboxy terminus, RFYVVMWK and IRVVM, support the attachment of melanoma, erythroleukemia, and endothelial cells (66,67). These sequences share homology with two cell-adhesive peptides from laminin (67). Sequences similar to IRVVM have also been found in von Willebrand factor, α_2-macroglobulin, and tenascin (67).

TSP1 contains one free thiol per monomer. The sulfhydryl is distributed among 12 cysteines in the type 3 repeats and in the carboxy terminal domain, possibly through disulfide isomerization. The reactivity of the thiols can be influenced by calcium concentration (150). This may be important for the ability of TSP to bind and inactivate the serine protease plasmin (54).

TSP Receptors

Numerous receptors specific for the different domains of TSP1 have been characterized and described. The amino terminal HBD and the GGWSHW sequence within the type 1 repeats bind to heparan sulfate proteoglycans, including syndecan, and sulfatides (63,128,153,154). Elegant studies by Murphy-Ullrich et al. showed that TSP binds to and is internalized by heparan sulfate proteoglycans present on the surface of parental Chinese Hamster Ovary cells (CHO) but not heparan-sulfate-deficient mutant CHO cells (104). In addition, TSP may bind with weaker affinity to chondroitin sulfate chains. The VTCG motif within the type 1 repeats binds to CD36 on endothelial cells, platelets, and some tumor cells (5,121,161). Within the type 3 repeats, the RGD sequence is responsible for binding to integrins (56). Finally, two cell attachment peptides were recently identified in the carboxy terminus of TSP (RFYVVMWK and FIRVVMYEGKK) and shown to bind to a 52 kDa integral membrane protein present in K562 cells (38). All of these receptors were identified using TSP1; binding to the other TSP isoforms has not been examined. However, both TSP1 and TSP2 bind heparin and cause the loss of focal adhesions in endothelial cells (105). Hence, they may share a common receptor. The high degree of homology at the carboxy terminus of the TSP family members suggests that all of the

isoforms may be capable of binding to platelets; an event mediated through the carboxy terminus.

TSP Functions

TSP and cell adhesion

Many extracellular matrix proteins are involved in cellular adhesion. TSP has been shown to be adhesive for many different cell types including melanoma cells and fibroblasts (13,70,71). However, cell adhesion is a multi-stage process that involves cellular attachment, spreading, and the organization of the cytoskeleton with the assembly of focal adhesions (17,24,172). Many cell types can bind/attach to adsorbed TSP, but do not spread or form focal contacts.

Murphy-Ullrich et al. showed that TSP1 and TSP2 stimulate the dissociation of focal adhesions formed by adherent endothelial cells and fibroblasts. These studies show that the addition of TSP1 or TSP2 to endothelial cell cultures results in the rearrangement of the actin cytoskeleton, associated with the loss of focal contacts. The fact that TSP protein levels are highest in migrating cells supports the role of TSP as an anti-adhesive factor. TSP may be a member of a new family of anti-adhesive matrix proteins that include SPARC and tenascin (105). Perhaps TSP would be better termed an attachment (but not adhesive) protein. These contrasting data suggest that the role of TSP in cellular adhesion is complex and may depend on the cell type and experimental conditions (1,63,71,153).

TSP and angiogenesis

TSP has been shown to be a potential regulator of smooth muscle cell proliferation through a synergistic effect with EGF (85). This has been postulated to

be due to the activation of S6 kinase via phosphoinositide turnover resulting in cell growth (136). TSP1 also stimulates the proliferation of normal fibroblasts in vitro (119) but, in contrast, inhibits the proliferation of endothelial cells in vitro (7,106,156).

The ability of TSP to modulate endothelial cell growth suggests a role in angiogenesis. Indeed, TSP has been shown to be anti-angiogenic both in vitro and in vivo. The anti-angiogenic activity of TSP has been partially localized to the CSVTCG sequence motif within the type 1 repeats of TSP1 (158). The levels of TSP are increased in cells adjacent to developing cords and tubules. This may act to limit the association of additional endothelial cells with the developing tubules and inhibit the proliferation of the endothelial cells (57). When used at low concentrations, TSP inhibits chemotaxis and proliferation of endothelial cells in the presence of the angiogenic factor, bFGF (156). Endogenous TSP (based on neutralization with antibodies) also blocks cord formation. A recent study by Dameron et al. showed that TSP secreted by fibroblasts inhibited corneal neovascularization and that the level of TSP mRNA is up-regulated by the tumor suppressor gene p53 (22). The p53 protein is mutated in human tumors more frequently than any other known oncogene or suppressor gene (31). Thus, one could speculate that in cancers where p53 is mutated, TSP1 levels would be decreased. This could lead to increased angiogenic activity and enhanced metastases and survival of the tumor.

Wound healing

The role of TSP1 in hemostasis has been the subject of intense research since TSP was first described as a thrombin-sensitive protein released from intact human

platelets by thrombin. TSP is involved in both the soluble and cellular phases of hemostasis, including fibrinolysis. TSP supports the procoagulant activity of platelets by binding to high molecular weight kininogen (26) and interacts with Factors IXa and Xa, although the exact consequences of these interactions are unknown (8). TSP is incorporated into the fibrin clot (111) and alters the physical properties of the clot (8). TSP also plays an important role in platelet aggregation (39,59). Thus, platelet-bound TSP may be involved in platelet aggregation at the injured site via several mechanisms.

In thrombolysis, TSP appears to be involved in clot-associated fibrin degradation. This is achieved by TSP's ability to complex with plasminogen and tPA (tissue plasminogen activator, 143). In addition, plasminogen bound to TSP is activated more rapidly as compared to plasminogen in solution (143). In contrast, TSP was shown to inhibit plasmin (54) and neutrophil elastase activity (55), although the kinetics of the inhibition are slow. Therefore, it appears that TSP enhances fibrinolysis by increasing conversion of plasminogen to plasmin. However, once plasmin is formed, TSP inhibits enzymatic activity by binding and inactivating plasmin. Perhaps TSP is involved in different stages of fibrinolysis, initially activating plasmin, then later inhibiting its activity.

Increased levels of TSP are secreted by proliferating cells in vitro as compared to quiescent cells. This correlates with the observed increase in TSP synthesis in wound margins in vivo (125). TSP is present in the wound margin for 2-7 days (125). Thereafter, it clears from the those sites and is found around vascular channels near the wound. In addition, high-levels of cell-secreted TSP are found at

regenerating areas in concert with the disappearance of the fibrin matrix (171). Thus, TSP appears to play a role in the normal physiology of wound repair.

TSP and transforming growth factor-ß (TGF-ß)

TSP inhibits the proliferation of endothelial cells (7,106). We observed that TSP-mediated inhibition of endothelial cell growth was not reversible with TSP specific antibodies. However, growth inhibition was reversible with TGF-ß neutralizing antibodies (106). We then showed that platelet-derived TSP is specifically complexed with TGF-ß (106) and that the TGF-ß bound to TSP retains biological activity. Therefore, the role of TSP in the modulation of TGF-ß activity was studied.

TGF-ß

TGF-ß is part of a family of multifunctional growth regulatory proteins derived from independent genes having different temporal and spatial expression in vivo, which appear to have somewhat overlapping functions (reviewed in 9,88,89,129). There have been five isoforms of TGF-ß isolated thus far, with TGF-$ß_1$ being the prototype and best studied member of the family. TGF-$ß_1$ is a 25,000 dalton disulfide-linked homodimer found in the α-granules of platelets. In addition, virtually all cells in culture secrete TGF-ß in a biologically inactive form that must be activated before it can bind to cellular receptors (93,120,166,167). The various members of the TGF-ß family are initially synthesized as larger precursor molecules with an amino terminal signal sequence that is cleaved intracellularly and a pro domain (latency associated peptide) of varying size (41,42). In TGF-$ß_1$, the best characterized isoform, the amino terminal 278 amino acid pro domain or latency

associated peptide (LAP), is cleaved intracellularly from the carboxy terminal mature TGF-ß at a dibasic site between residues 278 and 279 by proteinases active at acidic pH. However, unlike other growth factor proproteins, the LAP remains non-covalently associated with mature TGF-ß, rendering it inactive (41,42). The LAP also serves as a functional binding protein for mature TGF-ß (91,114). The LAPs from the different TGF-ß isoforms are poorly conserved. However, the LAPs are conserved from differing species within a specific isoform. Post-translational modifications of the LAP include N-linked glycosylation, and phosphorylation of three specific mannose-6-phosphate residues (15,43,122,138,139). The LAP is important for the proper folding and secretion of TGF-ß (138,139). The mature region of TGF-ß is highly conserved among isoforms, as well as among species. Mature TGF-ß contains seven critical (highly conserved) cysteine residues, which are characteristic of family members.

TGF-ß$s_{1,2,3}$ are the mammalian forms of TGF-ß. All of these isoforms are highly conserved within the mature region but differ in the LAP (64). The LAP varies in both length and sequence. In TGF-$ß_2$, the LAP forms an α-helix that is linked to the mature region by an additional disulfide bond. This bridge is missing in most other members of the family. Although TGF-$ß_1$ and TGF-$ß_2$ share ~80% sequence homolgy, there are differences. This structural difference leads to differing affinities for the cellular receptors, as well as to differing biological activity, for example, TGF-$ß_1$ is 100-fold more potent than TGF-$ß_2$ at inhibiting the proliferation of endothelial cells. Domain swap studies have shown that the different biological potencies map to the active TGF-ß domain (123). Although the sequence and tertiary

structure of LAPs differ among the TGF-ß isoforms, the biologically latent form of TGF-ß is always composed of an LAP domain non-covalently associated with its corresponding mature region. This form of latent TGF-ß is known as the "small latent complex."

In addition to small latent TGF-ß, many cell types secrete TGF-ß as a large latent complex (93,114,116,166). The large latent complex is composed of the LAP, mature TGF-ß, and a 135-180 kDa protein covalently attached to the LAP, known as the latent TGF-ß binding protein (LTBP). The LTBP is a unique calcium-binding protein containing 16 EGF-like repeats, an RGD sequence, and a unique cysteine motif that shares homology with the protein fibrillin (16,19,62,159). The size of the LTBP differs among cell types as a result of alternative splicing and proteolytic processing. This leads to the possibility that the LTBP is tissue and cell-type specific. Although the exact function of the LTBP is unknown, large latent TGF-ß is secreted more effectively than its smaller counterpart (95). In addition, the LTBP may serve to bind latent TGF-ß to the extracellular matrix, perhaps targeting latent TGF-ß to specific tissues. Latent TGF-ß bound to the matrix may also be more accessible to proteolytic and other activators (155). The LTBP itself is not sufficient to render TGF-ß inactive. TGF-$ß_1$ and TGF-$ß_2$ have been found as both large and small latent complexes; however, TGF-$ß_3$ is only found in the small form. Since expression of the known TGF-ß receptors is constitutive in most cell types (89), regulation of TGF-ß activity primarily resides in factors that modulate the activation of latent TGF-ß.

Regulation of latent TGF-ß activation

Latent TGF-ß can be activated in vitro by a variety of physiochemical agents, including heat, chaotrophic agents, extremes of pH, and deglycosylation (14,94,120). These agents are thought to activate latent TGF-ß by dissociating the LAP from mature TGF-ß. In addition, site-directed mutagenesis of conserved cysteines within the LAP also leads to the secretion of active TGF-ß (15,16). Biological activity can be abolished by adding a caboxy terminal extension to mature TGF-ß, suggesting the proper folding of both the LAP and mature TGF-ß are required for biological activity (169).

Activation of latent TGF-ß in vivo is not well understood. The best described model for activation of latent TGF-ß utilizes a co-culture model of endothelial cells and smooth muscle cells (pericytes). Activation of endogenous TGF-ß in this system requires cell-cell contact and proteases (2,126,133,134). In addition, the cells must be from the same species (reviewed in 126). The inhibition of either urokinase plasminogen activator (uPA) or plasmin in the co-culture model blocks TGFß activation (113,133,134). The generation of plasmin requires that uPA be bound to its receptor. Cells deficient in uPA receptors are unable to activate latent TGF-ß (113). It is speculated that the plasmin required for activation is derived from the serum used to culture the cells (126). Exogenous plasmin can also activate recombinant latent TGF-ß secreted by Chinese Hamster ovary Cells (CHO, 84), in addition to purified recombinantly-produced latent TGF-ß (83).

Activation in this system also requires binding to the cell surface. Several mechanisms facilitate the binding of latent TGF-ß to the cell surface. One such

interaction is via the mannose-6-phosphate residues on the LAP. The residues on the LAP bind to mannose-6-phosphate/insulin-like growth factor II cation independent receptors on the cell surface (28,68) with either subsequent activation by plasmin (133,134) or internalization and processing in acidic microenvironments (61). The LTBP can also bind to the cell surface. Although the exact binding site is unknown, it appears the LTBP mediates the binding of large latent TGF-ß to the extracellular matrix, thus allowing it to be processed by plasmin (155). In addition, an antibody specific for the LTBP blocks latent TGF-ß activation in the co-culture system (34). The LAP may also serve to bind latent TGF-ß to smooth muscle cells, through an unidentified receptor (135).

In homotypic cultures, active TGF-ß formation can also be induced. bFGF and retinoids induce latent TGF-ß activation in keratinocytes and endothelial cells (33,44,65), perhaps by increasing levels of plasminogen activator (PA). bFGF can also increase the levels of PA leading to increased TGF-ß activity (33). Independently of the co-culture system and retinoids, there are reports of autocrine TGF-ß activation occurring, especially in certain types of tumor cells (56).

TGF-ß receptors

Once activated, TGF-ß can bind to one of five proposed cellular receptors to elicit a biological response. Three of these receptors have been cloned and characterized. The type I TGF-ß receptor is a ~55,000 dalton serine/threonine kinase and is found in all TGF-ß-responsive cell types (6). The type II receptor is ~85,000 daltons and is also a serine/threonine kinase (6). The molecular weights of the receptors differ depending on the cell type, possibly a result of different levels of

glycosylation. Both receptors may be autophosphorylated on serine/threonine residues (29). A cell must contain both the type I and the type II receptors in order to be responsive to TGF-ß. Mutational analysis studies have shown that the type I receptor is required for the ability of TGF-ß to activate nuclear factors, while the type II receptor is required for inhibition of cell growth (89). These studies also showed that the type I receptor fails to bind TGF-ß in the absence of the type II receptor.

Recently, numerous studies have shown that the type I and type II receptors function as heterocomplexes and are dependent on one another for proper activity (173,175). The type II receptor forms stable homomeric complexes (presumably dimers) in the presence and absence of TGF-ß (6,18,53,175). The stoichiometry of the type I and type II receptor-complex may consist of a type II homodimer and two type I receptors (175). Both the type I and type II receptors have high affinities for TGF-ß, with binding constants in the picomolar range. However, the different TGF-ß isoforms have different affinities for these receptors.

In addition to the cell signaling receptors, a third receptor has been identified. Betaglycan is a 200,000 dalton proteoglycan, which contains heparan sulfate and chondroitin sulfate chains but lacks kinase activity (82,88,170). Betaglycan is thought to function as a storage site and is not found in all cell types responsive to TGF-ß (88). In the presence of betaglycan, the affinity of TGF-ß for the type II receptor increases (82). In addition, the presence of betaglycan is required for the binding of TGF-$ß_2$ to the type II receptor (82,100). Betaglycan appears to form homocomplexes in the absence of ligand, and transient heterocomplexes with the type II receptor in

the presence of ligand (18,53). The presence of high affinity, constitutively expressed receptors on virtually every cell type leads to the varied functions of TGF-ß.

Functions of TGF-ß

TGF-ß was discovered by its ability to stimulate the anchorage-independent growth of normally adherent fibroblasts (reviewed in 9). No epithelial cell types proliferate in response to TGF-ß; however, TGF-ß can indirectly act as a mitogen for AKR-2B fibroblasts by inducing c-*sis* mRNA, which in turn activates PDGF (79). TGF-ß is possibly the most potent growth inhibitory factor for a wide variety of cell types, including certain tumor cells. The extent of the growth inhibitory response depends on the cell type and the cellular environment. The growth inhibitory response of TGF-$ß_1$ is a result of halting various cell types in the early G1 phase of the cell cycle. Evidence shows in several cell lines that TGF-ß lengthens or arrests the cell cycle in early G1 phase (51,73,81,109,142). It is speculated that this is a result of TGF-ß preventing the phosphorylation of the retinoblastoma gene product, leading to the suppression of c-*myc* activity (73). C-*myc* is required for cell proliferation in a number of different cell lines (73). In addition, TGF-$ß_1$ down regulates the expression of cyclin A and E (40). The cyclins are the regulatory subunits for the cyclin-dependent kinases (cdk), which are required for the progression through the different phases of the cell cycle. Both cyclin A and E are G1 specific cyclins. TGF-ß inhibits both the mRNA and protein expression of cyclin E. However, once cyclin E transcription has begun in mid-to-late G1, TGF-ß loses its ability to inhibit gene expression. TGF-$ß_1$ also blocks the ability of cyclin E to complex with its specific cdk, also leading to a block in the G1 phase (32,147). The

ability of TGF-$ß_1$ to inhibit cellular proliferation is complex, and the specific components involved are dependent on the cell type examined.

One of TGF-ß's most impressive functions is the promotion of extracellular matrix formation in vivo. TGF-ß stimulates the synthesis of the majority of the extracellular matrix proteins by increasing transcription. In addition, TGF-ß inhibits the synthesis of enzymes that degrade the extracellular matrix (matrix metalloproteinases). TGF-ß also enhances the binding of cells to the matrix by increasing the synthesis of integrins, cell-surface receptors for different matrix proteins. All of these different activities lead to the accumulation of matrix and to the implication that TGF-ß may function in normal and abnormal wound and tissue repair, bone formation, embryogenesis and development, and metastasis.

TGF-$ß_1$ is stored in high concentrations in the α-granules of platelets, as well as expressed by cells in a latent form (reviewed in 88,89). Latent TGF-ß can also be found in the serum (110) and in plasma (168). Thus, TGF-ß can be delivered to wound sites in high concentrations. The activity of TGF-ß as a promoter of extracellular matrix deposition and a regulator of cell migration plays a major role in wound healing. Administration of TGF-ß into wound chambers or to incisional wounds stimulates the formation of granulation tissue and cellularization of the wound, accelerating the wound healing process (108,127,151). In addition, TGF-ß is found in the bone and may promote bone repair as well as recycling of bone marrow by stimulating osteoclasts (137,157). Although TGF-ß does increase wound healing, it could also lead to an increased amount of granulation tissue and contribute to a variety of fibrotic disorders (11,20). Shah et al. have shown in in vivo models of

wound healing that the application of a neutralizing antibody against TGF-$ß_1$ at the time of wounding reduces the accumulation of granulation tissue (140). Interestingly, the application of TGF-$ß_2$ increases the time of healing without causing the accumulation of granulation tissue, suggesting that both the concentration and isoforms of TGF-ß are important in TGF-ß-mediated biological responses.

TGF-$ß_1$ is highly angiogenic in vivo in a variety of assay systems, including stimulating a localized response at the site of TGF-ß injection (127). However, this angiogenic response is accompanied by inflammation. TGF-ß is a powerful chemoattractant for many inflammatory cells, including macrophages (165), which secrete the angiogenic factor bFGF. Therefore, the positive effects of TGF-ß on angiogenesis in vivo may be an indirect result of the migration of inflammatory cells. In vitro, TGF-ß inhibits the proliferation of endothelial cells in a reversible manner (51,52,85,101) and also inhibits the ability of endothelial cells grown on collagen gels to invade the gel and form tube-like structures in response to bFGF (101). Based on these conflicting data, the exact role of TGF-ß in angiogenesis is highly controversial. However, Moses et al. have proposed the following model for the role of TGF-ß in angiogenesis based on in vivo studies in the absence of an inflammatory response: TGF-ß is initially secreted by the wounded cells in sub-picomolar concentrations, sufficient to increase the migration of keratinocytes toward the wounded area. Once the cells reach the center of the wound, TGF-ß concentrations increase, and TGF-ß no longer acts as a chemoattractant but induces growth inhibition and the stimulation of extracellular matrix, leading to endothelial capillary tubule formation. This model shows the potential importance of TGF-ß in angiogenesis as well as wound healing.

Based on the ability of TGF-ß to affect cellular growth and differentiation, it is not unreasonable to speculate that TGF-ß plays a role in cancer progression and metastasis. TGF-ß has been shown to inhibit the growth of mammary epithelial cells, both normal and malignant, in vitro (3,33,65,124,162), suggesting the possibility that endogenous TGF-ß functions as a negative autocrine regulator of cell growth. This hypothesis is supported by studies demonstrating that anti-TGF-$ß_1$ and -$ß_2$ neutralizing antibodies stimulated the proliferation of hormone-independent human breast cancer cell lines (4). In addition, the loss of responsiveness to TGF-ß growth inhibitory signals either at the receptor or postreceptor level has been associated with progression to carcinogenesis (58). These data suggest that TGF-ß is a negative regulator of tumor growth.

However, there is also much evidence suggesting that TGF-ß is associated with increased tumorigenicity. High levels of TGF-ß activity have been found in the medium conditioned by hormone-independent, highly tumorigenic breast cancer lines (3,30). TGF-ß transcripts are more abundant in highly proliferative tumors than in non-tumor breast tissue (10), and intense staining for TGF-$ß_1$ is positively correlated with disease progression (46). Indeed, increased TGF-ß activity is detected at the advancing edge of mammary tumors and in lymph node metastases (22). The most striking data suggesting that TGF-ß may increase tumorigenicity in vivo are from the study that showed there is increased tumor formation in nude mice overexpressing active TGF-ß (3). Therefore, the role of TGF-ß in breast cancer is controversial, not unlike its role in other diseases and physiologic processes.

Relevance

TGF-ß, once activated, is able to elicit a number of cellular responses, both positive and negative. Activation in vivo is not understood and is currently being investigated. The co-culture model of activation is complex and does not explain the ability of certain tumor cells to secrete active TGF-ß. Our previous studies have shown that TSP specifically complexes with TGF-ß in the α-granules of thrombin-stimulated platelets. This may lead to a high concentration of active TGF-ß at wound sites upon platelet degranulation, which would serve to promote wound healing. Therefore, these studies will serve to characterize the interaction of TSP with latent TGF-ß, which results in the formation of biologically active TGF-ß. In this work, we show that TSP activates latent TGF-ß secreted by endothelial cells via a novel mechanism that does not involve cell surface binding or protease activity. Further studies with purified recombinant latent TGF-ß show that TSP activates latent TGF-ß in a chemically-defined system via binding interactions. In addition, we have identified a three amino acid sequence unique to TSP1 that activates latent TGF-ß as a consequence of interactions with a sequence in the LAP. We have also defined a second sequence in the type 1 repeats of both TSP1 and TSP2 that binds to the mature portion of TGF-ß: when present as a discrete peptide, this sequence can competitively inhibit the activation of latent TGF-ß by TSP1. These findings may be important in understanding how TGF-ß becomes activated in vivo. In addition, both the inhibitory and activating peptides from TSP have potential therapeutic importance in situations in which TGF-ß activity requires modulation, for example, wound healing.

The first four chapters of this thesis are a series of papers that have been published in peer-reviewed journals.

THROMBOSPONDIN CAUSES ACTIVATION OF LATENT TRANSFORMING GROWTH FACTOR-ß SECRETED BY ENDOTHELIAL CELLS BY A NOVEL MECHANISM

STACEY SCHULTZ-CHERRY AND JOANNE E. MURPHY-ULLRICH

Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-0019

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ABSTRACT

Thrombospondin (TSP) forms specific complexes with transforming growth factor-ß (TGF-ß) in the α-granule releasate of platelets, and these TSP-TGF-ß complexes inhibit the growth of bovine aortic endothelial cells (BAE). In these studies, we report that TSP stripped of associated TGF-ß (sTSP) retained growth inhibitory activity that was partially reversed by a neutralizing antibody specific for TGF-ß. Since BAE cells secrete latent TGF-ß, we determined whether sTSP activates the latent TGF-ß secreted by BAE cells. Cells were cultured with or without sTSP, and then the conditioned medium was tested for the ability to support TGF-ß-dependent NRK colony formation in soft agar. Medium conditioned with sTSP showed a dose and time-dependent ability to stimulate BAE-secreted TGF-ß activity reaching maximal activation by 1-2 h with 0.4 μg/ml (0.9 nM) sTSP. The sTSP-mediated stimulation of TGF-ß activity is not dependent on serum-factors and is not a general property of extracellular matrix molecules. The sTSP-mediated stimulation of TGF-ß activity was blocked by a monoclonal antibody specific for sTSP and by neutralizing antibodies to TGF-ß. Activation of BAE cell secreted latent TGF-ß by sTSP can occur in the absence of cells and apparently does not require interactions with cell surface molecules, because in conditioned medium removed from cells, and then incubated with sTSP, activation occurs with kinetics and at levels similar to what is seen when sTSP is incubated in the presence of cells. Serine proteases such as plasmin are not involved in sTSP-mediated activation of TGF-ß. Factors that regulate the conversion of latent to active TGF-ß are keys to controlling

TGF-ß activity. These data suggest that TSP is a potent physiologic regulator of TGF-ß activation.

INTRODUCTION

TGF-ß is a member of a family of growth, differentiation, and morphogenesis autocrine and paracrine factors (3,26). TGF-ß can affect diverse cellular functions in virtually all cell types. Depending on the cell type and its extracellular environment, these effects can be either positive or negative. TGF-ß inhibits the proliferation of endothelial cells in vitro (31) but stimulates angiogenesis in vivo (39). TGF-ß has also been shown to enhance or inhibit the proliferation of fibroblasts, depending on the nature of the substrate and the mitogens present (3). Myoblast differentiation can also be induced or blocked by TGF-ß, depending on the availability of mitogens (25,45).

TGF-$ß_1$ is a disulfide-linked homodimer that is synthesized as part of a latent precursor molecule (26). The latent precursor molecule is 390 amino acids in length and consists of an N-terminal 278 amino acid latency associated peptide (LAP) and the C-terminal 112 amino acids that constitute the active domain (15-17). The proregion of TGF-ß is unique in that it remains non-covalently attached to the active region after intracellular proteolytic processing and secretion (15). Association of the LAP with the mature peptide region confers latency: the LAP-associated growth factor is unable to interact with its cellular receptors. The LAP contains three N-linked glycosylation sites, two of which have mannose-6-phosphate residues (8,28,38). These carbohydrate structures may be important for latency since endoglycosidase F treatment leads to activation of TGF-ß (28). The disulfide-bonded

dimeric structure of LAP is critical for latency because site-directed mutagenesis of critical cysteine residues (cys 223,225) in the LAP abolishes activity (9). The active domain contains nine conserved cysteine residues that participate in interchain and intrachain disulfide bonding (27).

TGF-ß is secreted by most cell types as a latent complex (27,37). Because TGF-ß synthesis and TGF-ß receptor expression are not highly regulated, primary regulation of TGF-ß activity occurs by controlling conversion of the latent TGF-ß complex to the active molecule. Physiochemical activation can occur by extremes of pH, heat, chaotropic agents, and deglycosylation (6,27,28,37). Activation in vivo is more complex and not well understood. There is evidence from cell culture models that activation may occur through binding of the latent molecule to mannose 6-phosphate receptors (12,21), by plasmin-mediated proteolytic processing (4,23,40,41), and/or by processing in acidic cellular microenvironments (20). In some systems, activation of latent TGF-ß by plasmin is relatively inefficient (41). In addition, there are reports of TGF-ß activation occurring independently of these mechanisms (19). These results suggest that additional mechanisms of latent TGF-ß activation may exist.

We have shown previously that thrombospondin (TSP), a disulfide-linked trimer (450,000 daltons) present in connective tissues and in platelet alpha granules, is associated with TGF-ß as an active complex in the releasate of stimulated platelets (5,14,30,34). TSP is synthesized and secreted by cells in vitro, where it is then able to bind various cellular receptors and/or become incorporated into the extracellular matrix (reviewed in 5,14,30). TSP is present transiently in wound environments and

its synthesis is rapidly induced by growth factors, including TGF-ß. TSP, like TGF-ß, has varied effects on cell growth and differentiation. TSP stimulates the proliferation of fibroblasts (36) and smooth muscle cells (24), and in contrast, it inhibits the proliferation of endothelial cells (2,42). TSP may also serve as both an attachment protein and an anti-adhesive molecule, as shown by its ability to cause disassembly of focal adhesions in endothelial cells (33).

In the present studies, we show that TSP activates latent TGF-ß secreted by bovine aortic endothelial (BAE) cells in culture. TSP-mediated activation of TGF-ß occurs independently of plasmin activity and does not require interactions with cell surface molecules. Based on these data, we propose that TSP activates latent TGF-ß through a novel mechanism. Furthermore, TSP-TGF-ß interactions may act as a physiologic means of regulating TGF-ß activity.

MATERIALS AND METHODS

Thrombospondin Purification

TSP was purified as previously described (34). Briefly, 8-10 units of fresh human platelets were purchased from the Birmingham American Red Cross and washed with Hepes wash buffer (10% ACD, 0.05 M Hepes, 0.15 M NaCl, and 5 mM Dextrose, pH 7.6). The platelets were thrombin stimulated, and the platelet releasate was applied to a heparin-Sepharose CL-6B (Pharmacia, Piscataway, NJ) affinity column pre-equilibrated with TBS-C (0.01 M Tris-HCl, 0.15 M NaCl, 0.1 mM CaCl) pH 7.4. The bound TSP was eluted with 0.55 M NaCl/TBS with 1 mM CaCl and applied to a A0.5M gel filtration column (Bio-Rad, Richmond, CA) pre-equilibrated with TBS-C, pH 11, to remove associated TGF-ß. Purity was assessed by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie blue or silver staining. No contaminating TGF-ß activity was found associated with sTSP in NRK soft agar colony formation assays.

Cells

BAE cells were isolated from aortas obtained at a local abattoir and were characterized by Dil-AcLDL and by staining for Factor VIII antigen. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cell-Gro, Mediatech, Herndon, VA) supplemented with 4.5 g/l glucose, 2 mM glutamine, and 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), as previously described (33). Clone 49F of NRK cells (American Type Culture Collection, Rockville, MD, CRL 1570) was provided by Dr. Lynn-Allen Hoffmann, UW-Madison. Stocks were cultured in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, and 10% calf serum (CS; Hyclone Laboratories, Logan, UT) as described (1). Calf Serum was tested and chosen for low levels of active TGF-ß. Mink Lung Epithelial cells (CCL 64) (Mv1Lu) were cultured in minimum Eagle's medium (MEM; Cell-Gro, Mediatech, Herndon, VA) supplemented with 4.5 g/l glucose, 2 mM glutamine, and 10% FBS. Chinese Hamster Ovary cells (CHO) were provided by Dr. Jeff Esko, University of Alabama at Birmingham. Stocks were cultured in F-12 (Cell-Gro, Mediatech, Herndon, VA) supplemented with 10% FBS. All cells tested negative for mycoplasma contamination.

NRK Colony Formation in Soft Agar

TGF-ß activity was assayed by determining colony formation by NRK cells in soft agar assays as described (1) except assays were performed in 24-well tissue

culture plates. Briefly, 5% Noble agar (Difco, Detroit, MI) was diluted ten-fold in 10% calf serum/DMEM, and 0.5 ml of this 0.5% agar dilution was added per well to a 24-well tissue culture plate as a base layer and allowed to harden. A 0.2 ml sample containing 5 ng epidermal growth factor (EGF) was combined with 0.6 ml 0.5% agar and 0.2 ml (2×10^3) of an NRK cell suspension in 10% calf serum/DMEM. 0.5 ml of this 0.3% agar-sample solution was added to the cooled base layer, and the plates were incubated for 7 days at 37°C, 5% CO_2. The number of colonies greater than 62 μm (>8-10 cells) in diameter was counted. Experiments were performed in triplicate.

BAE Cell Proliferation Assays

BAE cells were plated at 5000 cells per well in 1 ml of DMEM with 20% FBS in 24-well tissue culture plates and incubated overnight at 37°C, 5% CO_2. The cells were rinsed once in serum-free DMEM. Test samples in 0.5 ml 2.5% FBS DMEM were added to each well in triplicate (day 0). On day 2, cells received fresh aliquots of test sample in 0.5 ml without removing the original media (to give a final volume of 1 ml). Cells were grown for another 2 days; then culture media were removed, and cells were trypsinized with 0.5 ml trypsin-EDTA (Gibco, Grand Island, NY) and harvested. The number of harvested cells was determined using a model ZM Coulter Counter (Coulter Electronics, Hialeah, FL).

Preparation of BAE Conditioned Media

BAEs were plated at a density of 100,000 cells in a 25 cm^2 flask in 20% or 0.2% FBS/DMEM and incubated overnight at 37°C, 5% CO_2. This density was determined by comparing the ability of sTSP to activate latent TGF-ß in sparse,

sub-confluent, and confluent BAE cultures. This cell density showed the greatest difference in levels of active TGF-ß between control and TSP-treated media. Flasks were rinsed once with 2 ml serum-free DMEM, and then test samples were added in 2.5 ml of DMEM with either 0.2% or 20% FBS as specified in the figure legends. The flasks were incubated for additional times at 37°C, 5% CO_2. Conditioned media was collected, centrifuged at 1200 rpm for 5 min to remove cellular debris, and stored at 4°C in polypropylene tubes for no more than 3 days before testing in NRK soft agar assay to determine TGF-ß activity.

Activation of Purified Recombinant Latent TGF-ß by sTSP

sTSP (13 nM) was incubated with 2 nM purified recombinant latent TGF-ß in a final volume of 0.5 ml in PBS for 2 h at 37°C or 4°C in siliconized microfuge tubes. Samples were then tested in NRK colony forming soft agar assays for TGF-ß activity.

Additional Materials

The following items were purchased: ϵ-aminocaproic acid (EACA), Aprotinin, pepstatin A, and $\alpha_2$anti-plasmin (Sigma Chemical, St. Louis, MO); E-64, carboxypeptidase, leupeptin (Calbiochem, San Diego, CA); cystatin (Boehringer-Manheim, Indianapolis, IN). Recombinant TGF-ß (rTGF-ß) and purified recombinant latent TGF-ß (LTGF-ß) were generous gifts of Dr. Daniel Twardzik (Bristol-Myers Squibb, Seattle, WA). Tenascin was a gift of Dr. Harold Erickson, Duke University. Laminin was obtained from Dr. Magnus Höök (Institute of Biotechnology, Houston, TX).

Antibodies

Mouse anti-TSP 133 was raised against stripped TSP and developed using the Monoclonal Antibody Core facility at University of Alabama at Birmingham. This antibody is an IgG_1, which recognizes the 50 kDa chymotryptic fragment of stripped TSP by Western blotting. Mab TSP-B7 ascites was raised against human platelet releasate and is specific for TSP (11) (Sigma Chemical, St. Louis, MO). Mouse monoclonal and rabbit polyclonal anti-TSP antibodies, raised against native TSP, were generous gifts of Dr. Deane Mosher, University of Wisconsin-Madison, and mouse anti-TSP antibodies to specific regions of native TSP were generous gifts of Dr. Vishva Dixit, University of Michigan.

A chicken anti-TGF-ß antibody was purchased from Oncomembrane, Seattle, WA, and a mouse monoclonal anti-TGF-ß antibody was purchased from Genzyme, Cambridge, MA. Anti-vitronectin monoclonal and polyclonal antibodies were purchased from Telios, San Diego, CA. A polyclonal anti-platelet factor 4 antibody was purchased from Atlantic Antibodies, Scarborough, ME. Mouse monoclonal anti-basic fibroblast growth factor (bFGF) was obtained from Upstate Biotech, Inc., Lake Placid, NY.

RESULTS

TSP Stripped of TGF-ß Activity Inhibits the Growth of BAE Cells

Previous results from our lab and others have shown that TSP inhibits BAE cell growth (2,42). We observed that active TGF-ß associated with the TSP was partially responsible for growth inhibition of BAE cells (34). In order to examine whether TSP stripped of associated TGF-ß (sTSP) activity inhibited BAE growth, we

repeated cell proliferation assays using sTSP. BAE cells were exposed to increasing concentrations of either native TSP (TGF-ß activity associated with TSP) or stripped TSP (sTSP, no associated TGF-ß activity) in media supplemented with 2.5% FBS for a period of 4 days, at which time cell number was determined. Native TSP and sTSP significantly inhibited the proliferation of BAE cells compared to 2.5% FBS alone (Fig. 1). Furthermore, the dose response curves were nearly identical for native and stripped TSPs. No significant cell death was observed. The inhibition of BAE proliferation by TSP was concentration dependent with 1 μg/ml sTSP inhibiting 75% of growth. Cells grown in the presence of sTSP assumed a more elongated, fibroblastic shape and had prominent nucleoli (Fig. 2 *a*) as compared to the polygonal cells in the 2.5% FBS media control (Fig. 2 *e*). Similarly, TGF-ß treated cells were elongated with numerous processes and prominent nucleoli (Fig. 2 *c*).

A neutralizing antibody to TGF-ß reversed the growth inhibitory effects of sTSP by 42% (Fig. 3). Addition of the neutralizing antibody to TGF-ß to wells containing sTSP also caused a partial reversion to a smaller, more polygonal cell, characteristic of normal BAE cells (Fig. 2 *b*). Similar results were obtained with both mouse and chick anti-TGF-ß antibodies. In contrast, a polyclonal antibody (gift of Dr. Deane Mosher, University of Wisconsin-Madison) and various monoclonal antibodies specific for native TSP were not able to neutralize sTSP-mediated growth-inhibition (data not shown). These results are similar to what was observed in growth inhibition experiments using native TSP (34). Antibodies alone did not affect cell growth. These data suggest that growth inhibition of BAE cells by TSP stripped

of associated TGF-ß activity is at least partially due to a TGF-ß-dependent component.

sTSP Activates TGF-ß in BAE Conditioned Media

Because sTSP-mediated BAE growth inhibition is partially TGF-ß dependent, it is possible that sTSP incubation with BAE cells is causing activation of endogenous latent TGF-ß. TGF-ß secreted from endothelial cells as an inactive molecule (18). It is not entirely clear how endothelial cell latent TGF-ß is activated. In order to test the hypothesis that sTSP activates latent TGF-ß secreted by endothelial cells, sTSP was added to BAE cells in DMEM with 0.2% FBS for 0-48 h. Aliquots of the conditioned media were tested in NRK colony forming soft agar assays for the presence of TGF-ß activity. sTSP at 0.4 μg/ml (0.9 nM) was able to increase colony forming activity in the conditioned media by two- to three-fold as compared to conditioned medium alone (Fig. 4). Increases in TGF-ß activity were observed as early as 15 min after addition of sTSP to cells and persisted above control levels for at least 48 h. Similar levels of activation were observed when cells were conditioned in media where serum levels were raised from 0.2% to 20%, suggesting that sTSP-mediated stimulation of TGF-ß activity is independent of serum factors.

Stimulation of TGF-ß Activity is Dependent on sTSP Concentration

In order to assess whether the stimulation of TGF-ß activity in BAE conditioned media was dependent on the concentration of sTSP present, varying doses of sTSP ranging from 10 ng to 10 μg were added to BAE cells in 2.5 ml of media. Concentrations of sTSP between 40-400 ng/ml (100-1000 ng added) were effective at stimulating NRK colony formation in soft agar (Fig. 5). The maximal response was

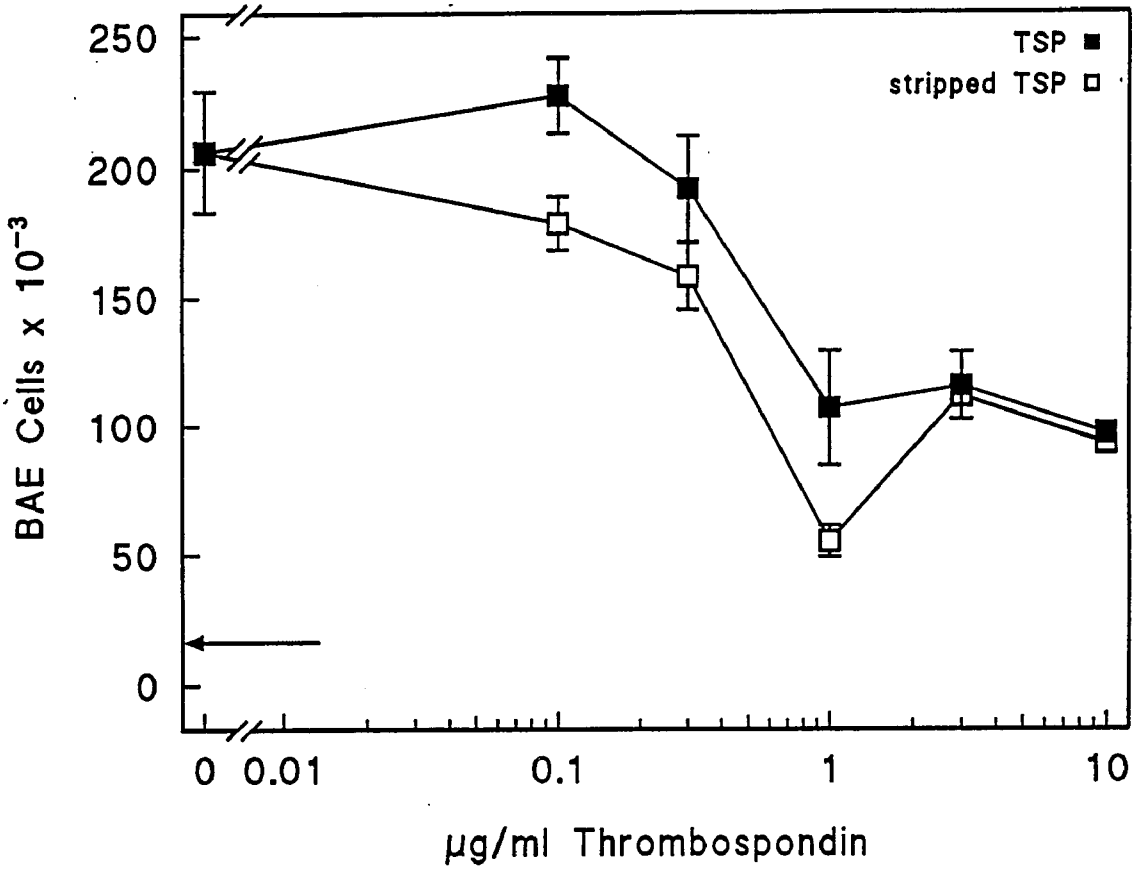

Figure 1. Inhibition of BAE cell proliferation by TSP. BAE cells were seeded at 5,000 cells per well in 24-well plates and incubated overnight in DME supplemented with 20% FBS. They were then washed with DME, and treated with varying concentrations of TSP (native or stripped) in DME containing 2.5% FBS on days 0 and 2. Final cell number was determined on day 4. Cell number on day 0 was 15,640 $\pm$ 2925 cells/well (arrow). Results are expressed as means of triplicate determinations $\pm$ SD.

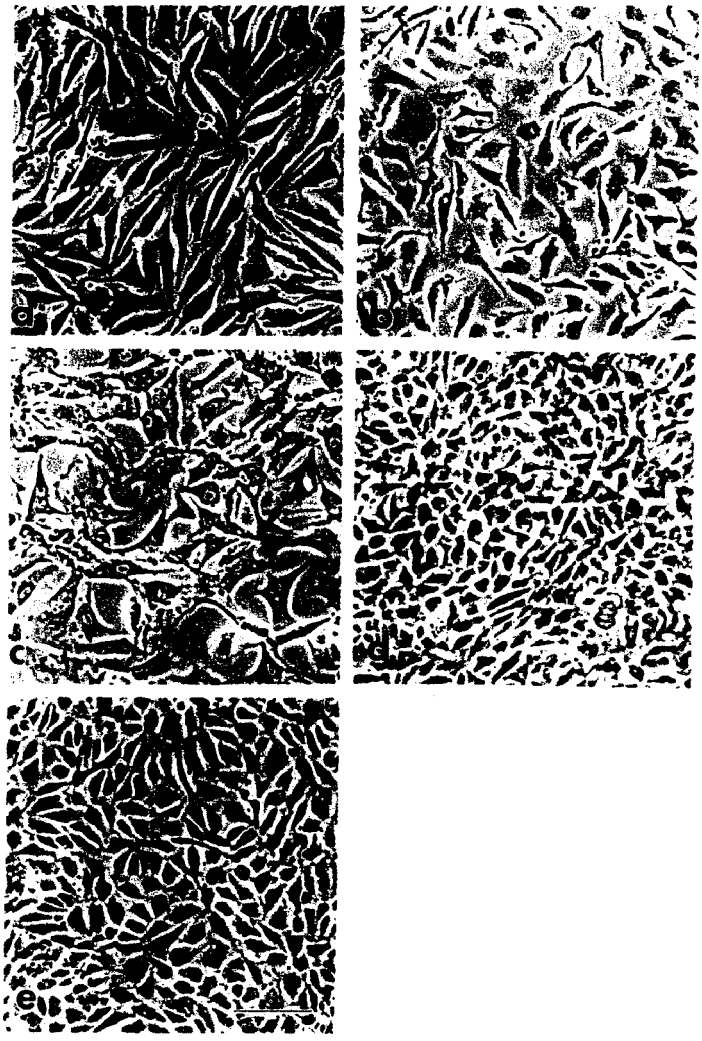

Figure 2. The altered shape of BAE cells grown in sTSP is partially reversed by anti-TGF-ß antibody. Cells were seeded at 5,000 cells per well in 24-well plates, grown overnight in DME with 20% FBS, washed, and refed on days 0 and 2 with DME with 2.5% FBS and (*a*) 3 μg/ml sTSP, (*b*) 3 μg/ml sTSP + 2.5 μg/ml mouse anti-TGF-ß, (*c*) 1 ng/ml recombinant TGF-ß, (*d*) 1 ng/ml recombinant TGF-ß + 2.5 μg/ml mouse anti-TGF-ß, or (*e*) no additions. Phase micrographs of these cells were photographed on day 4. Bar, 100 μm.

repeatedly observed with 1 ug sTSP/25 cm^2 flask (0.4 μg/ml, or 0.9 nM). When compared to recombinant TGF-ß (rTGF-ß), the level of maximal NRK colony formation induced by sTSP correlated to approximately 0.1 ng/ml of TGF-ß activity.

In order to rule out that the increase in TGF-ß activity in the NRK soft agar assays was due to sTSP acting at the level of the NRK cells, we tested whether sTSP affected mature TGF-ß activity and whether anti-sTSP antibody 133, which inhibits sTSP-stimulation of TGF-ß activity in the conditioned medium, affected TGF-ß activity in the NRK assay. As shown in Table I, there was no modulation of TGF-ß activity by either sTSP or anti-TSP antibodies, nor did sTSP by itself stimulate colony formation. sTSP also does not activate the latent TGF-ß present in the 0.2% FBS (Table I).

Stimulation of TGF-ß Activity in BAE Conditioned Media is Specific for sTSP

Other extracellular matrix proteins were tested for their ability to activate endothelial cell secreted latent TGF-ß. Equimolar amounts of tenascin, fibronectin, BSA, or laminin did not stimulate TGF-ß activation (Fig. 6). Basic FGF, in contrast to a previous report (13), did not stimulate increased TGF-ß activity in our system (Fig. 6). These results show that stimulation of TGF-ß activity in BAE conditioned medium is not a general property of extracellular matrix molecules, including TGF-ß-binding molecules such as fibronectin, and therefore is a specific property of sTSP.

Antibodies to sTSP Inhibit Stimulation of TGF-ß Activity

In order to eliminate the possibility that the observed increase in TGF-ß activity was due to potential components associated with sTSP, we attempted to block

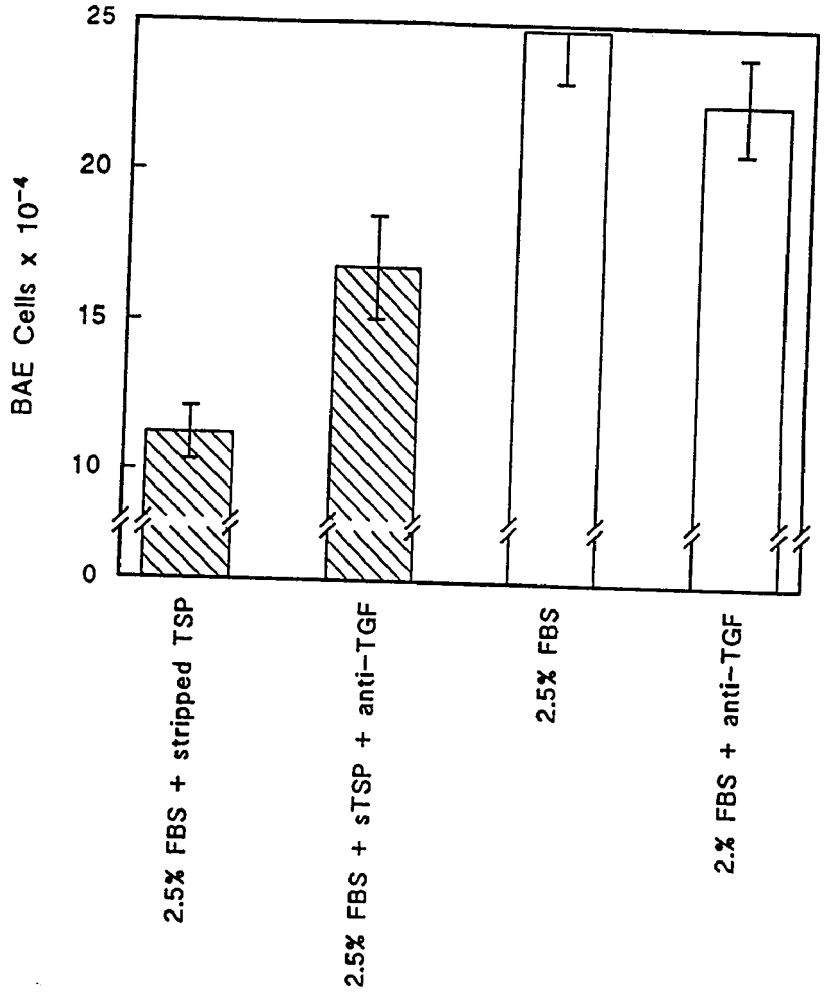

Fig. 3. Inhibition of BAE proliferation by sTSP is sensitive to anti-TGF-ß neutralizing antibody. BAE cells were seeded at 5,000 cells per well and incubated overnight in DME with 20% FBS, washed once with DME, and then grown in DME with 2.5% FBS supplemented on days 0 and 2 with either 3 μg/ml sTSP, 3 μg/ml sTSP + 1 μg/ml chicken anti-TGF-ß, or 1 μg/ml chicken anti-TGF-ß. Cell number on day 0 was 13,840 $\pm$ 600 cells per well. Cells were harvested and cell number per well was determined on day 4. Results are expressed as means of triplicate determinations $\pm$ SD.

stimulation with antibodies to TSP. Monoclonal antibody 133 ascites, which recognize an epitope in the 50 kDa chymotryptic fragment of sTSP, completely inhibited the stimulation of TGF-ß activity by sTSP (Fig. 7). Mab TSP-B7 ascites, which is specific for the 70 kDa core of platelet TSP (11), also blocked this effect of sTSP. However, another monoclonal antibody, which recognizes an epitope in the 70 kDa fragment of native TSP (gift of Dr. Deane Mosher), only inhibited sTSP activation of latent TGF-ß by 32%. Antibodies alone had no effect on these assays and did not interfere with the ability of rTGF-ß to form colonies in soft agar (data not shown).

Colony formation was also TGF-ß dependent since a polyclonal chicken anti-TGF-ß antibody and a monoclonal mouse anti-TGF-ß neutralizing antibody completely inhibited colony formation (Fig. 7). These results show that the factor activated by sTSP in BAE conditioned medium is TGF-ß.

In contrast, antibodies to vitronectin (both monoclonal and polyclonal), platelet factor-4, bFGF, and control ascites did not inhibit the stimulation by sTSP (Fig. 7 and data not shown). These data show that increases in TGF-ß activity observed in the NRK soft agar assays are not due to the presence of commonly associated matrix and platelet proteins, but are dependent on sTSP and TGF-ß.

sTSP Stimulation of TGF-ß Activity in BAE Conditioned Medium Occurs Independently of Binding to the Cell Surface

A proposed mechanism of latent TGF-ß activation <u>in vivo</u> is through binding to and internalization of latent TGF-ß by mannose-6-phosphate receptors and subsequent processing in acidifying vesicles or processing by plasmin at the cell

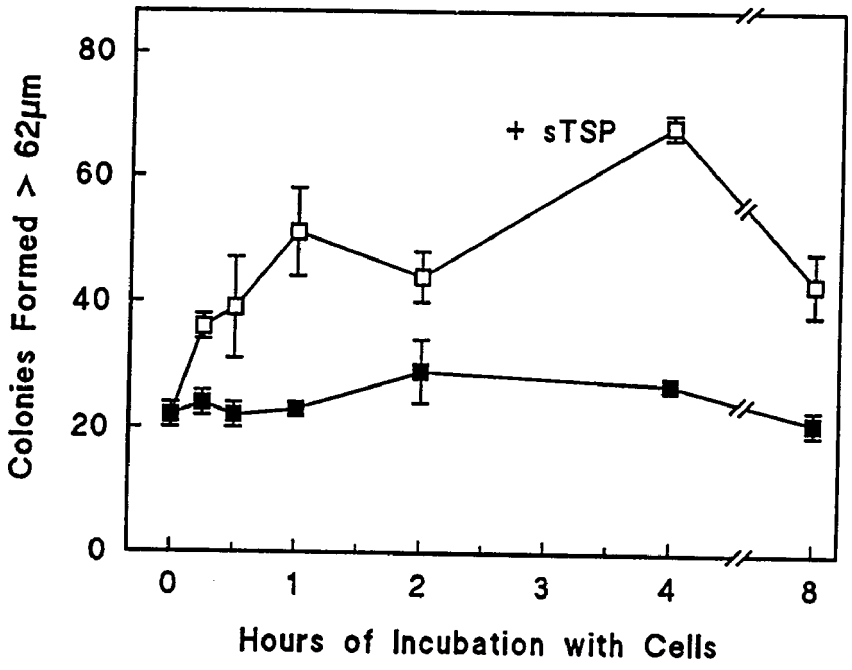

Figure 4. Kinetics of sTSP activation of TGF-ß in 0.2% BAE conditioned media (CM). BAE cells seeded at 1 x 10^5 cells/25 cm^2 flask were grown overnight in DME supplemented with 0.2% FBS and 2 mM glutamine. sTSP (0.4 μg/ml, 0.9 nM) was added to the flasks and incubated for 15 min to 8 h at 37°C, 5% CO_2. Aliquots (0.2 ml/sample) of conditioned medium with (open squares) or without (closed squares) added sTSP were tested in the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations ± SD.

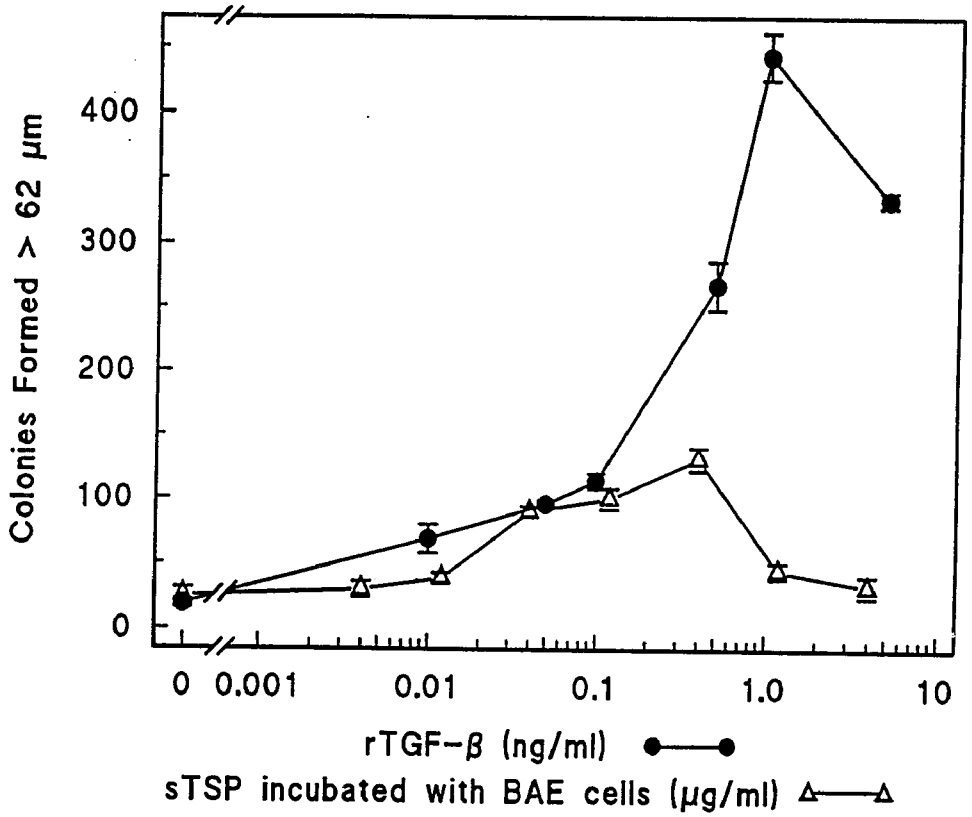

Figure 5. Activation of TGF-ß in BAE conditioned media by sTSP is dose dependent. BAE cells were seeded at 1 x 10^5 cells/25 cm^2 flask in DME with 20% FBS and incubated overnight. Flasks were rinsed once with DME, and then increasing amounts of sTSP in 2.5 ml of DME with 0.2% FBS were incubated an additional 48 h. Aliquots of these conditioned media were tested in the NRK soft agar colony forming assays to determine TGF-ß activity (open triangles). Different concentrations of rTGF-ß were also tested in the same set of NRK soft agar assays (closed circles). Results are expressed as means of triplicate determinations $\pm$ SD.

surface (12,20,21). Experiments were performed to determine whether sTSP requires interactions with cell surface molecules in order to activate latent TGF-ß. After incubating BAE cells in DMEM with 0.2% FBS overnight, the media were removed from the culture flasks and incubated in polypropylene tubes in the presence or absence of sTSP (0.4 μg/ml) for the indicated times. This was done in direct comparison with sTSP incubated in the presence of cells. Aliquots of the conditioned medium were then tested in NRK colony forming soft agar assays for sTSP-mediated activation of TGF-ß. These data show that sTSP is able to activate TGF-ß in the absence of cells to a similar extent and with similar kinetics as shown in Fig. 4, in which sTSP was incubated in the presence of cells (Fig. 4). Cell-conditioned media incubated with sTSP in the absence of cells demonstrated increased TGF-ß activity as early as 15 min after addition of sTSP. Maximal levels were reached by 2 h and persisted above baseline for at least 48 h (Fig. 8). Thus, in contrast to previously reported mechanisms of activation, TSP-mediated activation of latent TGF-ß does not require interactions with cell surface molecules. sTSP Mediated Stimulation of TGF-ß Activity is Insensitive to Serine Protease Inhibitors

Previous studies have shown that plasmin can activate latent TGF-ß in vitro (22,23). In co-cultures of endothelial and smooth muscle cells, plasmin levels have been shown to be upregulated, thus activating latent TGF-ß (40,41). The common motif is the involvement of a serine protease in the activation of latent TGF-ß. Therefore, we tested the effects of different serine protease inhibitors on the activation of TGF-ß by sTSP in BAE-conditioned medium. TGF-ß activation by sTSP was

TABLE I: sTSP does not affect the activity of mature rTGF-ß or stimulate TGF-ß activity in FBS

	# of Colonies Formed
EGF	39 ± 1
EGF + rTGF-ß	240 ± 3
EGF + rTGF-ß + sTSP	241 ± 2
EGF + sTSP	49 ± 12
EGF + sTSP	40 ± 4
EGF + 0.2% FBS	41 ± 5
EGF + 0.2% FBS + sTSP	41 ± 4

The NRK colony forming assay was performed as described in Materials and Methods. All samples contained 5 ng/ml EGF in 10% calf serum/DMEM, except the samples in which EGF was in 0.2% FBS as indicated. sTSP (1 μg/ml) was preincubated with 1 ng/ml rTGF-ß for 2 h at 37°C, and compared for relative activity versus 1 ng/ml rTGF-ß or 1 μg/ml sTSP. sTSP (3 μg/ml) was also incubated with 0.2% FBS for 2 h at 37°C and tested for relative activity versus sTSP (3 μg/ml) or 0.2% FBS. All samples were tested in the NRK soft agar assay for 7 days at 37°C, 5% CO_2. Results are expressed as the means of triplicate determinations ± S.D.

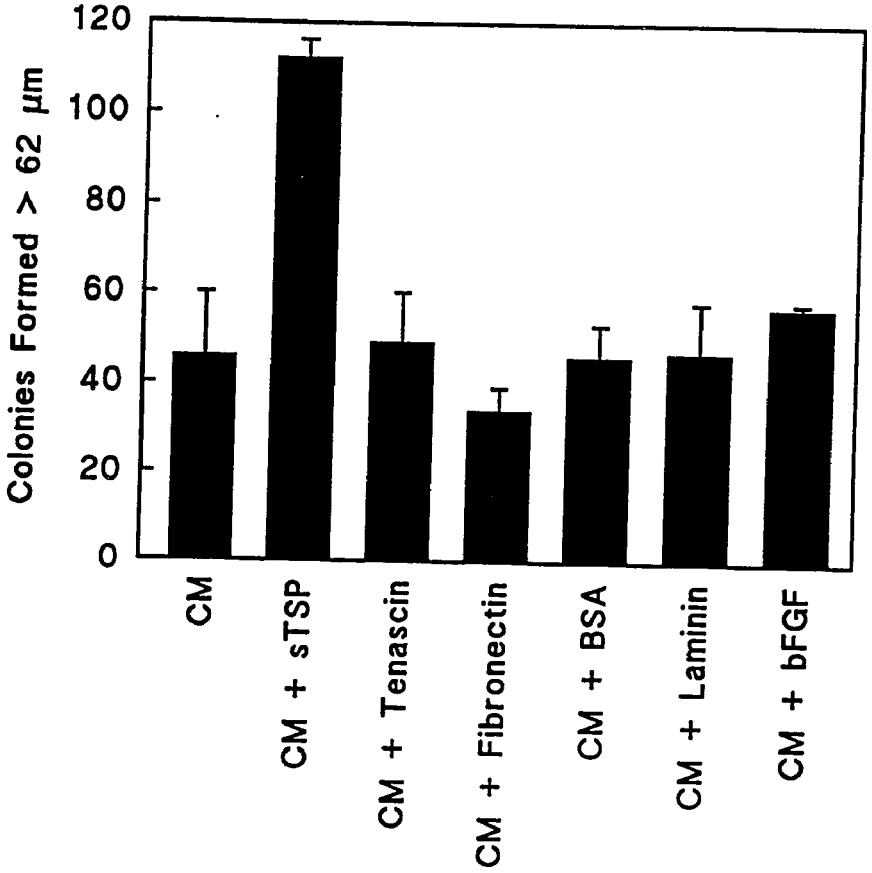

Figure 6. Stimulation of active TGF-ß in BAE conditioned media (CM) is specific for sTSP. BAE cells seeded at 1 x 10^5 cells/25 cm^2 flask were grown overnight in DME supplemented with 20% FBS and then washed with DME. Then 0.4 µg/ml sTSP or equimolar amounts (0.9 nM) of various proteins were then added to 2.5 ml of 20% FBS/DME and incubated with the cells for 48 h at 37°C, 5% CO_2. bFGF was used at 1 ng/2.5 ml media. Aliquots of conditioned medium (0.2 ml/sample) were tested in NRK colony forming soft agar assays to determine TGF-ß activity. Similar results were obtained when cells were conditioned in DME with 0.2% FBS. Results are expressed as the means of triplicate determinations $\pm$ SD.

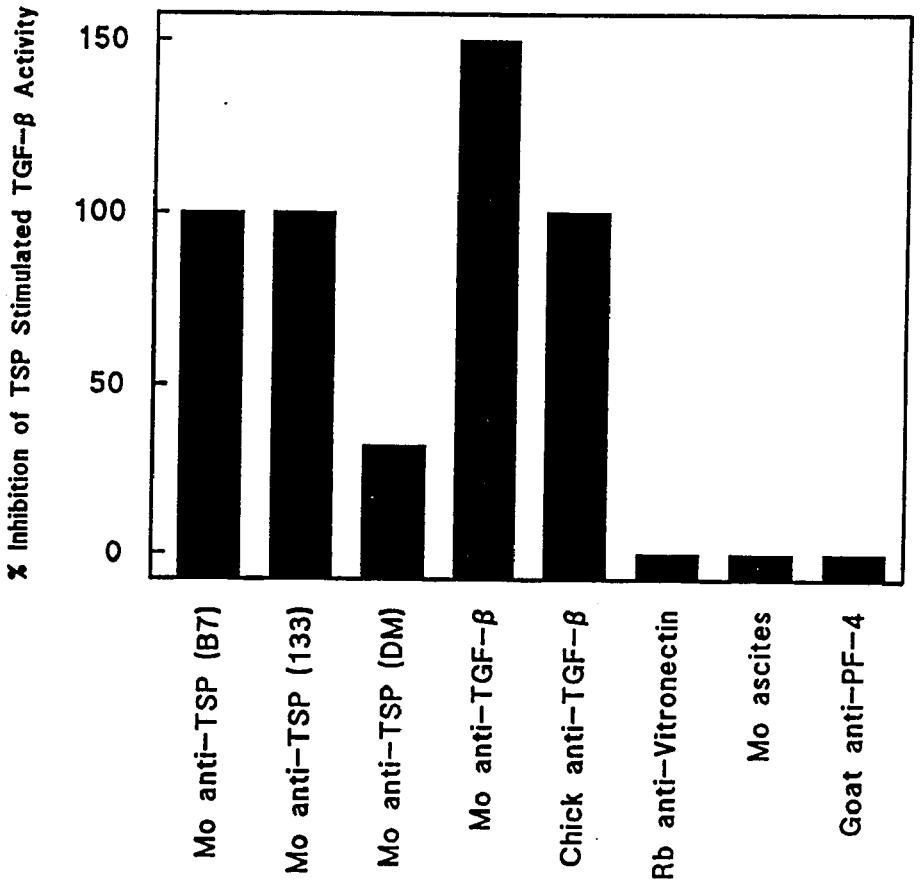

Figure 7. Percent inhibition of sTSP-stimulated TGF-ß activity is specific for antibodies to TGF-ß and sTSP. BAE cells were seeded at 1 x 10^5 cells/25 cm^2 flask in DME with 20% FBS and incubated overnight and then rinsed with serum-free DME. sTSP (1 μg), 1 μg sTSP + 1:100 mAb TSP-B7 ascites, 1 μg sTSP + 1:100 mAb 133 ascites, 1 μg sTSP + 10 μg mouse anti-TSP (DM, gift of Deane Mosher), 1 μg sTSP + 2.5 μg mouse anti-TGF-ß (Genzyme), 1 μg sTSP + 10 μg chick anti-TGF-ß (Oncomembrane), 1 μg sTSP + 1:100 control ascites, and 1 μg sTSP + 10 μg goat anti-platelet factor-4 (PF-4) were added in 2.5 ml DME with 0.2% FBS/flask and incubated 48 h at 37°C, 5% CO_2. Antibodies alone were used as controls and had no effect. Aliquots of conditioned medium (0.2 ml/sample) were tested in NRK colony forming soft agar assays to determine TGF-ß activity. Variations among triplicates was < 10%.

tested against a panel of serine protease inhibitors (Table II). sTSP (0.4 μg/ml) was incubated with ϵ-aminocaproic acid (EACA, 0.3 mM), aprotinin (6 mM), and α_2-antiplasmin (0.6 μM). The concentrations of these inhibitors were chosen based on previous studies (40) and dose response assays. These inhibitors were unable to inhibit sTSP-mediated activation of TGF-ß and had no effect on rTGF-ß activity in soft agar assay. Due to evidence that TSP can interact with these serine proteases (7), sTSP was also tested for associated plasmin and thrombin activity using enzyme assays measuring generation of chromogens from specific substrates (Boehringer-Mannheim, Indianapolis, IN). No associated plasmin or thrombin activity was detected in sTSP and no plasmin activity was generated in sTSP-conditioned medium as compared to control conditioned medium (data not shown). These data show that in contrast to activation of endothelial cell-derived latent TGF-ß by bFGF or in co-culture systems, latent TGF-ß activation by sTSP does not involve serine proteases such as plasmin.

sTSP Can Activate Purified Recombinant Latent TGF-ß

In order to determine if sTSP was activating latent TGF-ß without the involvement of cell-secreted factors, sTSP was incubated with purified recombinant latent TGF-ß for 2 h, then assayed for TGF-ß activity. sTSP was able to activate recombinant latent TGF-ß at both 37°C and 4°C (Fig. 9 and Schultz-Cherry et al., manuscript in preparation). sTSP at 13 nM could activate approximately half of the acid-activatable latent TGF-ß. These results show that sTSP is able to activate latent TGF-ß directly, without the involvement of cell-secreted factors such as proteases.

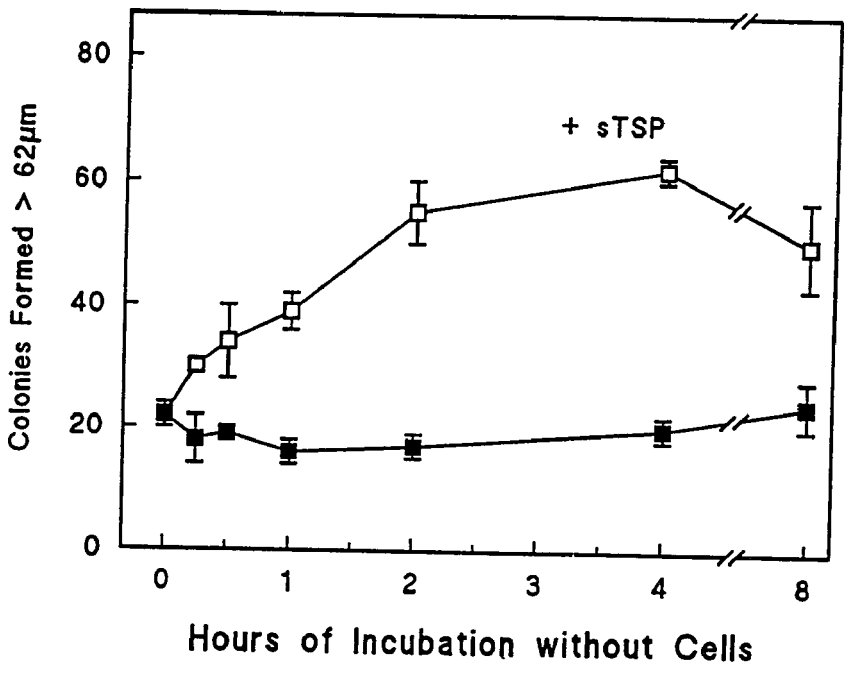

Figure 8. sTSP stimulation of TGF-ß activity in BAE conditoned medium occurs independently of binding to the cell surface. BAE cells were seeded at 1 x 10^5 cells/25 cm^2 flask and incubated overnight in DME supplemented with 0.2% FBS. The cell-conditioned medium was removed, centrifuged to remove debris, and then incubated in sterile polypropylene tubes in the presence or absence of 0.4 µg/ml sTSP for 15 min to 8 h. Aliquots of media with (open squares) or without (closed squares) sTSP were tested in the NRK soft agar assay to determine TGF-ß activity. All results are expressed as the means of triplicate determinations $\pm$ SD.

DISCUSSION

In these studies, we describe a novel mechanism for activation of latent TGF-ß secreted by endothelial cells that is dependent upon the platelet and extracellular matrix protein TSP. TSP stripped of associated TGF-ß activity (sTSP) inhibited BAE cell growth and caused morphological changes that were at least partially TGF-ß dependent. This led us to propose that these TGF-ß-dependent responses were due to activation of endogenous BAE latent TGF-ß by added sTSP. Indeed, these studies show that incubation of sTSP with BAE cells or BAE-conditioned medium generates TGF-ß activity as measured by EGF-dependent NRK colony formation in soft agar. Activation is related to the concentration of sTSP added to the cells and occurs fairly rapidly, within 15-120 min, and persists for at least 48 h. The increases in TGF-ß activity occurring with sTSP treatment are most likely due to conversion of latent to active TGF-ß and are not due to increased synthesis of TGF-ß, because activation of latent TGF-ß by sTSP can occur in BAE- conditioned media in the absence of cells.

It is interesting that levels of TGF-ß activity peak at 2-4 h and do not increase with time. It is possible that after the initial activation of latent TGF-ß by sTSP, which occurs within 15 min, the available binding sites for TGF-ß on sTSP become saturated. We have initial evidence suggesting the activation of latent TGF-ß and the binding of active TGF-ß are due to the same region of TSP (Schultz-Cherry et al.,manuscript in preparation). Once the latent TGF-ß is activated by TSP, this region could bind to the newly activated TGF-ß, potentially preventing further interactions. This may explain why the levels of active TGF-ß remain steady or are

TABLE II: sTSP-mediated stimulation of TGF-ß activity is insensitive to serine protease inhibitors.

Protease Inhibitor	Concentration	% Inhibition of sTSP-stimulated TGF-ß activity
rTGF-ß sTSP +		
EACA	0.3 mM	0
Aprotinin	7.0 μM	0
α_2-antiplasmin	0.6 μM	0

BAE cells were seeded at 1 x 10^5 cells/ 25 cm^2 flask in DMEM with 20% FBS and incubated overnight. Flasks were washed with DMEM, and 1 μg sTSP + protease inhibitor was added to each flask in DMEM with either 2.5 ml 0.2% or 20% FBS and then incubated with cells an additional 48 h. Aliquots of conditioned medium were tested in NRK colony forming soft agar assays to determine TGF-ß activity. Recombinant TGF-ß (5 ng/ml) was also incubated with the inhibitors and assayed for colony forming activity. Inhibitors alone were also added to the conditioned medium and had no effects on the assay (data not shown). Results are representative of several different experiments each performed in triplicate. Variations among triplicates were less than 10%.

even slightly decreased at incubation times greater than 4 h. sTSP may also play a role in prolonging the half-life of active TGF-ß.

Although several matrix proteins have been shown to bind active TGF-ß (29,34,35,44), activation of latent TGF-ß is not a general property of extracellular matrix molecules or of TGF-ß binding proteins because equimolar concentrations of tenascin, fibronectin, BSA, or laminin were unable to activate latent TGF-ß secreted by endothelial cells. Serum co-factors do not appear to be important in sTSP-mediated activation because activation occurs to a similar extent under high and low serum conditions. In these studies, sTSP is apparently activating latent TGF-ß secreted by BAE cells and not the latent TGF-ß present in the FBS used in the medium, because TGF-ß activity levels are not increased in FBS incubated with added sTSP as compared to control FBS. There is evidence that there are active TGF-ß-TSP complexes normally present at low levels in these fluids, because, when aliquots of FBS- or BAE-conditioned medium are applied to a monoclonal anti-TSP-CNBr Sepharose affinity column, active TGF-ß elutes with the TSP fractions (Ribeiro et al., manuscript in preparation). It is possible that the TSP present in the serum and the endogenous TSP secreted by the endothelial cells are already complexed with TGF-ß and therefore lack available binding sites for interactions with latent TGF-ß synthesized during the course of the experiments.

It is not likely the sTSP-mediated activation of latent TGF-ß is the result of sTSP acting as an adhesive protein or by causing increased internalization of TGF-ß by the NRK cells, because sTSP incubated with rTGF-ß causes no increase in colony number as compared to rTGF-ß alone and anti-sTSP antibody, which neutralizes

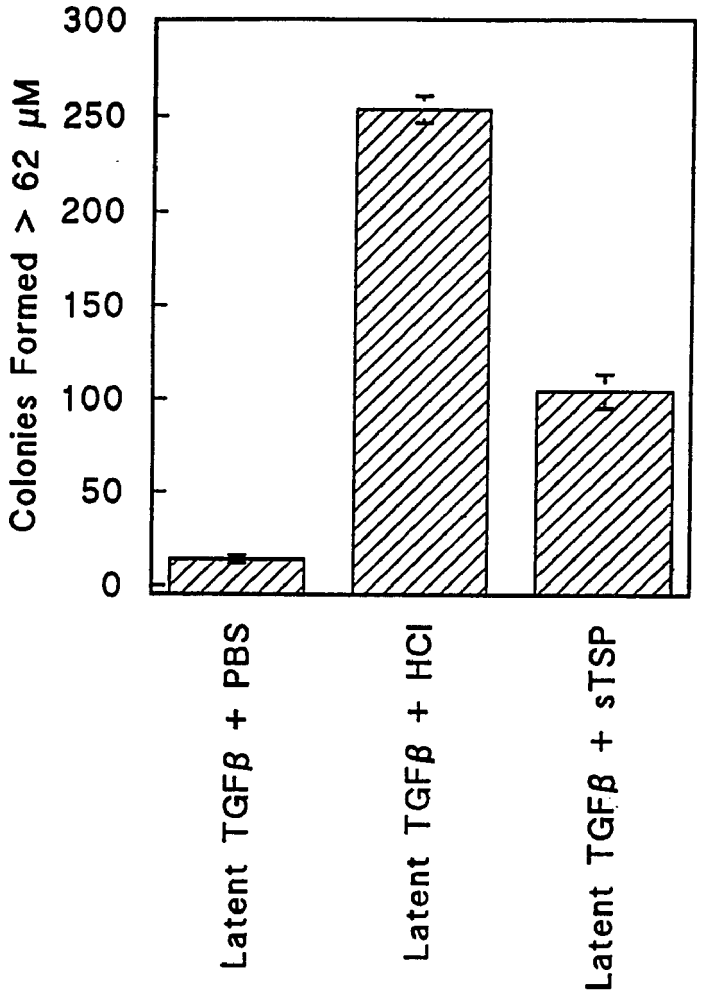

Figure 9. sTSP activates purified recombinant latent TGF-ß. sTSP (13 nM) was incubated with 2 nM purified recombinant latent TGF-ß for 2 h at 4°C in siliconizd microfuge tubes. HCl (4 mM) was used as a positive control. The final volume was brought to 0.5 ml with PBS. Samples were then tested in the NRK colony forming soft agar assay for TGF-ß activity. Results are expressed as the means of triplicate determinations $\pm$ SD.

TSP-mediated activation of latent TGF-ß in conditioned medium, has no effect on mature TGF-ß activity in the NRK assays.

The B7 and 133 monoclonal antibodies effectively inhibit TGF-ß activation by sTSP. These antibodies both recognize epitopes in the 50-70 kDa chymotryptic core of TSP (11 and see pages 102-132). This is consistent with recent data from our lab that show that the 50 kDa fragment of sTSP binds TGF-ß and, furthermore, that this 50 kDa fragment can activate TGF-ß in the conditioned media assays (pages 102-133).

The observation that most antibodies raised against native platelet TSP were unable to inhibit sTSP-mediated activation of TGF-ß, whereas a monoclonal raised against sTSP was able to completely block activation, suggests that bound TGF-ß blocks the epitope recognized by monoclonal antibody 133 in sTSP or, alternately, that there are conformational differences between native and stripped TSPs. There may be definite conformational requirements that determine TSP-TGF-ß interactions. These could involve disulfide-bond influenced tertiary structure because heat denaturation and reduction and alkylation of sTSP abolish sTSP activity in the conditioned medium assays (data not shown). These possibilities remain to be clarified.

It has been shown previously in co-culture systems (12,21) that latent TGF-ß requires interactions at the cell surface for activation. In contrast, sTSP does not require interactions with the cell surface to activate TGF-ß. Activation of latent TGF-ß in BAE-conditioned medium removed from cells occurs with kinetics and to levels similar to what is observed when sTSP is incubated in the presence of cells.

Furthermore, sTSP is able to activate latent TGF-ß secreted by cells (CHO mutants pgsA 745) that lack TSP receptors (10,32). In addition, data show that sTSP binds and activates purified recombinant latent TGF-ß (Fig. 9 and pages 63-102). These data show that activation most likely occurs through direct TSP-TGF-ß interactions.

The activation of latent TGF-ß by plasmin in endothelial cell systems is well documented (22,23,40,41). In co-cultures of endothelial cells and pericytes, activation of latent TGF-ß was related to increased production of plasmin and was blocked by plasmin/serine protease inhibitors (40,41). In contrast, activation of TGF-ß by sTSP in BAE-conditioned media was not sensitive to serine protease or specific plasmin inhibitors. Furthermore, we were unable to detect any plasmin activity associated with sTSP or any increases in plasmin activity in sTSP-treated BAE-conditioned medium as compared to control conditioned medium. When inhibitors of other classes of protease (cysteine, aspartyl) were tested, we found either no effect or that the cysteine protease inhibitors, cystatin and E-64, inhibited TSP-mediated stimulation of TGF-ß. However, cystatin was determined to be cytotoxic to the endothelial cells and E-64 was shown to interfere with the binding of TGF-ß to sTSP. Furthermore, E-64 had no inhibitory effect on sTSP-mediated stimulation of BAE-conditioned medium removed from the cells. In addition, calpastatin, a specific inhibitor of the cysteine protease calpain, did not affect sTSP-stimulation of TGF-ß activity in BAE-conditioned medium. We also observed that sTSP was able to activate purified recombinant TGF-ß with similar kinetics when tested at both 4°C and 37°C (Figure 9 and pages 63-102). Together these data strongly suggest that sTSP activation of latent TGF-ß occurs independently of proteolytic activity.

sTSP-mediated activation of TGF-ß is not restricted to endothelial cells since incubation of sTSP with Mv1Lu and CHO cells (wildtype and glycosaminoglycan-deficient mutants) results in increased TGF-ß activity (data not shown). Thus, activation of latent TGF-ß by sTSP may be a general mechanism for paracrine control of TGF-ß activity by cells that synthesize TGF-ß in the latent form. It will be important to examine the processing of the TGF-ß once it is activated by sTSP. It is not known whether the pro-region of the latent molecule remains associated with the sTSP.

Activation of TGF-ß by sTSP is efficient because levels of colony formation in sTSP-treated conditioned medium were 80% of the total alkaline-activatable TGF-ß present in control BAE-conditioned medium (data not shown). The levels of TGF-ß activity generated by sTSP-treatment of BAE cells are physiologically significant ($\sim$250 pg TGF-ß activity per $\sim 8 \times 10^5$ cells over 48 h). These two observations strongly suggest that TSP interactions with latent TGF-ß may play a substantive role in activation of TGF-ß in vivo.

These results show that TSP is a potent modulator of TGF-ß activity, acting through novel mechanisms. In addition to activating TGF-ß, TSP may serve as a carrier molecule to prolong the otherwise extremely short half-life of active TGF-ß in body fluids (43). TSP may also serve to locally deposit active TGF-ß at appropriate sites in tissues. Because TSP is released in large quantities from stimulated platelets and its synthesis is rapidly induced by growth factors, it is reasonable to suggest that TSP may enhance the ability of TGF-ß to stimulate wound healing. Alternately,

TSP-TGF-ß interactions may serve to facilitate the excessive fibrosis stimulated by TGF-ß in some pathological states. These possibilities remain to be explored.

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ABBREVIATIONS

BAE: Bovine aortic endothelial cells; CM: conditioned medium; DMEM: Dulbecco's modified Eagle's medium; EACA: e-aminocaproic acid; EGF: Epidermal growth factor; lTGF-ß: latent TGF-ß; LAP: latency associated peptide; Mv1Lu: Mink Lung epithelial cells; TBS-C: tris-buffered saline with 0.1 mM CaCl; sTSP: Thrombospondin stripped of associated TGF-ß activity; TSP: Thrombospondin; TGF-ß: Transforming growth factor type-beta

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THROMBOSPODNIN BINDS AND ACTIVATES THE SMALL AND LARGE FORMS OF LATENT TRANSFORMING GROWTH FACTOR-ß IN A CHEMICALLY-DEFINED SYSTEM

STACEY SCHULTZ-CHERRY*, SOLANGE RIBEIRO*, LARRY GENTRY#, AND JOANNE E. MURPHY-ULLRICH*

*Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-0019, #Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, Ohio, 43699.

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ABSTRACT

Transforming growth factor-ß (TGF-ß) is a potent growth regulatory protein normally secreted by cells in a latent form. Primary regulation of TGF-ß activity occurs through factors that control the processing of the latent to the biologically active molecule. Thrombospondin (TSP), a platelet α-granule and extracellular matrix protein, forms specific complexes with active TGF-ß in platelet releasate, and activates endogenous latent TGF-ß secreted by endothelial cells via a cell- and protease-independent mechanism. In order to better understand TSP-mediated activation of cell-secreted latent TGF-ß, we examined the consequences of interactions of the large (platelet-derived) and small (recombinant) forms of latent TGF-ß with TSP in a chemically-defined system. Data from these studies show that interactions between TSP and both forms of latent TGF-ß result in the generation of biologically active TGF-ß as assayed by the ability of NRK-49F cells to form colonies in soft agar, by the ability to compete for binding to TGF-ß receptors on endothelial cells, and by an ELISA selective for the active form of TGF-ß. Activation of latent TGF-ß by TSP stripped of associated TGF-ß activity (sTSP) is time and concentration dependent, but temperature independent. The mechanism whereby sTSP activates latent TGF-ß appears to involve the direct binding of sTSP to the latent molecule as shown by gel permeation chromatography. In addition, a polyclonal antibody specific for the amino-terminal region of the latency-associated peptide (LAP, amino acids 81 - 94) inhibits sTSP-mediated activation of latent TGF-ß in both the chemically-defined system and in endothelial cell conditioned media. These data and the observation that similar concentrations of sTSP activate latent TGF-ß in both the chemically-defined

system and in the endothelial cell system indicate that there is a common mechanism by which TSP activates the small, large, and endothelial-cell derived latent TGF-ß complexes. The ability of TSP to convert latent TGF-ß to biologically active TGF-ß suggests that TSP is a major regulatory factor in the control of TGF-ß activity.

INTRODUCTION

Transforming growth factor-ß (TGF-ß) is a family of small polypeptide growth and differentiation factors (4,16,20,36,42). Most of our knowledge about TGF-ß function comes from studies utilizing TGF-$ß_1$ (16,36,42). TGF-ß is involved in a variety of cellular functions and can have seemingly paradoxical effects on the same cell type. For example, TGF-ß inhibits growth of endothelial cells in vitro (26), while it promotes angiogenesis in vivo (35). TGF-ß plays a role in tissue morphogenesis, wound healing, angiogenesis, and inflammation (20).

TGF-ß is secreted by virtually all cell types as a biologically inactive molecule (4,20). Latent TGF-ß is composed of an amino-terminal latency-associated peptide (LAP) that remains non-covalently associated with the carboxy-terminal mature TGF-ß molecule (11,12,13). The LAP is glycosylated on asparagine residues, and phosphorylated on mannose-6-phosphate residues (7,34,41). The LAP is necessary for latency and the proper secretion of TGF-ß (13). The release of mature TGF-ß from LAP is thought to be necessary in order for TGF-ß to interact with its cellular receptors and elicit biological effects.

In addition, latent TGF-ß is present in the α-granules of platelets, and it exists in certain cell types as a "large" latent complex (9,22,30,31,45). The large latent complex contains a protein, known as the latent TGF-ß binding protein (LTBP), that

is covalently bound to the LAP. The function of the LTBP is not known, although it may be important for the proper folding of the latent molecule (24). The LTBP itself is not needed to maintain latency.

Since expression of the known TGF-ß receptors is constitutive in most cell types (20), regulation of TGF-ß activity resides primarily in the activation of the latent TGF-ß molecule. Activation of latent TGF-ß occurs in vitro by extremes of pH, heat, chaotropic agents, and deglycosylation (6,22,23,33). Activation in vivo is not well understood. In co-culture systems, plasmin activates latent TGF-ß in a cell-surface dependent manner that requires contact between different cell types (2,37,38). Latent TGF-ß can bind to the cell surface through several potential binding sites. The insulin-like growth factor II/mannose-6-phosphate cation-independent receptor on the cell surface can bind mannose-6-phosphate residues on the LAP (8,17). In addition, the LAP has been reported to bind to the smooth muscle cell surface by an unidentified receptor (39). Binding to the cell surface is followed by subsequent activation by plasmin (37,38) or by internalization and processing in acidic microenvironments (15).

The LTBP has also been proposed to be involved in latent TGF-ß activation because an antibody to LTBP blocks latent activation in the co-culture system (9). The role of LTBP may be to target the large latent complex to sites where activation by plasmin can occur (9). In addition, there are reports of autocrine TGF-ß activation occurring independently of these mechanisms, especially in certain types of tumor cells (14). Furthermore, we recently showed that thrombospondin (TSP), an extracellular matrix protein, activates latent TGF-ß secreted by bovine aortic

endothelial (BAE) cells via a novel mechanism, which occurs in the soluble phase, and does not require proteolytic activity (40).

The TSPs are a family of multifunctional glycoproteins that are present in connective tissues, the α-granules of platelets, wound fluids, and in embryonic tissues (5,10,18,25). TSP is secreted and incorporated into the extracellular matrix by a variety of cell types in vitro. TSP, like TGF-ß, has varied effects on cellular behavior. TSP inhibits the growth of endothelial cells (3,43,44) and, in contrast, enhances smooth muscle cell and dermal fibroblast growth (19,32). Unlike most other extracellular matrix proteins, such as fibronectin and vitronectin, TSP has anti-adhesive properties and mediates disassembly of focal adhesions in endothelial cells (27,29).

In previous work, we showed that TSP associates with active TGF-ß in the releasate of activated platelets (28) and that soluble TSP activates endogenous endothelial cell latent TGF-ß (40). In order to better understand the mechanisms underlying this observation, we utilized a system in which we could study the activation of latent TGF-ß by TSP without the complication of cells or cell-derived components. In the present studies, we report that TSP binds both the small and large forms of the latent complex, and that this binding interaction is sufficient to generate biologically active TGF-ß. Furthermore, we show that the LTBP does not appear to be involved in sTSP-mediated activation of latent TGF-ß. Based on these data, we propose that activation of latent TGF-ß by sTSP occurs via interactions between these two proteins, and that the formation of latent TGF-ß-TSP complexes

potentially represents a physiologically important means of activating latent TGF-ß in vivo.

MATERIALS AND METHODS

Thrombospondin Purification

TSP was obtained from fresh human platelets purchased from the Birmingham American Red Cross and was purified in the presence of 0.1 mM $CaCl_2$ according to the modified protocol described (28) using heparin-affinity and gel permeation chromatography in buffer at pH 11. Purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue or silver staining. No contaminating TGF-ß activity was found associated with stripped TSP (sTSP; free of associated TGF-ß) in NRK soft agar colony formation assays.

Cells

BAE cells were isolated from aortas obtained at a local abattoir, and were characterized by the uptake of acetylated low density lipoproteins and by staining for Factor VIII antigen. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cell-Gro, Mediatech, Herndon, VA) containing 2 mM glutamine and 4.5 g/l glucose, and supplemented with 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT), as previously described (27). Clone 49F of NRK cells (American Type Culture Collection, Rockville, MD. CRL 1570) was provided by Dr. Lynn Allen-Hoffmann, UW-Madison, or purchased from the ATCC. Stocks were cultured in DMEM containing 2 mM glutamine and 4.5 g/l glucose, and supplemented with 10% calf serum (CS; Hyclone Laboratories, Logan UT) as previously described (1). All cells tested negative for mycoplasma.

Production of Purified Recombinant Latent TGF-ß and Active TGF-ß

Recombinant latent simian TGF-$ß_1$ was expressed in Chinese Hamster Ovary cells and purified from culture supernatant as described (11; a generous gift of Dr. Daniel Twardzik and Ms. Jane Ranchalis, Bristol-Myers Squibb, Seattle, WA). The recombinant latent TGF-ß is the small form of the latent complex that lacks the latent TGF-ß binding protein.

Large latent TGF-ß was purified from human platelets by multiple chromatographic steps as described (22) and was a generous gift of Dr. Kohei Miyazono and Dr. Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden).

NRK Colony Formation in Soft Agar

TGF-ß activity was assayed by determining colony formation by NRK cells in soft agar assays as described (28). Colonies greater than 62 μm (>8-10 cells) in diameter were counted. Experiments were performed in triplicate. sTSP incubated with EGF had no colony formation above EGF control levels. TSP does not appear to increase the binding of active TGF-ß to NRK cellular receptors. sTSP incubated with recombinant active TGF-ß did not increase the number of colonies formed, as compared to TGF-ß alone (40). In addition, TSP did not increase the amount of ^{125}I-active TGF-ß bound to NRK cellular receptors as monitored by cellular cross-linking studies (data not shown).

Activation of Small and Large Latent TGF-ß by sTSP

Various concentrations of sTSP were incubated with 2 nM (200 ng/ml) recombinant small latent TGF-ß or 0.85 pM (200 pg/ml) large latent TGF-ß in a final

volume of 0.5 ml PBS in siliconized microfuge tubes for different times, at either 37°C or 4°C, as indicated in the figure legends. Optimal concentrations of large and small latent TGF-ß were chosen based on the results of dose response assays (Fig. 1). HCl (4 mM) was used as a positive control for activation. BSA (0.1%) was added to the samples and controls to decrease non-specific binding of TGF-ß to tubes. Samples were then assayed for TGF-ß activity by evaluating NRK colony formation in soft agar. At least four different preparations of recombinant small latent TGF-ß were used for these experiments, with only slight variations in activation by HCl and TSP.

ELISA for TGF-ß Activity

Recombinant latent TGF-ß (1 μg/300 μl) was incubated with sTSP (3 μg/300 μl) for 5 min at 37°C, in a total volume of 300 μl PBS in siliconized microfuge tubes. The samples were then tested for active TGF-ß using a TGF-ß1 ELISA Predicta kit (Genzyme, Cambridge, MA). The ELISA is selective for active TGF-ß and only minimally recognizes latent TGF-ß.

Radioreceptor Assay for TGF-ß

BAE cells were seeded at 3.5 x 10^4 cells/well in a 96-well plate (Falcon 3072, Becton Dickinson, Lincoln Park, NJ) in 200 μl DMEM with 20% FBS and allowed to attach overnight. The wells were washed one time with 300 μl binding buffer (3.7 g sodium bicarbonate, 10.67 g BES (N,N-bis[2-Hydroxyethyl]-2-aminoethane- sulfonic acid, Sigma Chemical, St. Louis, MO), 1 g BSA/l DMEM), and incubated for 1 h at 37° in 300 μl binding buffer. ^{125}I-TGF-ß (0.125 ng, specific activity = 3.7 x 10^5 cpm/ng) was added to the wells along with the test samples in a final

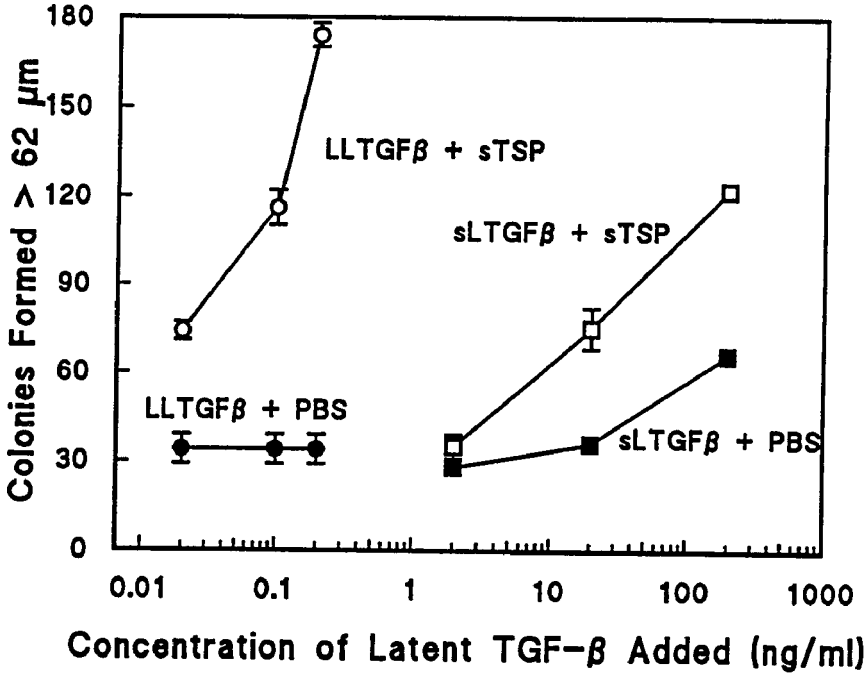


Figure 1. Activation of increasing concentrations of large and small latent TGF-ß by sTSP. Large latent TGF-ß (LLTGFß) concentrations ranging from 20 pg/ml (0.09 pM) to 200 pg/ml (0.85 pM) or small latent TGF-ß (sLTGFß) concentrations ranging from 2 ng/ml (0.02 nM) to 200 ng/ml (2 nM) were incubated with 6 μg/ml (13 nM) sTSP for 2 h at 37°C. Samples were then assayed by NRK colony forming soft agar assay for TGF-ß activity. Activation of 200 pg/ml large latent TGF-ß by HCl represented 128 ± 5 colonies. Activation of 200 ng/ml small latent TGF-ß by HCl represented 206 ± 16 colonies. Results are expressed as the means of triplicate determinations ± SD.

volume of 100 μl, and incubated for 2 h at 37°C or 4°C. Plates were then incubated for 5 min on ice, and washed twice with cold binding buffer. Cells were solubilized using 200 μl solubilizing buffer (10% glycerol, 1% Triton X-100, 1% BSA in PBS), and the amount of radioactivity bound to the cells was determined using a gamma counter. Experiments were performed in triplicate.

Chemical Crosslinking of sTSP to Recombinant Latent TGF-ß

sTSP (1 μg) was incubated with 1 μg recombinant latent TGF-ß or 10 μl metabolically labelled ^{3}H-latent TGF-ß for 2 h at 37°C. DSS (disuccinimidyl suberate; Pierce, Rockford, IL), a crosslinking agent, was added to the samples at 0.1 - 0.25 mM and incubated for 15 min at 4°C. Sample buffer containing DTT was added and the samples were heated for 5 min at 100°C. The proteins were fractionated on either 8% (^{3}H-metabolically-labeled latent TGF-ß) or 6.5% polyacrylamide gels. The radioactive gel was treated with EnHance (Amersham, Arlington Heights, IL) and then exposed to Kodak AR film (International Biotechnologies, Inc., New Haven, CT) for 7 days.

Analysis of sTSP-latent TGF-ß Complex Formation by Gel Permeation Chromatography

sTSP (0.47 μM) and small latent TGF-ß (2.7 μM) were incubated for 2 h at 37°C in a final volume of 50 μl. Incubation was carried out in siliconized microfuge tubes to minimize loss of proteins to the tube. DSS (disuccinimidyl suberate, 0.25 mM, Pierce, Rockford, IL) was then added to the samples for 15 min at 4°C, and samples were loaded onto a Hydropore 5-SEC HPLC gel permeation column (4.6 mm x 25 cm, Rainin, Emeryville, CA) pre-equilibrated in PBS + 0.2% Zwittergent 3-12 (Calbiochem Corp., San Diego, CA). The column was run at a flow rate of 0.5

ml/min (0.90 Kpsi) and 100 μl (12 second) fractions were collected. Total run time was 10 min. Fractions were pooled as indicated and tested for TGF-ß activity in the NRK colony forming assay. Controls included sTSP and latent TGF-ß incubated separately at 37°C, followed by cross-linking, subjected to HPLC, and then tested for TGF-ß activity. These experiments were repeated at least two times. The presence of detergent in the buffer was necessary to decrease protein retention by the column.

Antibodies

Rabbit polyclonal antibodies to amino acids 81-94 (1125), to the amino-terminus of LAP, and amino acids 225-236 (7251), to the carboxy-terminus of LAP, were prepared as described (11). Rabbit polyclonal antibody 39 was raised against purified LTBP as described (23). In addition, a pre-immune rabbit serum was used as a control.

Additional Materials

The following items were purchased: low molecular weight heparin, BSA fraction V, ε-aminocaproic acid, soybean trypsin inhibitor, pepstatin A, aprotinin, α2-antiplasmin (Sigma Chemical, St. Louis, MO); siliconized microfuge tubes (United Plastic Labware, St. Louis, MO); ^{125}I-TGF-ß (Dupont-NEN, Wilmington, DE); E-64, leupeptin (Calbiochem Corp. San Diego, CA); cystatin (Boehringer-Mannheim Corp., Indianapolis, IN).

RESULTS

sTSP Activates the Small Form of Latent TGF-ß

We showed previously that sTSP added to BAE cells in culture or BAE culture supernatant activates endogenous latent TGF-ß (40). In order to determine whether

the interaction of the latent complex and TSP results in generation of bioactivity, the ability of TSP to activate latent TGF-ß was further characterized in a chemically-defined system. sTSP was incubated with a recombinant form of small latent TGF-ß, and then tested for TGF-ß biological activity. Increases in TGF-ß activity are seen as early as 2 min after addition of sTSP and activity plateaus within 5 min (Fig. 2). Activity is stable for at least 2 h. Levels of colony formation in samples treated with TSP are comparable to those treated with HCl. These results show that sTSP activates the small latent TGF-ß complex in a chemically-defined system.

The kinetics of activation of small latent TGF-ß by sTSP were examined at both 37°C and 4°C. Increases in colony formation are seen as early as 5 min after addition of TSP, however, the kinetics of activation are slightly slower at 4°C as compared to 37°C, and activity does not plateau until 15 min after TSP addition (Fig. 2). However, maximal levels of colony forming activity are identical at both 37°C and 4°C. These data can be interpreted to mean that enzymatic activity (proteases) is not the primary mechanism of sTSP-mediated activation of latent TGF-ß. This interpretation is further supported by the observation that activation of small latent TGF-ß by sTSP was not diminished in the presence of protease inhibitors selective for different classes of proteases, including serine (α_2-antiplasmin, ϵ-aminocaproic acid, soybean trypsin inhibitor, leupeptin), cysteine (cystatin, E-64), and aspartic proteases (pepstatin A) (data not shown).

In addition to the NRK biological assay, we also utilized an immunological assay to evaluate activation of latent TGF-ß by TSP. This ELISA system preferentially recognizes the active form of latent TGF-ß. In these assays, we

observed that incubation of latent TGF-ß with sTSP for 5 min at 37°C increased the amount of active TGF-ß recognized by antibodies specific for the active domain. Active TGF-ß levels in the latent TGF-ß preparation increased from a basal level of 0.2 ng to 1.04 ng upon incubation with TSP (Table I). Similarly, treatment of latent TGF-ß with HCl increased the amount of immunoreactive TGF-ß by > 4 ng (Table I). These results indicate that latent TGF-ß interactions with TSP result in exposure of a previously cryptic epitope in the active portion of the TGF-ß molecule. The antibodies do not recognize TSP because samples of TSP alone had background levels of absorbance (Table I). The observed increases in absorbance are unlikely to be due to a carrier effect of TSP because incubation of latent TGF-ß with BSA, a known carrier protein, did not increase immunoreactivity above baseline levels in samples of latent TGF-ß in PBS (Table I). Furthermore, the results of the ELISA are consistent with the data from the NRK biological assay that show that protease inhibitors fail to block TSP-mediated activation of latent TGF-ß. Aprotinin (60 μg/300 μl) did not inhibit the sTSP-mediated increase in TGF-ß activity in the ELISA (Table I). In contrast, the same concentration of aprotinin decreased plasmin activation of latent TGF-ß by > 60% (data not shown).

sTSP Activates the Large Latent TGF-ß Complex

The large latent TGF-ß complex is found in platelets and in certain cell types. Because previous studies showed large latent TGF-ß to be present in endothelial cell conditioned medium (9), it was important to determine if TSP could also activate the large form of the latent complex. Because the LTBP is covalently linked to the LAP, it is possible that the large latent TGF-ß complex is conformationally distinct from

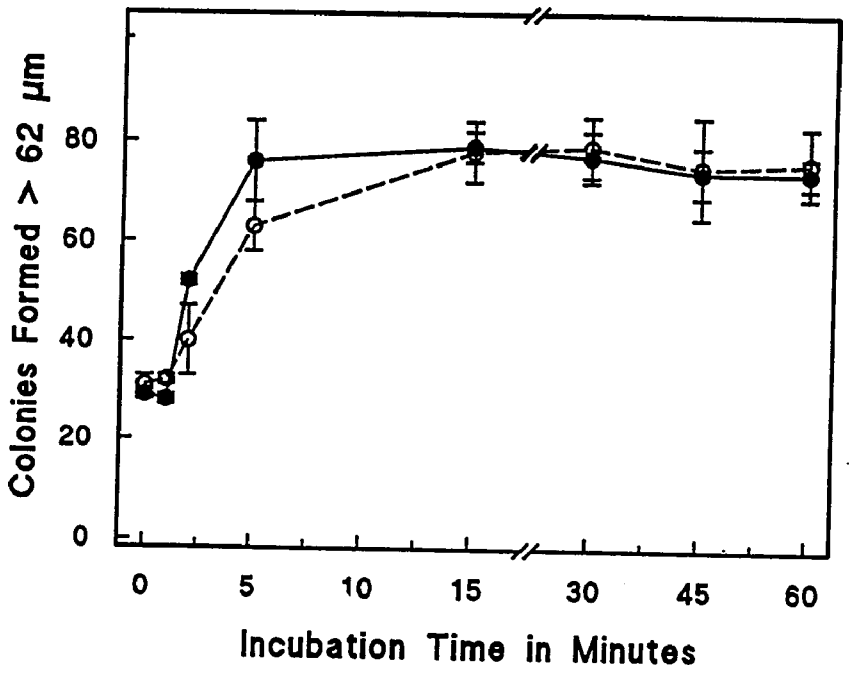


Figure 2. Activation of recombinant small latent TGF-ß by sTSP is time-dependent and temperature-independent. Recombinant small latent TGF-ß (2 nM, 200 ng/ml) was incubated with ~4 nM (2 µg/ml) sTSP for 1 min to 2 h at either 37°C (closed circles) or 4°C (open circles). Samples were then assayed for TGF-ß activity by NRK colony forming soft agar assays. Results are expressed as the means of triplicate determinations ± SD. Zero incubation time represents TGF-ß incubated in PBS for 1 hour at 37°C. Latent TGF-ß activity in PBS remained stable between 1 min and 1 h.

small latent complex and therefore may not be susceptible to activation by TSP. In order to test this, sTSP was incubated with platelet-derived large latent TGF-ß complex at 37°C, and samples were tested for TGF-ß activity. sTSP activated large latent TGF-ß at levels comparable to those achieved using the small latent complex (Fig. 3). Increases in colony forming activity were seen as early as 2 min after addition of sTSP, and activity plateaued within 5 min, and remained stable for 2 h (Fig. 3). These data show that sTSP activates the large form of latent TGF-ß.

The kinetics of activation of the large latent complex by sTSP was also examined at 4°C. In these assays, increases in colony forming activity above baseline are not seen until 15 - 30 min after the addition of sTSP (Fig. 3), and activity plateaus at 45 min and remains stable for 2 h. Although the kinetics of the response is slightly delayed at 4°C, similar levels of colony forming activity are observed at both 37°C and 4°C. In addition, sTSP-mediated activation of large latent TGF-ß was insensitive to aprotinin (data not shown). The difference in activation of large latent TGF-ß by sTSP at 37°C versus 4°C may be indicative of the dynamics of the interaction. These results indicate that sTSP activates large and small latent TGF-ß complexes at similar levels and with similar kinetics at 37°C.

Activation of Latent TGF-ß Complexes is Dependent on the Concentration of sTSP

In previous experiments, we showed that activation of latent TGF-ß secreted by endothelial cells is dependent on sTSP concentration (40). In order to compare the chemically-defined system to the endothelial cell system, increasing concentrations of sTSP were incubated with either small or large latent TGF-ß. sTSP doses ranged

TABLE I: TSP increases TGF-ß immunoreactivity

Sample	TGF-ß Immunoreactivity (ng/well)
LTGF-ß + PBS	0.2
LTGF-ß + BSA	0.2
LTGF-ß + HCl	> 4
LTGF-ß + sTSP	1.0
LTGF-ß + sTSP + aprotinin	0.96
sTSP	0

Recombinant latent TGF-ß (1 μg/300 μl) was incubated with equimolar concentrations of sTSP (3 μg/300 μl) or BSA for 5 min at 37°C in a total volume of 300 μl PBS in siliconized microfuge tubes. HCl (1 N) was used as a positive control for activation. Aprotinin was added at a concentration of 60 μg/300 μl. TGF-ß activity was monitored by an ELISA selective for active TGF-ß (Genzyme, Cambridge, MA). Levels of TGF-ß activity were determined using a TGF-ß standard curve. Results are expressed as TGF-ß activity in ng/well. Experiments were performed in triplicate (n=4).

from 200 ng/ml to 20 μg/ml (0.4 - 44 nM). A maximal response was observed with 0.6 μg/ml (1 nM) sTSP in the presence of either 2 nM small latent TGF-ß or 0.85 pM large latent TGF-ß (Fig. 4). In the endothelial cell system, 0.4 μg/ml (0.9 nM) TSP was optimal for activating cell-secreted latent TGF-ß (40). These data suggest that the mechanism of sTSP-mediated activation in the defined system is similar to the endothelial cell system.

Latent TGF-ß Activated by sTSP can Compete for Binding of ^{125}I-TGF-ß to Cells

The functional consequence of activation of TGF-ß is that the growth factor is capable of binding to its receptors, whereas the latent precursor is unable to interact with its signaling receptors (20). Therefore, we tested whether latent TGF-ß incubated with sTSP could compete for binding of active ^{125}I-TGF-ß to BAE cells. Small latent TGF-ß was pre-incubated with either 4 mM HCl or sTSP. The samples were then added to BAE cells along with ^{125}I-TGF-ß at 4°C, in order to examine competition for receptor binding in the absence of internalization. sTSP-activated latent TGF-ß inhibited >90% of the specific binding of ^{125}I-TGF-ß to BAE cells, while HCl-activation of latent TGF-ß resulted in total inhibition of specific binding (Fig. 5). As expected, recombinant latent TGF-ß incubated with 0.1% BSA in PBS was not able to compete for binding of iodinated active TGF-ß to BAE cells (Fig. 5). The decrease in binding of ^{125}I-TGF-ß to BAE cells is the apparent result of activation of the latent complex by sTSP and not due to stearic blocking of ^{125}I-TGF-ß binding because neither sTSP nor HCl had any effect on the binding of ^{125}I-TGF-ß to BAE cells. These results show that the incubation of latent TGF-ß with sTSP results in the generation of a TGF-ß species that is recognized by TGF-ß receptors.

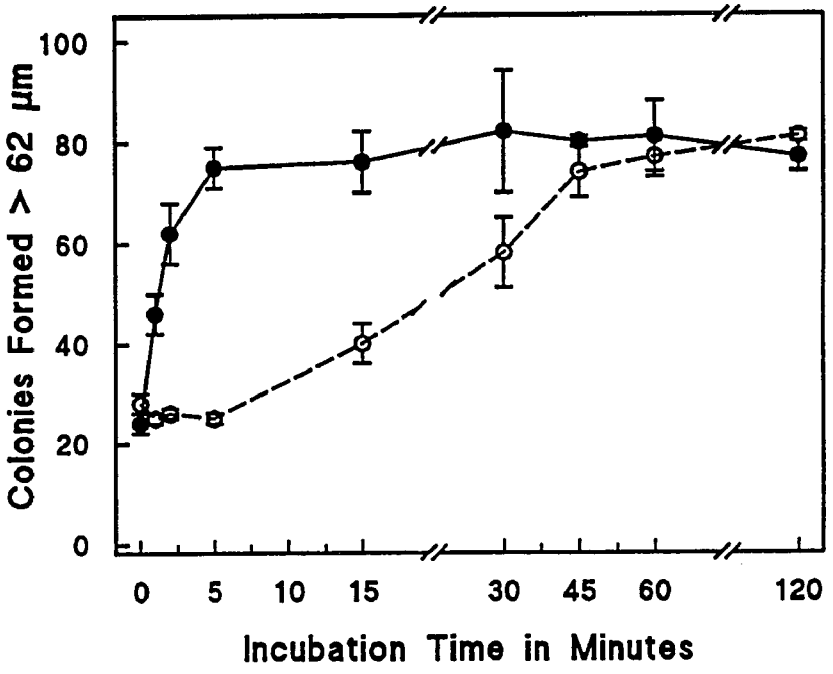


Figure 3. Activation of platelet-derived large latent TGF-ß by sTSP is time-dependent. Purified platelet-derived large latent TGF-ß (0.85 pM, 200 pg/ml) was incubated with ~4 nM (2 µg/ml) sTSP for 1 min through 2 h at either 37°C (closed circles) or 4°C (open circles). The samples were then assayed for TGF-ß activity by NRK colony forming soft agar assays. Results are expressed as the means of triplicate determinations ± SD. Zero incubation time represents large latent TGF-ß incubated in PBS for 2 h at 37°C.

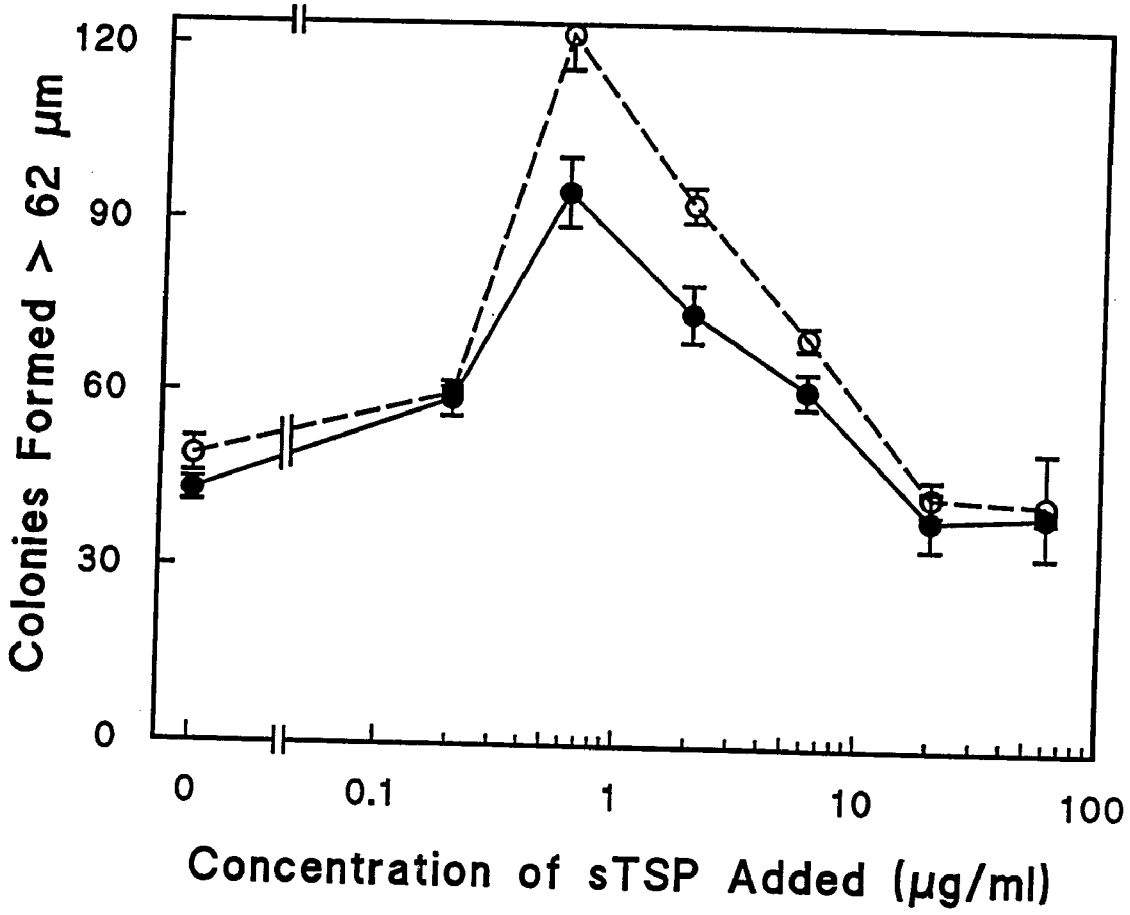


Figure 4. Activation of latent TGF-ß complexes is dependent of sTSP concentration. Recombinant small latent TGF-ß (2 nM, 200 ng/ml, closed circles) or 0.85 pM (200 pg/ml) platelet-derived large latent TGF-ß (open circles) was incubated at 37°C for 2 h with sTSP concentrations ranging from 0.2 μg/ml (0.44 nM) to 20 μg/ml (44 nM). Samples were then assayed by NRK colony forming soft agar assay for TGF-ß activity. Results are expressed as the means of triplicate determinations ± SD.

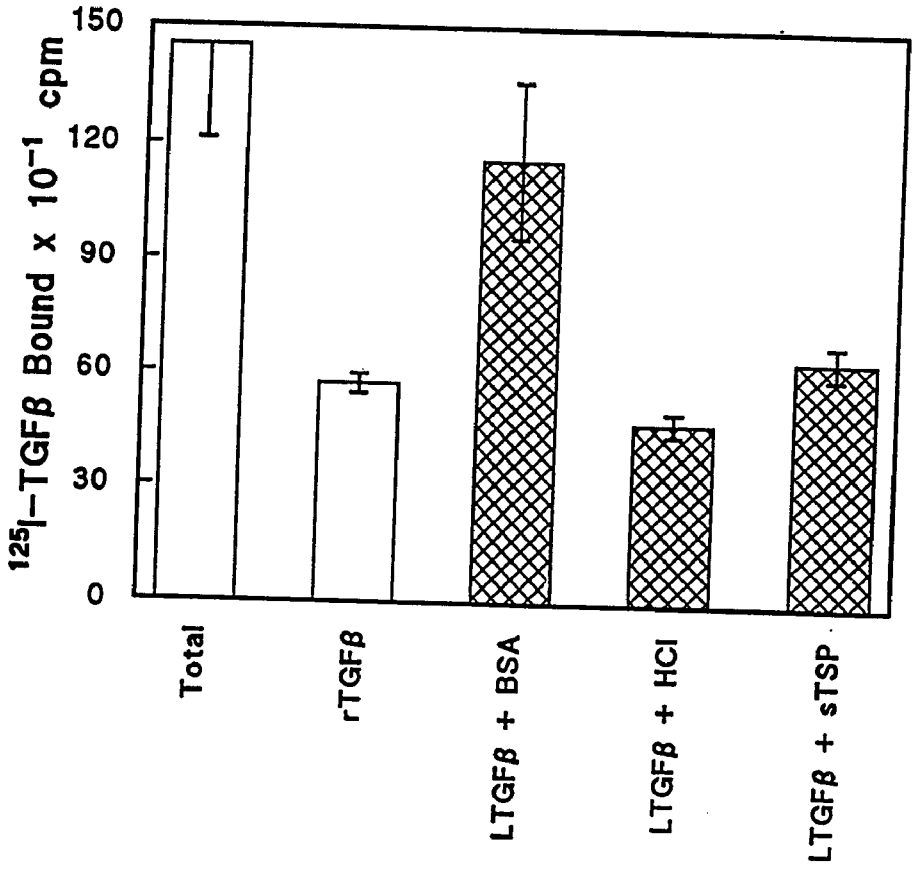


Figure 5. sTSP-activated latent TGF-ß competes for binding of ^{125}I-TGF-ß to BAE. BAE were seeded at 3 x 10^4 cells/well in a 96-well plate and incubated at 37°C for 16 h in 20% FBS/DME. Wells were washed with binding buffer, and nonspecific sites were blocked with 1% BSA/PBS for 1 h at 37°C. During this time, 1 μg of small latent TGF-ß was incubated with 1 μg sTSP in a final volume of 1 ml or an equal volume of PBS or 4 mM HCl for 2 h at 37°C. All samples contained 0.1% BSA to reduce non-specific binding to tubes. Samples were then added to the cells in the presence of 0.125 ng of ^{125}I-labeled active TGF-ß, and the mixture was incubated with the cells for 2 h at 4°C. Cells were then solubilized with 200 μl of solubilizing buffer, and bound radioactivity was determined with a counter. Binding in the presence of excess (100 ng) cold recombinant TGF-ß (rTGFß) was designated as nonspecific binding. Specific binding was calculated as the difference between total binding minus nonspecific binding. Controls included ^{125}I-TGF-ß + sTSP (1124 $\pm$ 81) and ^{125}I-TGF-ß + HCl (1156 $\pm$ 84).

sTSP Binds to Latent TGF-ß

Previous data showed that sTSP binds active TGF-ß (28). It is possible that sTSP may be activating latent TGF-ß through changes elicited as the result of direct interactions with the latent precursor. Therefore, in order to determine if sTSP binds to the latent form of TGF-ß, binding interactions were examined by chemical cross-linking assays. Small latent TGF-ß was incubated with sTSP, and the appearance of DSS cross-linked complexes was monitored by SDS-PAGE. sTSP-latent TGF-ß complexes formed in the presence of DSS migrated as high molecular mass complexes (> 200 kDa). The formation of high molecular mass complexes was accompanied by the disappearance of the free TSP (180 kDa, reduced) and latent TGF-ß (55 and 45 kDa, reduced) bands (Fig. 6 *a*). These complexes were not observed when either TSP or latent TGF-ß alone were incubated with DSS or in the absence of DSS. The bands at ~120 kDa and 200 kDa may represent aggregated or covalently-bound oligomers of misfolded recombinant latent TGF-ß. The latent TGF-ß is indeed present in these high molecular weight complexes, as seen by the appearance of high molecular weight radioactive bands of latent TGF-ß-sTSP in experiments performed with ^{3}H-metabolically labeled preparations of latent TGF-ß (Fig. 6 *b*). These data show that sTSP binds to latent TGF-ß.

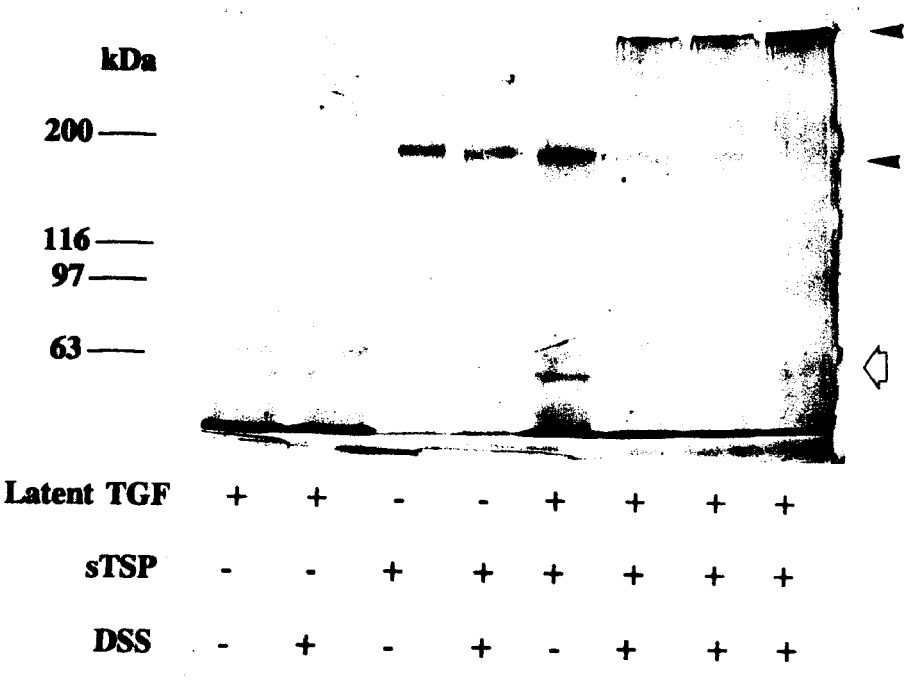
In order to determine if sTSP-latent TGF-ß complex formation results in TGF-ß biological activity, sTSP was pre-incubated with small latent TGF-ß, chemically cross-linked, and then analyzed on a high performance liquid chromatography (HPLC) gel permeation column. sTSP and latent TGF-ß alone were run as controls. sTSP eluted at 3.5 min (pool 1), while latent TGF-ß eluted at 4.9 min (pool 2). A third

peak (pool 3) was consistently found at 6.5 min. The identity of the material eluted in pool 3 has not been determined; it may represent cross-linked small peptides or degradation products. Fractions corresponding to the individual peaks were pooled and monitored for TGF-ß activity in the NRK colony forming assay. No activity was recovered from pool 2, corresponding to the latent complex, or from pool 1, corresponding to sTSP (Fig. 6 *c*). These data indicate that detergent did not activate latent TGF-ß, and they are consistent with the observation that Triton X-100 does not destroy the latent complex (21). In contrast, when small latent TGF-ß was pre-incubated with sTSP, colony forming activity was eluted at the position corresponding to pool 1 (Fig. 6 *c*). These data show that sTSP activates latent TGF-ß as the result of binding interactions. Furthermore, since little if any activity eluted in pool 3, these results suggest that it is not necessary that the complex (sTSP-LAP-TGF-ß) be dissociated for TGF-ß activity to be observed.

An Antibody to the Amino-Terminus of the LAP Inhibits Activation of Latent TGF-ß by sTSP

We show in the previous results that sTSP binds to small latent TGF-ß. The latent TGF-ß complex consists of the LAP, mature TGF-ß, and, in the large form, the LTBP. In order to attempt to determine how sTSP activates latent TGF-ß, we examined which portions of the latent complex might be involved in activating interactions with sTSP. Therefore, we tested the ability of polyclonal antibodies specific for the different components of the latent complex to interfere with activation of latent TGF-ß by sTSP. Only antibody 1125, specific for the amino-terminus of the LAP (amino acids 81 - 94), inhibited sTSP-mediated activation of small and large

Figure 6. sTSP binds to the large and small forms of latent TGF-ß. A, Recombinant latent TGF-ß (1 μg, lanes 5,6,7, and 8) was incubated with 1 μg sTSP (lanes 5-8) for 2 hours at 37°C. DSS, 0.1 mM (lane 6), 0.15 mM (lane 7), or 0.25 mM (lane 8) was added to samples for 15 min at 4°C, SDS-PAGE denaturant was added, and samples were heated and run on a 6.5% SDS-PAGE slab gel under reducing conditions and stained with Coomassie Blue. Controls include latent TGF-ß (lane 1), latent TGF-ß + 0.25 mM DSS (lane 2), sTSP (lane 3), and sTSP + 0.25 mM DSS (lane 4). B, metabolically ^{3}H-labeled recombinant latent TGF-ß (10 μl, 4 x 10^4 cpm) was incubated with 1 μg of sTSP for 2 hours at 37°C (lanes 2,3, and 4). The samples were then chemically cross-linked with 0.15 mM (lane 3) or 0.25 mM DSS (lane 4) for 15 min at 4°C as in Figure 6a. ^{3}H-labeled latent TGF-ß is shown in lane 1. Samples were run on an 8% SDS-PAGE slab gel under reducing conditions. Autoradiography of the gel was examined after 7 days. C, TGF-ß activity profile of eluate from a Hydropore 5-SEC HPLC gel permeation column. Top panel, sTSP (0.47 μM); middle panel, small latent TGF-ß (2.7 μM); bottom panel, sTSP (0.47 μM) incubated with small latent TGF-ß (2.7 μM) for 2 h at 37°C, in a final volume of 50 μl. Samples were incubated with 0.25 mM DSS for 15 minutes and then chromatographed as described. Fractions were pooled and tested for TGF-ß activity. Results are expressed as the means of triplicate determinations $\pm$ SD.


kDa
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116
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sTSP - - + + + + + +
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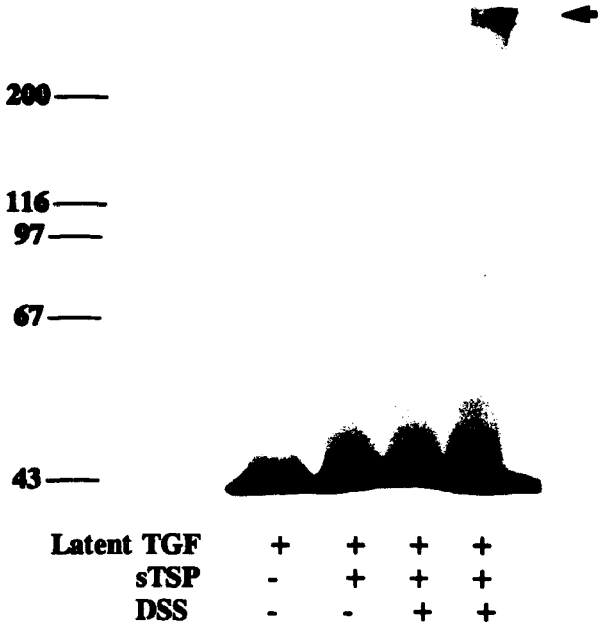
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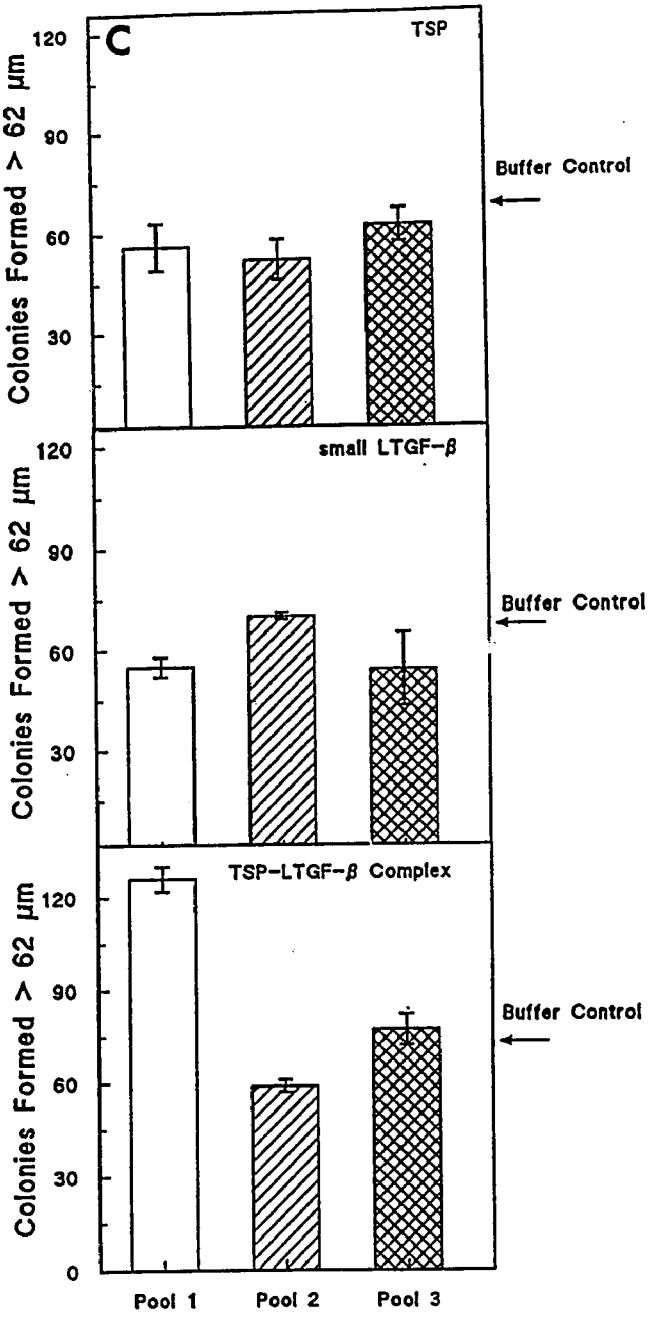
C
TSP
small LTGF-β
TSP-LTGF-β Complex
Colonies Formed > 62 µm
120
90
60
30
0
Buffer Control
Pool 1
Pool 2
Pool 3

latent TGF-ß (Figs. 7 *a* and 7 *c*). No inhibition of sTSP-mediated activity was observed using antibodies specific for the C-terminus of the LAP (antibody 7251), the LTBP (antibody 39) or a pre-immune rabbit sera control (Figs. 7 *a* and 7 *b*). Antibody 1125 does not appear to be interfering with colony formation because it had no effect on the ability of rTGF-ß to form colonies in the NRK assay. However, antibody 1125 may react with or near an epitope on the LAP that is critical for maintenance of latency because incubation of latent TGF-ß with antibody 1125 resulted in activation to levels comparable to those achieved with TSP-treatment (data not shown).

In order to examine if sTSP binds to a similar component in the endothelial cell secreted latent TGF-ß, these antibodies were tested in the BAE-conditioned medium assays. As observed in the chemically defined system, only antibody 1125 interferes with the activation of cell-secreted latent TGF-ß by sTSP (Figure 7c). Antibody 39, specific for the LTBP, had no affect on sTSP-mediated activation of latent TGF-ß. In addition, exogenously added purified LTBP did not effect the activation of cell secreted latent TGF-ß by sTSP (data not shown). These data suggest that the amino-terminus of the LAP contains or is proximal to the site(s) on the latent molecule involved in sTSP-mediated activation.

DISCUSSION

In earlier studies, we reported that incubation of sTSP with either BAE cells or their conditioned medium resulted in the activation of endogenous latent TGF-ß secreted by BAE cells (40). The sTSP-mediated activation of endothelial cell latent TGF-ß occurs in the soluble phase and is apparently independent of protease activity.

These data suggested that activation of latent TGF-ß occurred as a result of interactions with sTSP. We now report that in biological and immunological assays, both the small (recombinant) and large (platelet-derived) forms of latent TGF-ß can be activated by this novel mechanism that is dependent upon the binding of the platelet and extracellular matrix protein, sTSP.

The precise mechanism of latent TGF-ß activation by sTSP is unknown at this time. However, it appears that there is a mechanism common to both the chemically-defined system and the endothelial cell systems. This is indicated by the similar concentration of sTSP needed to activate small, large, and endothelial cell-secreted latent TGF-ß and by the inhibition of activation by antibody 1125 to the N-terminal portion of the LAP, in both the chemically-defined and endothelial cell systems. As in the endothelial cell system, activation of the small and large latent complexes does not obviously involve protease activity, as indicated by the similar kinetics at 37°C and 4°C and by the insensitivity to protease inhibitors. These data indicate that activation involves the binding of sTSP to the latent complex.

Our data show that the latent complex binds to soluble sTSP. The use of gel permeation HPLC showed that cross-linked sTSP-latent TGF-ß forms a high molecular mass complex (larger than 10^6 kDa), explaining the inability of cross-linked complexes to be resolved by SDS-PAGE. Given the tendency for TSP to oligomerize, this is not unexpected, but no meaningful conclusions about the molecular weight of sTSP-latent TGF-ß complexes can be inferred from these data. The appearance of TGF-ß activity in this high molecular weight pool shows that binding of the latent TGF-ß complex to sTSP results in TGF-ß biological activity.

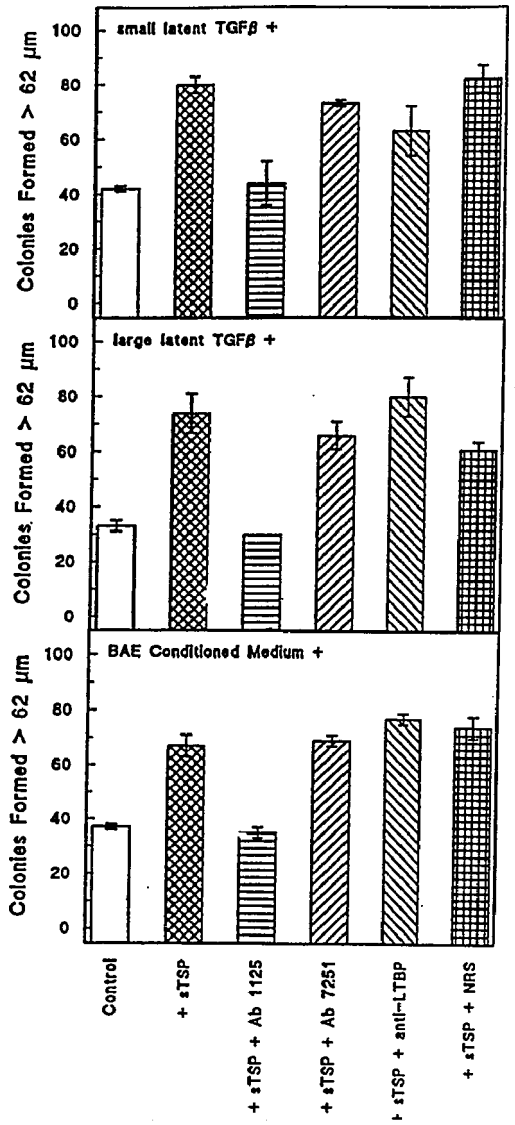

Figure 7. An antibody specific for the LAP inhibits activation by sTSP. A, Recombinant small latent TGF-ß (2 nM) or B, 0.85 pM large latent TGF-ß was incubated with either 13 nM (6 µg/ml) sTSP, 6 µg/ml sTSP + 1:1000 rabbit anti-sera 1125 (LAP residues 81-94), 6 µg/ml sTSP + 1:1000 rabbit anti-sera 7251 (LAP residues 225-236), 6 µg/ml sTSP + 1:100 rabbit anti-sera antibody 39 (LTBP), or 6 µg/ml sTSP + 1:100 preimmune rabbit sera (NRS). Samples were incubated for 2 h at 37°C and then tested for TGF-ß activity in the NRK colony forming soft agar assay. Antibodies 7251 and 39 had no effect on TGF-ß activity in the NRK assay (data not shown). Results are expressed as the means of triplicate determinations ± SD. C, BAE cells were seeded at 1 x 10^5 cells/25 cm^2 flask and incubated for 4 h at 37°C with 0.9 nM (0.4 µg/ml) sTSP, 0.4 µg/ml sTSP + 1:1000 anti-sera 1125, 0.4 µg/ml sTSP + 1:1000 anti-sera 7251, 0.4 µg/ml sTSP + 1:100 anti-LTBP, 0.4 µg/ml sTSP + 1:100 nonimmune sera. Conditioned media represents serum-free DME incubated in the presence of the cells for 4 h at 37°C. Results are expressed as the means of triplicate determinations ± SD.

It is not clear at this time how the binding of sTSP to the latent molecule results in TGF-ß activity. One possibility is that the binding of sTSP to the latent complex may induce a conformation change exposing otherwise cryptic portions of active TGF-ß. This would enable TGF-ß to bind to its cellular receptors. Data from the ELISA are consistent with the possibility that TSP is inducing a conformational rearrangement of the latent complex, involving exposure of an otherwise cryptic epitope on the mature part of the growth factor molecule. An alternative explanation is that the binding of sTSP to the latent complex causes active TGF-ß to dissociate, as observed with plasmin. However, our observation, that the latent TGF-ß chemically cross-linked to sTSP is biologically active, suggests that TGF-ß does not have to be dissociated from TSP in order to have activity.

We are also investigating what sites in the latent complex are important for TSP binding and activation of the complex. The LTBP does not appear to be required for activation by sTSP because sTSP activates the small complex that lacks the LTBP. Furthermore, sTSP does not bind to free LTBP, as analyzed by SDS-PAGE in the presence of a cross-linking agent (data not shown), and the antibody specific for the LTBP did not inhibit sTSP-mediated activation. However, we can not exclude the possibility that the LTBP maintains the latent complex in a more activation-sensitive conformation. This would explain why ~1000-fold less of the large latent TGF-ß is required for observable colony forming activity by either sTSP or HCl as compared to the small latent complex. On the other hand, the high proportion of anomalous disulfide-linkages in the recombinant form of small latent

TGF-ß may account for the high concentrations of the small complex needed for activation, as compared to the naturally-derived large form.

Studies with antibody 1125 indicate that the amino-terminus of the LAP is somehow involved in activation of the latent complex by sTSP. It is possible that sTSP may be binding on/or near the amino-terminal portion of the LAP. Alternatively, sTSP may be binding to a site on the mature portion of the growth factor that is proximal to the N-terminus of the LAP in the folded molecule. Studies investigating the potential binding of sTSP to purified LAP should resolve this question.

From the previous data and the results presented here, there is strong evidence for the existence of a mechanism for the activation of latent TGF-ß involving thrombospondin. In a system utilizing purified proteins, we demonstrated activation of latent TGF-ß resulting from interactions with sTSP.

Control of autocrine and paracrine activation of latent TGF-ß is crucial for regulating the physiologic consequences of TGF-ß. The identification of this defined mechanism of activation presents us with new possibilities for designing therapeutic agents to either facilitate or abrogate inappropriate TGF-ß activity.

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Squibb, Seattle, WA). The authors also thank Dr. Carl-Henrik Heldin and Dr. Kohei Miyazono at the Ludwig Institute for Cancer Research, Uppsala, Sweden, for the generous gifts of purified large latent TGF-ß, purified LTBP, antibody 39 and pre-immune sera, and for critical review of this manuscript

ABBREVIATIONS

BAE: Bovine aortic endothelial cells; DMEM: Dulbecco's modified Eagle's medium; EGF: epidermal growth factor; LAP: latency-associated peptide; LTBP: latent TGF-ß binding protein; LTGF-ß: latent TGF-ß, NRK: normal rat kidney cells; TBS: tris buffered saline; TGF-ß: transforming growth factor-ß; TTBS: tris buffered saline containing Tween-20; rTGF-ß: recombinant mature TGF-ß, sTSP: TSP stripped of associated TGF-ß activity; TSP: thrombospondin

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THE TYPE 1 REPEATS OF THROMOBOSPONDIN 1 ACTIVATE LATENT TGF-ß

STACEY SCHULTZ-CHERRY[#], JACK LAWLER[*], AND JOANNE E. MURPHY-ULLRICH[#]

[#]Department of Pathology, Division of Cellular and Molecular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294; and [*]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.

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ABSTRACT

Thrombospondin (TSP) is complexed with transforming growth factor-ß (TGF-ß) in the alpha granules of stimulated platelets. TSP stripped of associated TGF-ß activity (sTSP) activates latent TGF-ß secreted by bovine aortic endothelial cells (BAE) in culture. In order to better understand the interactions of TSP with TGF-ß we investigated which region of sTSP interacts with TGF-ß. The chymotrypsin-resistant core of TSP, which contains the procollagen-like region and the properdin-like type 1 repeats, activated both latent TGF-ß secreted by BAE cells and a recombinant form of the small latent TGF-ß complex at levels similar to or better than sTSP. The core fragment bound ^{125}I-TGF-ß in solution and shifted the elution profile of ^{125}I-TGF-ß in gel permeation chromatography. Fusion constructs of the type 1, 2, and 3 repeats and the carboxy terminus of TSP1 were tested for their ability to activate latent TGF-ß. Only the type 1 construct, containing the three properdin-like repeats of TSP found in the 50 kDa fragment, activated latent TGF-ß. In addition, a polyclonal antibody against the type 1 construct inhibits activation of latent TGF-ß by intact TSP, suggesting that this region is exposed in the intact molecule.

These results show that the type 1 properdin-like repeats of TSP are responsible for activating recombinant and endothelial cell-derived latent TGF-ß and that this site is exposed in intact TSP.

INTRODUCTION

Thrombospondins (TSP) are a family of multidomain glycoproteins that are present in connective tissues, the α-granules of platelets, wound fluid, and in

embryonic tissue (6,12,21,31). TSP1 is the prototypical molecule to which the other isotypes are compared. TSP1 is secreted and incorporated into the extracellular matrix by a variety of cells in culture (6,12,21,31). A number of functions for TSP1 have been suggested. Among these functions are inhibition of endothelial cell growth (3,34,46), stimulation of smooth muscle cell growth in synergy with epidermal growth factor (EGF) (29), stimulation of fibroblast growth (37), and inhibition of angiogenesis (14,48). We have found that TSP1 activates latent TGF-ß secreted by bovine aortic endothelial cells (BAE) by a novel mechanism (40). TSP1 also activates a small form of recombinant latent and platelet-derived large latent TGF-ß in a chemically-defined assay (see pages 63-102). The molecular interaction of TSP1 with latent TGF-ß is sufficient to elicit biological activity of latent TGF-ß. In order to further characterize this interaction, we investigated whether a specific region of TSP1 is responsible for activation of latent TGF-ß.

TSP1 contains a heparin binding domain at the extreme amino terminus of the molecule (6,12,31). This 25 kDa region is responsible for the ability of TSP to bind to cell surface heparan sulfate proteoglycans (32), sulfatides (38), and soluble heparin (8,21,25). This heparin binding region of TSP1 and TSP2 is also responsible for the disassembly of focal contacts in endothelial cells (33,35).

Carboxy-terminal to the heparin binding domain there are two cysteine residues, Cys-252 and Cys-256, that are responsible for the interchain disulfide-bonding of trimeric TSP1 (22,45).

The interchain disulfide-bond is followed by the procollagen-like domain. This region consists of ~90 residues, including 10 cysteines, that are homologous to

a propeptide that is cleaved from the amino terminus of human collagen I (52), and may be responsible for TSP1 binding to collagen (12). A peptide from within this region inhibits angiogenesis in vitro and in vivo (48).

The properdin-like (type 1) domain contains three repeats of ~60 residues each of which contains six conserved cysteines (15). Each repeat is ~47% identical to similar repeats found in the human complement component properdin and to the malarial parasite circumsporozoite protein (15,39). A central repeating motif from within these repeats, CSVTCG, was shown to be an attachment factor for melanoma cells and endothelial cells (37,49,50). Also within the type 1 repeats is the consensus sequence WSXW. This is highly conserved within the TSP gene family (5,19), as well as within members of the cytokine receptor (4) and transforming growth factor-ß superfamilies (53). Within TSP1 WSXW serves as a second heparin binding domain (16,17), and also binds to the gelatin-binding domain of fibronectin (42). The peptide KRFKQDGGWSHWSPWS, from the second type 1 repeat, has been shown to modulate aortic endothelial cell growth (51). This region may also be responsible for the binding of GPIIb to TSP in the absence of calcium (7).

Following the type 1 repeats there are three type 2 repeats of ~60 residues, which are homologous to epidermal growth factor. Soluble molecules, such as plasminogen and fibrinogen, may bind to TSP1 via this region (10,28,41). The eight type 3 repeats form the primary calcium binding domain of TSP1 and are responsible for the structural changes in TSP1 upon binding to calcium (11). Calcium binding regulates the tertiary conformation of TSP (11,23,24). An arginine-glycine-aspartic

acid (RGD) site is found within this region (26), as is the hemagglutinating activity of TSP1 (18).

The extreme carboxy terminal of TSP1 is a globular region containing a free sulfhydryl, which is shared with the cysteine residues in the type 3 repeats (46). Monoclonal antibodies to the extreme carboxy terminal end of TSP1 block the binding of GPIIb to TSP1 in the presence of calcium (7).

We showed previously that TSP1 interacts with the latent and the active forms of TGF-ß (34,40, pages 63-102). TGF-ß is secreted from most cells in a biologically latent form. In its active form TGF-ß is involved in a number of physiologic, as well as pathologic, processes including wound healing and angiogenesis. In order to better understand the interaction between TSP1 and TGF-ß, we have localized the TGF-ß-binding site of TSP1. We found that the chymotrypsin-resistant core binds and activates BAE cell TGF-ß, that a fusion protein containing the type 1 repeats within the fragment binds and activates TGF-ß, and that antibodies to the type 1 repeats inhibit activation by both the fusion protein and TSP1.

MATERIALS AND METHODS

Purification of Thrombospondin

Thrombospondin (TSP) was purified as described (34), except that the gel filtration column was equilibrated with TBS-C pH 11, in order to dissociate TGF-ß activity from TSP (40). Purity was assessed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using Coomassie blue or silver staining (20). No contaminating TGF-ß activity was found associated with sTSP in normal rat kidney (NRK) soft agar colony formation assays.

Cells

BAE cells were isolated from aortas obtained at a local abattoir and were characterized by the uptake of acetylated low density lipoproteins and staining for Factor VIII antigen. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Cell-Gro, Mediatech, Herndon, VA) supplemented with 4.5 g/l glucose, 2 mM glutamine, and 20% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT) as previously described (17). NRK cells, clone 49F (American Type Culture Collection, Rockville, MD, CRL 1570), were cultured in DMEM supplemented with 4.5 g/L glucose, 2 mM glutamine, and 10% calf serum (CS; Hyclone Laboratories, Logan UT) as described (2). All cells tested negative for mycoplasma.

NRK Colony Formation in Soft Agar

TGF-ß activity was assayed by determining colony formation by NRK cells in soft agar assays as described (34). The number of colonies greater than 62 μm (>8-10 cells) in diameter was counted. Experiments were performed in triplicate.

Preparation of BAE Conditioned Media

Bovine aortic endothelial cells (BAE), passage 3-12, were seeded at 1 x 10^5 cells/ 25 cm^2 flask and incubated for 16 h at 37°C, 5% CO_2. Flasks were washed, and samples were incubated in 2.5 ml 0.2% FBS with 2 mM glutamine/ DMEM for 4 h at 37°C, 5% CO_2. Conditioned medium was collected, centrifuged at 1500 rpm for 5 min to remove cellular debris, and stored for no longer than 3 days at 4°C in polypropylene tubes. Aliquots of conditioned media were tested in NRK colony forming soft agar assay for TGF-ß activity. Experiments were performed in triplicate.

Digestion and Purification of sTSP Fragments

sTSP in TBS-C pH 9 was digested with TLCK-treated α-chymotrypsin (Sigma Chemical, St. Louis, MO) at 37°C to yield previously characterized fragments (11,13). Briefly, to generate the core fragment, 1% (wt/wt) chymotrypsin was added to sTSP in 10 mM EDTA for 4 h. To generate the 70 kDa fragment, 0.1% (wt/wt) chymotrypsin was added to sTSP in 10 mM EDTA for 1 h. The 120 kDa fragment was generated with 0.1% (wt/wt) chymotrypsin was added to sTSP in 1 mM $CaCl_2$ for 15 min. Digestions were stopped with a final concentration of 0.1% PMSF (vol/vol, in anhydrous ethanol) and then applied to a heparin-Sepharose affinity column pre-equilibrated with TBS-C, pH 7.4. The 50, 70, and 120 kDa fragments eluted in the flow through. The heparin-binding fragment was eluted from the heparin column with 0.55 M NaCl. Purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using Coomassie blue or silver staining. The fragments from the stalk region (50 kDa, 70 kDa, and 120 kDa) were trimeric on non-reduced SDS-PAGE and free of intact TSP (data not shown).

Activation of Recombinant Latent TGF-ß by sTSP

Equimolar amounts of sTSP or fragments were incubated with 2 nM (200 ng/ml) purified recombinant latent TGF-ß in a final volume of 0.5 ml PBS for 2 h at 37°C in siliconized microfuge tubes. HCl (4 mM) was used as a positive control for activation of latent TGF-ß. BSA (0.1%) was added to the samples to reduce non-specific binding to the tube. Samples were then tested in NRK colony forming soft agar assay to determine TGF-ß activity. Experiments were performed in triplicate.

Fusion Protein Preparation

Fusion proteins were prepared exactly as described (27). Briefly, fusion proteins were prepared using endothelial cell cDNA. The portion of the cDNA that encodes for the type 1 repeats is between Gly (385) an Ile (522). The type 2 region is encoded by Pro (559) to Ile (669). The type 3 region is encoded by Asp (784) to Val (932) and the entire C-terminal portion of TSP starting with Asp (894). The DNA was packaged into the λ gt11 phage, infected into Escherichia *coli* strain Y1089 and lysogens were selected. The fusion proteins with ß-galactosidase were purified and used to produce antibodies as described previously (27). The cDNA for the various regions was also subcloned into the *Eco*R1 site of the pGEX1 expression vector. Fusion proteins with glutathione S-transferase were produced and purified on glutathione-agarose as described (44). The type 1 repeat fusion protein migrated as a 41 kDa band under both reducing and nonreducing conditions on SDS-PAGE (data not shown).

^{125}I-TGF-ß Binding Assays

Binding of ^{125}I-TGF-ß to immobilized sTSP was determined using a solid-phase binding assay (30). Briefly, 96-well Immulon 2 (Becton-Dickinson, Lincoln Park, NJ) plates were coated with 2 pmoles/well (0.9 μg) sTSP or BSA in 50 μl TBS-C pH 7.4 and incubated for 16 h at 37°C. Additional protein binding sites were blocked by incubation with 1% BSA in binding buffer (10 mM phosphate buffer, 0.1 M NaCl, pH 7.4) for 60 min at 37°C. ^{125}I-TGF-ß (0.125 ng, DuPont NEN, Wilmington, DE, specific activity = 3.7 x 10^5 cpm/ng) was incubated in binding buffer with the samples in the wells for 2 h at 37°C. Wells were washed three to four times with

wash buffer (binding buffer with 0.1% Triton-X 100), and bound radioactivity was counted in the isolated wells by gamma counter. Experiments were performed in triplicate.

^{125}I-TGF-ß Binding to sTSP or Fragments in Solution

Equimolar concentrations of sTSP (0.3 μM, 54 μg/ml) or sTSP fragments were incubated with 2.1 ng/ml (623,700 cpm) ^{125}I-TGF-ß (specific activity = 6 x 10^4 cpm/ng) for 2 h at 37°C in siliconized microfuge tubes in a final volume of 1 ml. The ^{125}I-TGF-ß-sTSP complex was separated from free ^{125}I-TGF-ß by gel filtration chromatography using a A0.5M column (bed volume = 99 ml), pre-equilibrated with TBS-C, pH 7.4. Complex formation was assessed by determining the elution position of the ^{125}I-TGF-ß using a Packard Cobra II gamma counter.

Additional Materials

The following reagents were purchased: α-chymotrypsin TLCK treated, PMSF (Sigma Chemical, St. Louis, MO). Recombinant TGF-ß and purified recombinant latent TGF-ß were kind gifts of Dr. Daniel Twardzik (Bristol Myers-Squibb, Seattle, WA).

RESULTS

A Domain within the Stalk Region of sTSP Competes for Binding of ^{125}I-TGF-ß to sTSP

We showed previously that TSP binds both active TGF-ß and a recombinant form of the small latent TGF-ß complex (34, pages 63-102). The molecular interactions between TSP and latent TGF-ß result in activation of the growth factor (40, pages 63-102). In order to determine which region of TSP1 is responsible for the binding to active TGF-ß, a solid-phase binding assay was performed using

purified TSP1 fragments and intact TSP1. The ability of 10-fold molar excess TSP1, 70, 50, or 25 kDa fragments to compete for ^{125}I-TGF-ß binding to immobilized TSP1 was examined. Soluble sTSP inhibited ^{125}I-TGF-ß binding to immobilized sTSP by 75% (Fig. 1). All of the fragments from the stalk region of TSP1 similarly competed for binding of TGF-ß to immobilized TSP1. However, the 25 kDa fragment from the amino-terminal did not compete for binding of TGF-ß to TSP1. This suggests that active TGF-ß binds to the stalk region of TSP1. The fact that the fragments from the stalk-region compete nearly equally suggests that the binding site is common to all of the fragments. Since the 50 kDa fragment was the minimum size fragment that competed for binding, we performed subsequent assays with the 50 kDa fragment.

The 50 kDa Fragment of sTSP Binds to TGF-ß in Solution

Under physiological conditions the interaction of TSP1 with latent/active TGF-ß most likely occurs in the soluble phase. The conformation of soluble TSP1 or fragments may differ from that of the immobilized proteins, thus the binding to TGF-ß may differ. We showed previously that TSP1 complexes with TGF-ß in solution and that complex formation can be monitored by a shift in the elution profile of ^{125}I-TGF-ß during gel permeation chromatography (34). Therefore, in order to determine if a specific domain within the stalk region preferentially bound TGF-ß in solution we performed gel permeation chromatography. We compared the abilities of sTSP or fragments of TSP1 to complex with ^{125}I-TGF-ß. ^{125}I-TGF-ß was incubated with sTSP or its proteolytic fragments for 2 h at 37°C and then applied to an A 0.5M

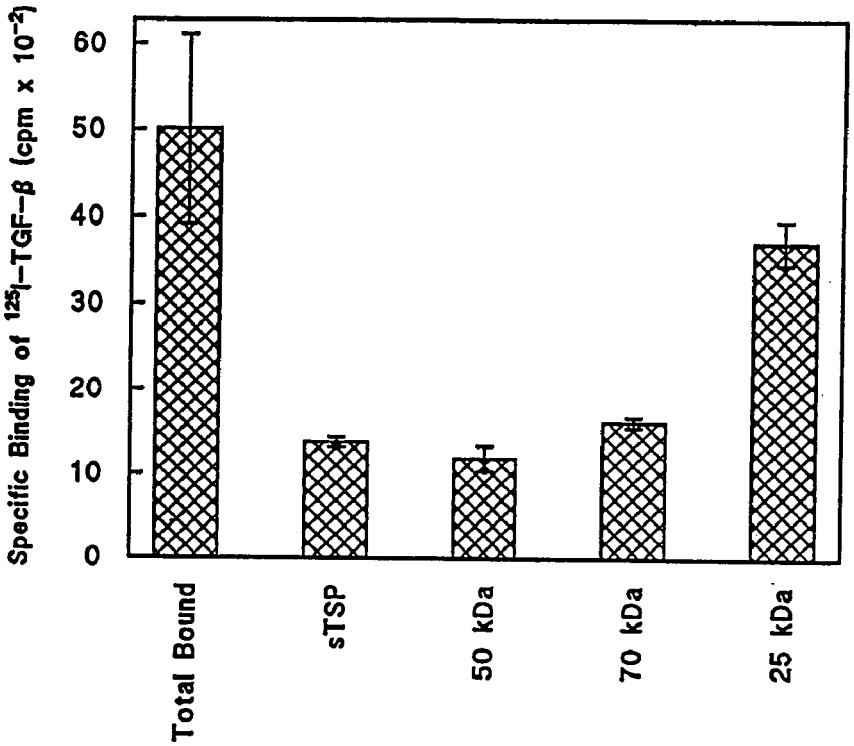


Figure 1. The 50-kDa fragment competes for binding of ^{125}I-TGF-ß to immobilized sTSP. sTSP (2 pmoles, 0.9 μg/well) were incubated in a total volume of 50 μl TBS-C, pH 7.4, in a 96-well plate overnight at 37°C. ^{125}I-TGF-ß (0.125 ng) was incubated in binding buffer with the samples for 2 h at 37°C. Samples include: total binding (100 μl binding buffer); sTSP (9 μg/100 μl); fragments 50, 70, and 25 kDa, equimolar to TSP. Wells were washed three to four times and counted in a counter to determine total binding. Results are expressed as the number of specifically bound counts. This was determined by subtracting the number of counts bound to BSA (657 $\pm$ 71) from the samples. Results are expressed as the means of triplicate determinations $\pm$ SD.

gel filtration column. The elution profile was obtained by counting the total radioactivity present in each sample.

When equimolar amounts of TSP1, 50 kDa or 70 kDa fragments were pre-incubated with ^{125}I-TGF-ß, a shift in the elution profile of ^{125}I-TGF-ß from K_{av} 0.9 to 0.05 was observed (Table I). The 50 kDa fragment was the most efficient at binding and shifting ^{125}I-TGF-ß (74% of counts shifted). In contrast, BSA and the 25 kDa heparin-binding domain had little to no binding activity. Further indication of specificity is shown by the percent of total applied counts recovered. When ^{125}I-TGF-ß was preincubated with BSA or the 25 kDa fragment, only 9-10% of the total counts added to the column were recovered (Table I) as compared to a 16% recovery when incubated with TSP1. Furthermore, the 50 kDa fragment resulted in recovery of 25% of the applied counts. These results show that the 50 kDa binds ^{125}I-TGF-ß in the soluble phase more efficiently than sTSP or the 70 kDa fragment. These data are supported by the ability of the 50 kDa fragment and TSP1 to shift the mobility of ^{125}I-TGF-ß when assayed by SDS-PAGE after cross-linking with DSS (data not shown).

The 50 kDa Fragment of sTSP Activates Latent TGF-ß Secreted by Bovine Endothelial Cells

Because these data show that the 50 kDa fragment is involved in binding active TGF-ß, we determined whether the 50 kDa region of sTSP activates latent TGF-ß secreted by endothelial cells. sTSP or equimolar amounts of the 50 kDa, 70 kDa, 120 kDa, and 25 kDa fragments were added to BAE cells for 4 h and aliquots of conditioned media were tested for TGF-ß activity in the NRK colony forming soft agar assay. sTSP increased NRK colony forming activity in the soft agar assay

two-fold as compared to conditioned medium alone (Fig. 2 *a*). The 70 kDa fragment stimulated colony formation at levels similar to TSP. The heparin binding domain of sTSP (25 kDa) and the 120 kDa fragment had little to no activity. The 50 kDa fragment at an equimolar concentration, was the only fragment able to increase TGF-ß activity as compared to intact sTSP.

sTSP can activate a recombinant form of the small latent complex through molecular interactions. In order to determine if the 50 kDa fragment is activating latent TGF-ß via the same mechanism, we tested the ability of the 50 kDa fragment to activate a recombinant form of the small latent TGF-ß complex in a chemically-defined system. Latent TGF-ß was incubated with equimolar amounts of sTSP or the 50 kDa and 25 kDa fragments. The concentration of TSP or the fragments ranged from 0.13 nM - 130 nM (0.06 μg/ml - 60 μg/ml) based on trimeric TSP. At an equimolar concentration, the 50 kDa fragment activates greater amounts of latent TGF-ß as compared to intact TSP (Fig. 2 *b*). The 25 kDa fragment has no activity. These results suggest that the 50 kDa fragment of sTSP is responsible for activation of both latent TGF-ß secreted by BAE cells and a recombinant form of latent TGF-ß. The chemically-defined system also shows that activation of latent TGF-ß by the 50 kDa fragment is due to molecular interactions between the fragment and latent TGF-ß and not due to non-specific interactions of the 50 kDa fragment with other components in the BAE conditioned media.

TABLE I: The 50 kDa region of sTSP binds to ^{125}I-TGF-ß in solution

Sample	Percent of total sample recovered	Percent of total recovered at K_{av} = 0.05	Percent of total recovered at K_{av} = 0.9
sTSP	16	56	26
50 kDa	25	74	18
70 kDa	13	47	34
25 kDa	10	33	35
BSA	9	26	43

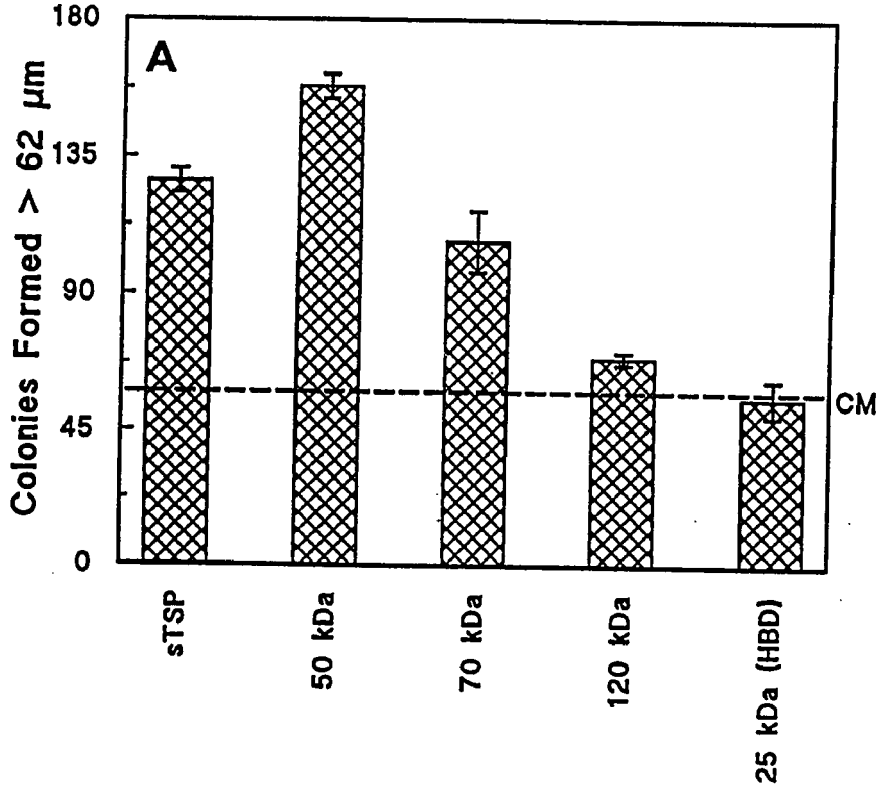
Intact sTSP or equimolar (0.3 μM) amounts of the 50 kDa, 70 kDa, 25 kDa, or BSA were incubated with ^{125}I-TGF-ß (specific activity = 6 x 10^4 cpm/ng) for 2 h at 37°C in siliconized microfuge tubes. The complex was applied to an A0.5M gel filtration column (total volume = 99 ml, void volume = 35 ml) pre-equilibrated with TBS-C, pH 7.4 and 1.35 ml samples were collected. Total radioactivity eluted was determined by summing the radioactivity present in all samples. Percentages of total counts eluted were calculated from the cpm eluted in the eluted peaks divided by the total number of counts recovered x 100. This figure is representative of two different experiments and the range is < 5%.

The Type 1 Repeats are Responsible for Activation of Endothelial Cell Secreted Latent TGF-ß

The 50 kDa chymotryptic fragment of TSP1 is composed of the intermolecular disulfide bond, the procollagen-like domain, the three properdin-like TSP type 1 repeats, and possibly a portion of the EGF-like type 2 repeats. In order to determine which domain within the 50 kDa fragment of TSP1 activates endothelial cell-secreted latent TGF-ß and recombinant latent TGF-ß, fusion constructs containing the different repeats were tested. Fusion constructs containing amino acids 385-522 (type 1 repeats), amino acids 559-669 (type 2 repeats), amino acids 784-932 (type 3 repeats) and to the entire C-terminal of TSP1 (starting at residue 894) were produced in E. coli and tested for their ability to activate latent TGF-ß (44). Equal molar concentrations of sTSP or the fusion proteins (based on monomers of TSP) were added to BAE conditioned media and incubated in the presence or absence of cells for 4 h at 37°C. sTSP caused a two-fold increase in colony formation above basal levels in conditioned media. Only the fusion protein containing the properdin-like, type 1 repeats stimulated colony formation (Fig. 3 *a*). The level of activation was similar to that seen with sTSP. This suggests that a sequence or conformation within the type 1 repeat of TSP1 is responsible for the activation of latent TGF-ß and that activation is specific for the type 1 repeats.

These results are further supported by the ability of the properdin-like repeats to activate recombinant latent TGF-ß. Equimolar concentrations of sTSP or the fusion constructs were incubated with a recombinant form of the small latent TGF-ß

Figure 2. The 50 kDa fragment activates latent TGF-ß secreted by BAE and recombinant latent TGF-ß. A, BAE were seeded at 1 x 10^5 cells/25 cm^2 flask in 20% FBS/DME and allowed to attach overnight at 37°C, 5% CO_2. The flasks were washed and 0.9 nM sTSP (0.4 μg/ml) or equimolar amounts of the 50, 70, 120, or 25 kDa fragments were added to BAE cells in 2.5 ml of 0.2% FBS/DME and allowed to incubate for 4 h at 37°C, 5% CO_2. Conditioned medium (CM) represents 2.5 ml of 0.2% FBS/DME incubated with the cells for 4 h at 37°C. Aliquots of the samples were tested in the NRK colony forming soft agar assay for TGF-ß activity. Results are expressed as the means of triplicate determinations $\pm$ SD.
B, Recombinant latent TGF-ß (2 nM, 200 ng/ml) was incubated with equimolar amounts of TSP or the 50 or 25 kDa fragments for 2 h at 37°C. The concentrations of TSP ranged from 0.13 to 130 nM (0.06 to 60 μg/ml). Samples were tested in NRK colony forming soft agar assay for TGF-ß activity. Results are expressed as the means of triplicate determinations $\pm$ SD.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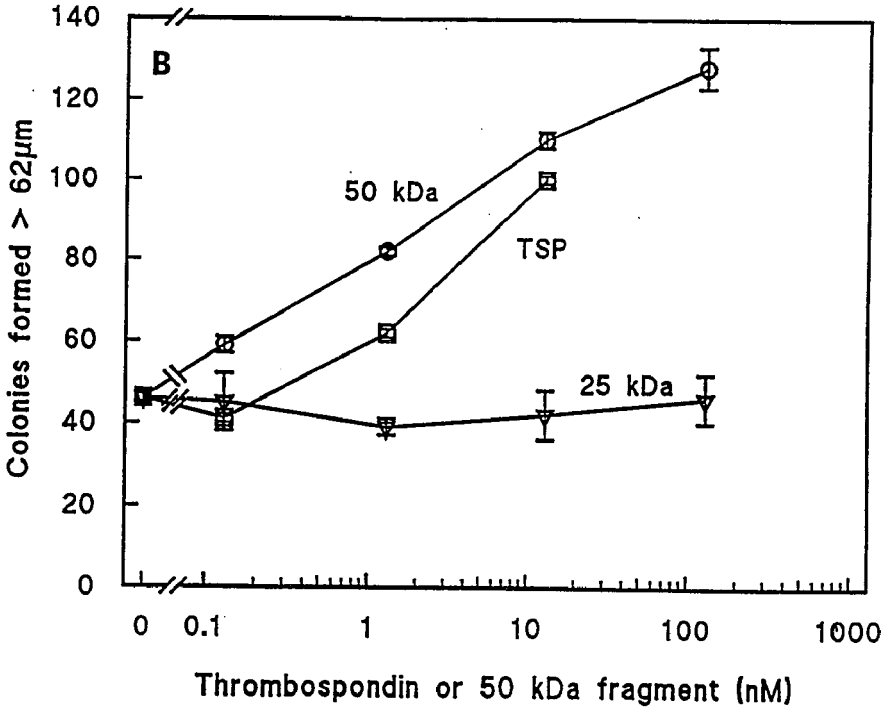
25 kDa (HBD)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140
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25 kDa
0
0.1
1
10
100
1000
Thrombospondin or 50 kDa fragment (nM)

complex and tested for TGF-ß activity in the NRK soft agar assay. sTSP incubated with latent TGF-ß showed a three-fold increase in colony forming activity in the NRK soft agar assay as compared to the latent TGF-ß alone (Fig. 3 *b*). Only the fusion protein containing the properdin-like type 1 repeats stimulated TGF-ß activity. The levels of activation were similar to that observed with intact TSP1. These results show that the site involved in the activation of TSP is located within the type 1 repeats and does not involve trans-acting sites.

An Antibody Specific for the Type 1 Fusion Construct Inhibits TSP-Mediated Activation of Latent TGF-ß

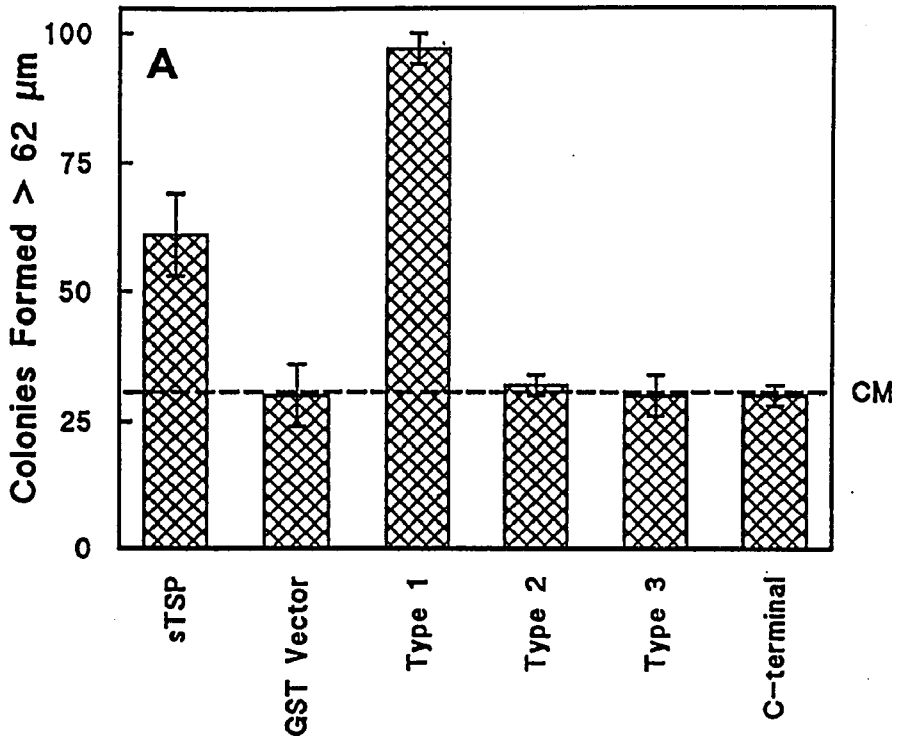
We have shown that the isolated type 1 repeats of TSP1 are responsible for the activation of latent TGF-ß by sTSP. In order to determine if the activation site is accessible for TGF-ß interactions in intact TSP1, we tested the ability of a polyclonal antibody produced against the type 1 fusion construct to block activation of TGF-ß by the TSP1 trimer. Equimolar amounts of TSP1 or the type 1 fusion construct were incubated with recombinant latent TGF-ß in the presence of polyclonal antiseras produced to the type 1, type 2, or type 3 constructs. Only the antibody specific for the type 1 construct inhibited the activation of latent TGF-ß by intact TSP1 and the type 1 construct (Fig. 4). Pre-immune rabbit sera had no effect on activation. In addition, mouse monoclonal antibody 133, which recognizes an epitope in the 50 kDa fragment of sTSP (40), inhibits activation of BAE cell-secreted latent TGF-ß by either sTSP or the type 1 construct. These data show that the type 1 repeats of TSP1 are responsible for activating latent TGF-ß and are exposed in the intact molecule.

DISCUSSION

The thrombospondins are a family of multidomain molecules that have been implicated in a number of biological functions (6,12,21,31). There is a relationship between the structure of the TSP1 domains and the proposed biological functions of each domain. We showed previously that TSP1 released from the alpha-granules of stimulated platelets is complexed to TGF-ß (34). We also showed that sTSP activates latent TGF-ß secreted by endothelial cells (40). Further experiments showed that activation of latent TGF-ß in a chemically-defined system occurs as the result of binding of TSP1 to latent TGF-ß (pages 63-102). In the present studies, we identify the region of TSP1 that mediates the binding and activation of transforming growth factor-beta (TGF-ß).

The trimeric 50 kDa chymotryptic fragment (containing the interchain disulfide bond, procollagen-like repeats, the properdin-like repeats, and a portion of the EGF-like repeats) bound TGF-ß in solution and competed for binding of ^{125}I-TGF-ß to intact sTSP in an immobilized form. Furthermore, the trimeric 50 kDa fragment activated both endothelial cell secreted latent TGF-ß and a recombinant form of the small latent TGF-ß complex. This is consistent with previous data showing that molecular interactions between latent TGF-ß and TSP1 are sufficient for activation (pages 63-102). The 50 kDa fragment was more efficient at binding and activating TGF-ß than was the entire TSP1 molecule. This may be due to the possibility that the binding site in the type 1 repeats may be less accessible or that less than all three potential binding sites are available on the intact trimer. There would be no stearic hindrance in the 50 kDa fragment from the type 3 repeat region or the globular

Figure 3. The type 1 repeats activate latent TGF-ß. A, BAE cells were seeded at 1 x 10^5 cells/25 cm^2 flask in 20% FBS/DME and allowed to attach overnight at 37°C, 5% CO_2. The flasks were washed and 0.9 nM sTSP (0.4 μg/ml), or equimolar amounts (to the subunit of sTSP) of the fusion constructs, were added to the BAE in 2.5 ml of 0.2% FBS/DME and allowed to incubate for 4 h at 37°C. Conditioned medium (CM) represents 0.2% FBS/DME incubated with the cells for 4 h at 37°C. Aliquots of the conditioned medium were tested for TGF-ß activity in the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations $\pm$ SD. B, Recombinant latent TGF-ß (2 nM, 200 ng/ml) was incubated with equimolar amounts of TSP or the fusion constructs for 2 h at 37°C. Samples were tested in NRK colony forming soft agar assay for TGF-ß activity. Results are expressed as the means of triplicate determinations $\pm$ SD.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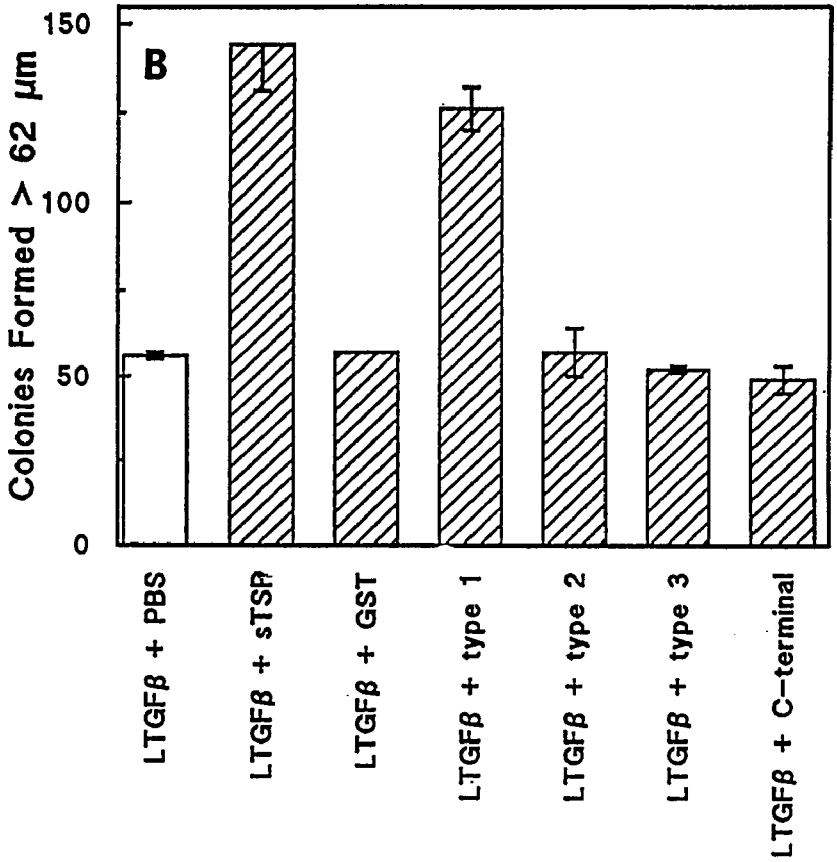
B
Colonies Formed > 62 µm
150
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LTGFβ + sTSP
LTGFβ + GST
LTGFβ + type 1
LTGFβ + type 2
LTGFβ + type 3
LTGFβ + C-terminal

carboxy terminal. These regions may partially mask the type 1 repeats in the intact molecule.

The use of bacterially-expressed fusion constructs encoding the different domains of human TSP1 aided in the determination of the activation site. Only the fusion construct corresponding to the type 1 repeats activated latent TGF-ß. This shows that the type 1 repeats are responsible for activation of latent TGF-ß and activation is not dependent on cooperativity between two domains. The ability of the type 1 fusion construct to activate latent TGF-ß suggests that monomeric forms of TSP1 are sufficient for latent TGF-ß activation. It also suggests that the potential N-linked glycosylation site within the 50 kDa fragment is not important for activation of latent TGF-ß.

The type 1 repeats are partially exposed in intact TSP1, since an antibody whose epitope is localized to the 50 kDa fragment (Mab 133) inhibits the activation of latent TGF-ß by TSP1. This antibody also blocks activation by the 50 kDa fragment and the type 1 construct (40 and data not shown). In addition, a polyclonal antibody specific to the type 1 fusion construct also inhibits activation of latent TGF-ß by sTSP. The type 1 repeat region contains two known consensus sequences, CSVTCG and WSXW. CSVTCG is an important attachment region for a number of cells, including endothelial cells and melanoma cells, to TSP (37,49,50). In addition, Tolsma et al. showed that this peptide inhibits neovascularization in vitro as well as in vivo. The WSXW motif binds with high affinity to heparin (16,17), as well as to sulfatides and fibronectin (16,17,42). This region may be responsible for the ability

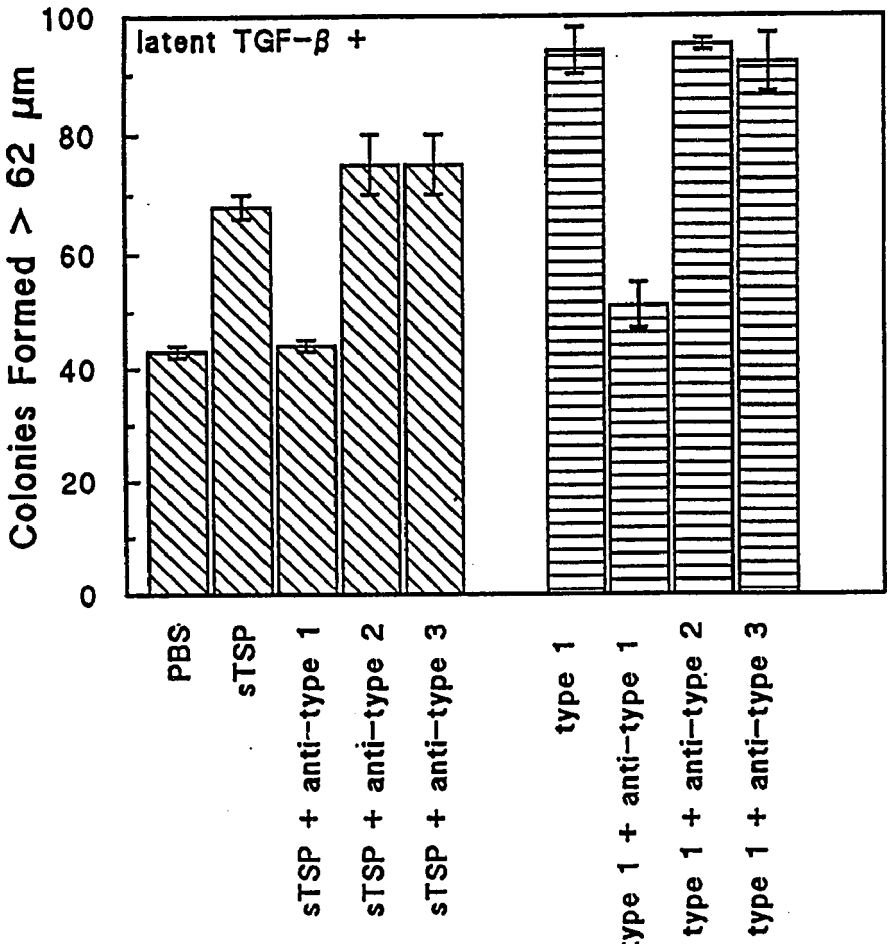

Figure 4. Antibodies to the type 1 repeats block TSP-mediated activation of latent TGF-ß. Latent TGF-ß (2 nM, 200 ng/ml) was incubated with equimolar amounts of sTSP (1.3 nM) or the type 1 fusion construct in the presence of 1:100 rabbit polyclonal antisera specific for the types 1, 2, or 3 constructs for 2 h at 37°C. Samples were tested in NRK colony forming soft agar assay for TGF-ß activity. The antibodies alone had no activity. Results are expressed as the means of triplicate determinations $\pm$ SD.

of the 70 kDa fragment of endothelial-cell derived TSP to bind heparin (9). We have obtained these and other peptides from the type 1 repeat region and are currently determining the peptide sequence responsible for the activation of TGF-ß by TSP (see pages 133-165).

TGF-ß is able to bind to a number of different proteins. Determination of the site within TSP1 where TGF-ß binds and is activated may lead us to a family of proteins that play a role in the regulation TGF-ß activity. This may aid in the formation of peptides and peptide antibodies that could be used in vivo in controlling TGF-ß activity.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

BAE: bovine aortic endothelial cells, DMEM: Dulbecco's modified Eagle's medium, sTSP: thrombospondin free of associated TGF-ß, TBS-C: tris buffered saline containing calcium, TGF-ß: transforming growth factor-beta, TSP: thrombospondin

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REGULATION OF TGF-ß ACTIVATION BY DISCRETE SEQUENCES OF THROMBOSPONDIN1

STACEY SCHULTZ-CHERRY*, CHEN HUI^, DEANE F. MOSHER^, TINA M. MISENHEIMER^, HENRY C. KRUTZSCH#, DAVID D. ROBERTS#, AND JOANNE E. MURPHY-ULLRICH*

*Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-0019; ^Departments of Biomolecular Chemistry and Medicine, University of Wisconsin, Madison, WI 53706; #Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892-1500.

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ABSTRACT

Transforming growth factor-ß (TGF-ß) is a potent growth regulatory protein secreted by virtually all cells in a latent form. A major mechanism of regulating TGF-ß activity occurs through factors that control the processing of the latent to the biologically active form of the molecule. We have shown previously that thrombospondin-1 (TSP1), a platelet α-granule and extracellular matrix protein, activates latent TGF-ß via a protease- and cell-independent mechanism and has localized the TGF-ß binding/activation region to the type 1 repeats of platelet TSP1. We now report that recombinant human TSP1, but not recombinant mouse TSP2, activates latent TGF-ß. Activation was further localized to the unique sequence (K)RFK found between the first and the second type 1 repeats of TSP1 (amino acids 412-415) by the use of synthetic peptides. A peptide with the corresponding sequence in TSP2, (T)RIR, was inactive. In addition, a hexapeptide GGWSHW, based on an adjacent sequence present in both TSP1 and TSP2, inhibited the activation of latent TGF-ß by TSP1. This peptide bound to ^{125}I-active TGF-ß and inhibited interactions of TSP1 with latent TGF-ß. Activation of latent TGF-ß by TSP1 was also inhibited by TSP2. These results indicate that activation of latent TGF-ß is mediated by two adjacent sequences present in TSP1. Peptides based on these active sites have potential therapeutic applications for modulation of TGF-ß activation.

INTRODUCTION

Transforming growth factor-ß (TGF-ß) is a multifunctional growth regulatory protein that is involved in such diverse biological activities as wound healing, growth and differentiation, and angiogenesis (4,18,24,30). TGF-ß is secreted by virtually all cells in culture as a biologically inactive molecule (4,18,24,30). An essential means

of regulating TGF-ß activity occurs through factors that control the processing of the latent to the active form of the molecule (7). Once activated, TGF-ß can bind to high affinity cellular receptors and elicit cellular responses. Thrombospondin 1 (TSP1) activates cell-secreted latent TGF-ß, as well as purified forms of small and large latent TGF-ß in a chemically-defined system via binding interactions (25-27).

The thrombospondins are a family of large multidomain glycoproteins involved in modulating cell growth, adhesion, migration, and angiogenesis (6,10,16,19). Our laboratory recently determined that the TGF-ß binding/activation site was localized to the type 1 (properdin-like) repeats of TSP1 (27). Within the type 1 repeats of TSP1 there are two well-defined consensus sequences, CSVTCG and WSXW. Both sequences are highly conserved within TSP family members containing the properdin-like type 1 repeats. CSVTCG was shown to be an attachment factor for melanoma and endothelial cells (3,23,32,33), as well as being anti-angiogenic in vivo (31). The sequence WSHW binds heparin and sulfated glycoconjugates with different affinities (12,13) and inhibits the interaction of TSP with the gelatin-binding domain of fibronectin (28). The WSXW motif is also found within the TGF-ß and cytokine receptor superfamilies (5,34). These motifs are present in both TSP1 and TSP2 (17). In order to further localize the site within the TSP type 1 repeats that is involved in the activation of latent TGF-ß, we compared the activities of TSP1 and TSP2 and tested activities of synthetic peptides for the ability to activate latent TGF-ß.

In this work, we now report localization of an activation site to a three amino acid sequence between the first and second type 1 repeats of TSP1. In addition, we have also identified a second sequence in the type 1 repeats that binds active TGF-ß.

This second sequence, while unable to activate latent TGF-ß, inhibits activation of latent TGF-ß by the trimeric TSP1 molecule.

MATERIALS AND METHODS

Purification of Thrombospondin

TSP was purified as described, performing gel permeation chromatography at pH 11 in order to dissociate TGF-ß (25). TSP purity was assessed by SDS-PAGE using Coomassie blue or silver staining. No contaminating TGF-ß activity was found associated with stripped TSP (sTSP) in NRK soft agar assays.

Peptide Synthesis

Peptides were synthesized as described (12,13). Briefly, peptides were synthesized corresponding to sequences of human TSP1 using standard Merrifield solid-phase synthesis protocols and *t*-butoxycarbonyl chemistry. Peptides were analyzed by reverse-phase high pressure liquid chromatography. The structure of the active peptides was verified by mass spectrometry.

Recombinant TSP1 and TSP2 Production in a Baculovirus System

The methods for baculovirally driven expression of mouse TSP2 (9) and human TSP1 (Misenheimer and Mosher, in preparation) in Spodoptera frugiperda cells are described in more detail elsewhere. Full length cDNA clones were kind gifts from Vishva Dixit, University of Michigan. Convenient restriction sites were used to truncate the 5' ends so that the cDNAs could be cloned into baculoviral transfer vectors with a minimal sequence 5' to the translational start sites, 42 base pairs in the case of TSP2 and 29 in the case of TSP1. Cotransfection of transfer vectors was carried out with linearized mutant viral genome DNA so that there was

positive selection of recombinant viruses. These viruses were plaque-purified prior to expansion. Recombinant TSPs were secreted into serum-free medium containing 0.2% BSA for 48-72 h after infection in spinner flasks. Purification was by affinity chromatography on heparin-agarose.

Cells

BAE cells were isolated from aortas obtained at a local abattoir and characterized by Dil-AcLDL uptake and staining for Factor VIII antigen. Stocks were maintained in Dulbecco's modified Eagle's medium (DMEM; Cell-Gro, Mediatech, Herndon, VA) supplemented with 4.5 g/l glucose, 2 mM glutamine, and 20% FBS (Hyclone Laboratories, Logan UT). NRK-49F cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, CRL 1570), and stocks were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, and 10% CS (Hyclone Laboratories, Logan UT) as described (2). All cells tested negative for mycoplasma contamination.

NRK Colony Formation in Soft Agar

TGF-ß activity was assayed by determining the growth and colony formation of NRK cells in soft agar as described (20). After 7 days incubation, the number of colonies greater than 62 μm (~8-10 cells) in diameter were counted. Experiments were performed in triplicate.

Activation of Recombinant Latent TGF-ß by sTSP

Equimolar amounts of sTSP or peptides (based on the molecular mass of TSP monomers) were incubated with 2 nM (200 ng/ml) recombinant latent TGF-ß in a final volume of 0.5 ml PBS for 1 h at 37°C. HCl (4 mM HCl) was used as a positive

control for activation. Samples were then tested for TGF-ß activity in the NRK colony forming soft agar assay. Experiments were performed in triplicate.

ELISA for TGF-ß Activity

The ELISA assay was performed as described (26). Briefly, 30 nM of recombinant latent TGF-ß (1 μg/300 μl) was incubated with 56 nM sTSP (3 μg/300 μl) or equimolar amounts of peptides in a total volume of 300 μl PBS for 5 min at 37°C. Samples were then tested for increased TGF-ß activity using TGFß Predicta kit (Genzyme Corporation, Cambridge, MA) following manufacturer's instructions. The ELISA utilizes monoclonal and polyclonal antibodies specific for an epitope within active TGF-ß. Increased TGF-ß activity was quantitated using a TGF-ß standard curve. Latent TGF-ß incubated with 1 N HCl for 1 h at 4°C was used as a positive control for latent TGF-ß activation.

Peptide Binding Assays

Direct binding of ^{125}I-TGF-ß to immobilized GGWSHW was determined as previously described (12,13). Briefly, the peptide was adsorbed on 96-well polyvinyl chloride microtiter plates (Falcon, Becton Dickinson, Oxnard CA) for 2 h at 37°C in a total volume of 100 μl PBS. Non-specific sites were blocked with 200 μl/well 1% BSA in PBS for 30 min at 37°C. Wells were washed four times and then incubated with 0.125 ng ^{125}I-TGF-ß (DuPont-NEN, Wilmington, DE), and test samples in 1% BSA/PBS in a total volume of 100 μl for 1.5 h at room temperature. The wells were washed three to four times and the bound radioactivity was counted in the isolated wells using a gamma counter. Experiments were performed in triplicate.

Additional Materials

Recombinant latent simian TGF-$ß_1$ was generously provided by Jane Ranchalis (Bristol-Myers Squibb, Seattle, WA). Fusion proteins corresponding to the intron-exon boundaries of the second TSP1 type 1 repeat were prepared by PCR amplification and expressed in E. coli by Glutathionine-S-transferase (29). These proteins were generously provided by Dr. Jack Lawler, Harvard University. Heparin (disodium salt and low molecular weight forms) was purchased from Sigma Chemicals (St. Louis, MO).

RESULTS AND DISCUSSION

Within the TSP family, only TSP1 and TSP2 contain the type 1 repeats and share ~60% homology (1). Therefore, in order to determine whether a sequence motif or tertiary structure common to the type 1 repeats of both TSP1 and TSP2 is responsible for activation of latent TGF-ß, human platelet TSP1, recombinant TSP1, and recombinant TSP2 were tested for their ability to activate latent TGF-ß. A recombinant form of the full-length TSP2 trimer is inactive (Fig. 1). The lack of activity of the TSP2 molecule does not appear to be due to anomalies of the expression system because 0.1 nM of a recombinantly-expressed form of full-length TSP1 trimer (rTSP1) activates latent TGF-ß two-fold above baseline with a response identical to that observed for human platelet TSP1 (Fig. 1). Although TSP2 is unable to activate latent TGF-ß, it does inhibit activation of latent TGF-ß by TSP1 (Table I). This suggests, that in a system where TSP1 and TSP2 are both present, there may be competition for binding to TGF-ß. This is a reasonable speculation given the ability of TSP2 to compete for the binding of ^{125}I-active TGF-ß to TSP1 in a defined system

(data not shown). TSP1 and TSP2 have different regulatory elements within their promoter regions suggesting that their expression is differentially controlled (1). TSP1, in contrast to TSP2, is an early response gene, similar to *jun* and *fos* and is upregulated by serum. In addition, TSP1 and TSP2 have distinct temporal and spatial patterns of expression. Thus, it is interesting to speculate that elements that regulate the relative expression of TSP1 and TSP2 influence levels of TGF-ß activity.

In order to localize the region within the type 1 repeats of TSP1 that activates latent TGF-ß, synthetic peptides corresponding to known sequence motifs were obtained and tested for their ability to activate latent TGF-ß. Peptides correlating to the CSVTCG motif include Mal I (amino acids 368-386, from the first type 1 repeat), Mal II (amino acids 424-442, from the second type 1 repeat), Mal III (amino acids 481-499, from the third type 1 repeat) (23) and the peptide VTCGGGVQKRSRL (amino acids 488-500). In addition, a peptide corresponding to amino acids 412-428, peptide 246 (KRFKQDGGWSHWSPWSS), from the second type 1 repeat of TSP1, was tested to examine the WSHW motif for potential activity. Increasing concentrations of the CSVTCG-containing synthetic peptides, ranging from 11 nM - 11 μM, were incubated with recombinant latent TGF-ß and then tested for TGF-ß activity in the NRK soft agar assay. The CSVTCG-containing peptides were inactive at all concentrations tested (Fig. 2 *a*). In contrast, peptide 246 activated latent TGF-ß as monitored by increased colony formation (Fig. 2 *b*). Maximal activation was observed using 0.03 nM peptide 246, resulting in a 4-fold increase in colony formation (Fig. 2 *b*), although increased colony formation was observed using concentrations up to 11 μM (data not shown). Dose response curves show an

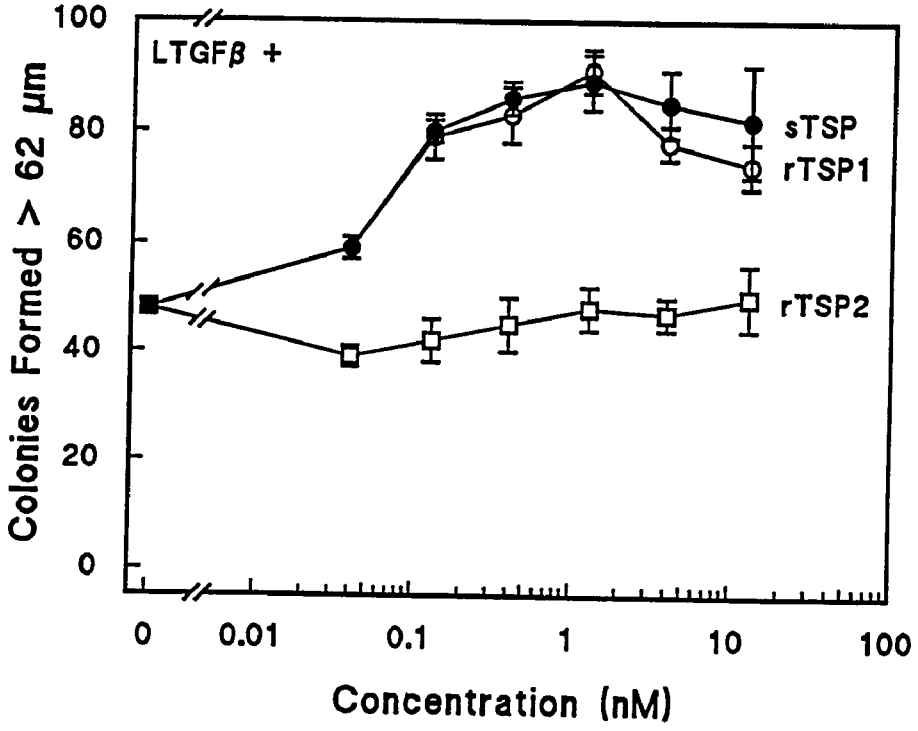


Figure 1. Activation of latent TGF-ß is specific for TSP1. Recombinant latent TGF-ß (2 nM) was incubated with increasing concentrations (0.04 - 13 nM) of sTSP1, recombinant TSP1 (rTSP1), and recombinant TSP2 (rTSP2) for 1 h at 37°C in a total volume of 0.5 ml PBS. BSA (0.1%) was added to all samples to decrease non-specific binding to the tubes. Samples were tested for TGF-ß activity in the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations $\pm$ SD.

TABLE I: TSP2 inhibits activation of latent TGF-ß by TSP1

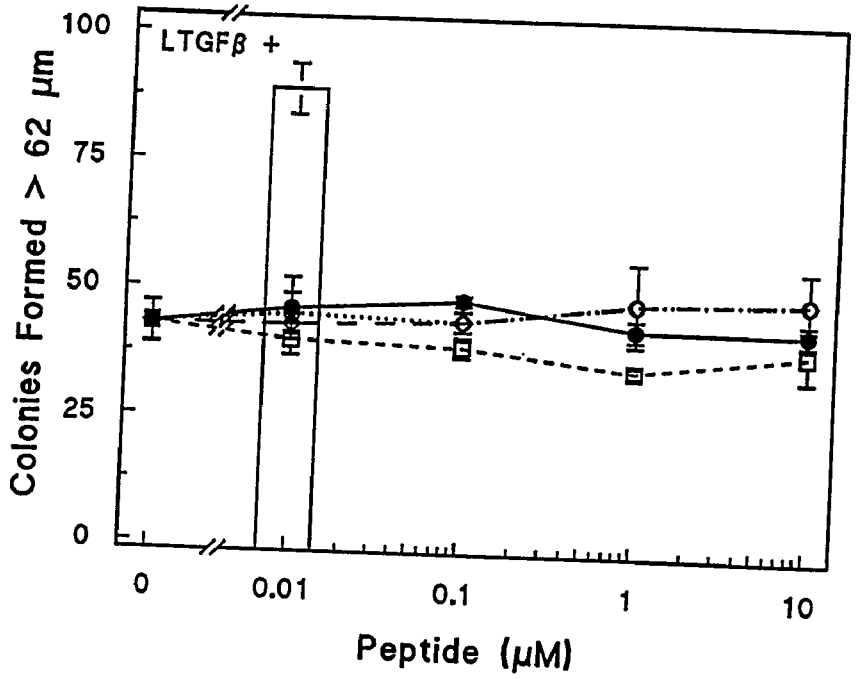
Protein	Colonies Formed
EGF	49 ± 5
Latent TGFß + PBS	51 ± 3
+ sTSP1	77 ± 5
+ sTSP1 + TSP2	52 ± 7

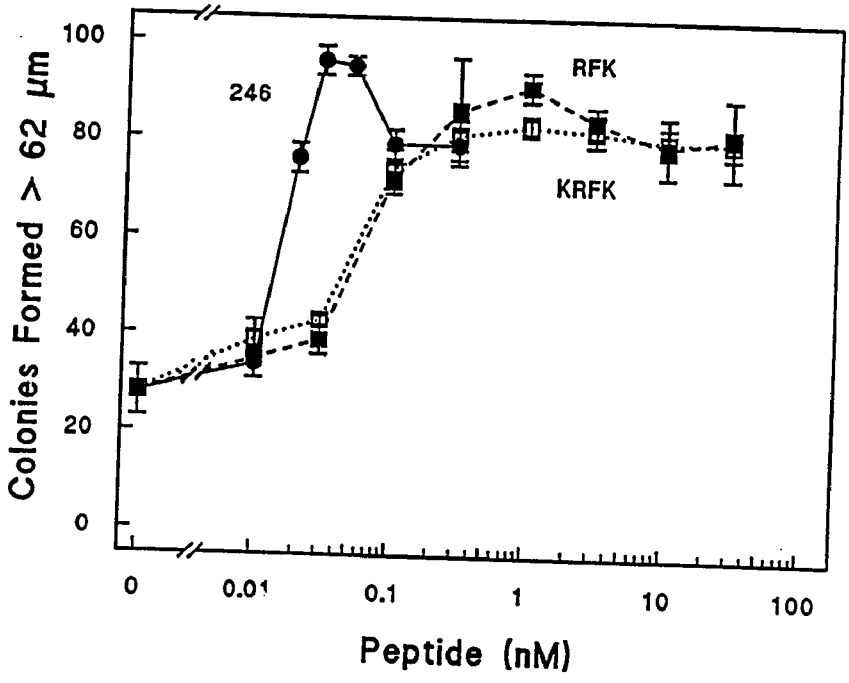
Small latent TGF-ß (2 nM) was incubated with sTSP1 (4 nM) or with sTSP1 + recombinant TSP2 (4 nM each) for 1 h at 37°C in a total volume of 0.5 ml PBS. Samples were assayed for TGF-ß activity by NRK colony formation in soft agar in the presence of EGF. Results are expressed as the means of triplicate determinations ± S.D.

estimated EC_{50} of 0.02 nM for peptide 246 suggesting that this peptide is slightly more effective at activating latent TGF-ß than the TSP trimer (EC_{50} of ~0.067 nM) (26). These data show that the activation site is within peptide 246, KRFKQDGGWSHWSPWSS, and that the CSVTCG motif is not involved. The amount of latent TGF-ß (2nM) used in these assays is in significant molar excess to either TSP or the peptides, suggesting that activation by peptide 246 may be a catalytic event. However, the effective molar concentration of the recombinant latent TGF-ß is significantly less as a result of misfolding of the recombinant small latent molecule, with only ~10% of the protein mass activatable (D. Twardzik, personal communication); thus the concentration of "native" or activatable latent TGF-ß may actually be closer to 0.02 nM. This suggests that TSP (and its peptides) may at least be acting at a molar equivalency to latent TGF-ß, if not a molar excess.

To further localize the activation site, deletions were made to the C-terminus of peptide 246. TSP1 (11 nM, based on TSP monomers) and equimolar concentrations of peptide 246 or the truncated peptides were incubated with latent TGF-ß and then tested for TGF-ß activity in an NRK assay. These experiments showed that the sequence KRFK, amino acids 412-415, is sufficient to activate latent TGF-ß (Table II). Although the peptides were used at 11 nM in order to compare activation to that achieved using full-length TSP, dose response assays show that the EC_{50} for these peptides varied (Table II and Fig. 2 *c*). Peptides 246, 263 (KRFKQDGGWSHWSP), 352 (KRFKQDGGWSHW), and 402 (KRFK) have activities with EC_{50s} of ~0.02 and ~0.06 nM (Table II). Although the difference between 0.02 and 0.06 nM does not seem dramatic when compared directly, the

Figure 2. Peptides containing the sequence RFK activate latent TGF-ß. A, Recombinant latent TGF-ß (2 nM) was incubated with 11 nM sTSP (bar) or increasing concentrations of peptides for 1 h at 37°C in a total volume of 0.5 ml PBS. Peptides include Mal I (residues 368-386, closed circles), Mal II (residues 424-442, open circles, dotted line), Mal III (residues 481-499, open diamond, long dashed line), or VTCGGGVQKRSRL (residues 488-500, open squares, short dashed line). Samples were then tested for TGF-ß activity using the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations $\pm$ SD. B, Latent TGF-ß (2 nM) was incubated with increasing concentrations (0.01 nM - 60 nM) of peptide 246 (residues 412-428, closed circles), peptide 402 (KRFK, residues 412-415, open squares, dotted line), or peptide 412 (RFK, residues 413-415, closed squares, hatched line) for 1 h at 37°C in a total volume of 0.5 ml PBS. Samples were then tested for TGF-ß activity in the NRK soft agar assay. Results are expressed as the means of triplicate determinations $\pm$ SD.


LTGFβ +
Colonies Formed > 62 μm
100
75
50
25
0
0
0.01
0.1
1
10
Peptide (μM)


246
RFK
KRFK
Colonies Formed > 62 μm
100
80
60
40
20
0
0
0.01
0.1
1
10
100
Peptide (nM)

results indicate that the full-length 246 peptide is much more active then peptide 402 (KRFK, Fig. 2 *c*). In addition, altering the spacing of the WSXW motif (peptide 262) or substituting Ala for Trp 420, 423, 426 (peptide 388) or Trp 420, 423 (peptide 266) in the larger peptides reduces the EC_{50s} to 0.2 nM and 200-300 nM (Fig. 2 *b* and Table II). These results suggest that the full-length peptide 246 is the most active, however, activity can be localized to the tetrapeptide KRFK. In addition, the Trp residues appear to be crucial for activity in peptide 246 and 263.

In order to confirm the results of our biological assay, we also tested the peptides for their ability to activate latent TGF-ß in an immunological assay using an ELISA that selectively recognizes the active domain of TGF-ß (26). Incubation of latent TGF-ß with either sTSP1, peptide 246 or KRFK resulted in an ~2-fold increase in TGF-ß activity above baseline (Table III). The VTCGGGVQKRSRL peptide was inactive in the ELISA. These results are consistent with the results of biological assays. The results with the ELISA eliminate the possibility that activation of latent TGF-ß by TSP or the peptides is due to increased protease activity or receptor binding in the biological assays. The ELISA also shows that the peptides are capable of activating latent TGF-ß within 5 min, which is similar to the kinetics observed for intact TSP (26). In addition, activation of latent TGF-ß by KRFK also occurs in a complex cellular mileau because KRFK activates bovine aortic endothelial cell-secreted TGF-ß in the presence of cells in serum (20% FBS)-containing medium as assayed by the NRK soft agar biological assay (data not shown). This shows that the KRFK peptide is active in an <u>in vitro</u> cellular system.

To determine if the sequence KRFK is accessible in the full-length repeat, fusion proteins corresponding to the intron-exon boundaries of the first and second type 1 repeat of TSP1 were constructed and tested for activity (29). The fusion protein containing KRF (11 nM) increased colony formation two-fold above baseline (Table II), similar to the levels achieved using an equimolar concentration of sTSP (Table II). The glutathionine-S-transferase vector control and the fusion protein lacking the N-terminal KRF (amino acids 412-414) had no activity (Table II). These results indicate that the KRFK sequence is functional within the context of the second type 1 repeat.

To determine which amino acids within KRFK are required for activity, modifications were made to KRFK. The corresponding sequence in TSP2, TRIR, was completely inactive (Table IV). Although TSP2 lacks the KRFK sequence between the first and second type 1 repeats, a similar sequence KKFK is located within the procollagen-like domain. However, the results with the full-length TSP2 trimer suggest this site is potentially inaccessible within TSP2. Lys 412 is not required for activity because it can be substituted with other amino acids (His or Gln) or deleted without a loss of activity (Table IV). RFK has an EC_{50} of ~0.06 nM, which is identical to the EC_{50} for KRFK, further verifying that the first lysine is not required for activity (Fig. 2 *c*). In contrast, Phe 414 is essential for activity (Table IV), because it cannot be substituted with other aromatic residues (Tyr, Trp) or with an Ala or Ile. Phe 414 must be flanked by charged basic residues because substituting either Arg 413 or Lys 415 with Gln abolishes activity (Table IV). The

TABLE II: The sequence KRFK is sufficient for activation of latent TGF-ß

Peptide #	Residues	Sequence	Colonies Formed	Increase in TGF-ß Activity
	LTGFß + PBS		50 ± 1	1.0 ± 0
	+ sTSP		106 ± 5	2.0 ± 0
246	412-428	KRFKQDGGWSHWSPWS	98 ± 2	2.0 ± 0
388	412-428	KRFKQDGGASHASPASS	58 ± 9	1.0 ± 0
263	412-425	KRFKQDGGWSHWSP	122 ± 1	2.3 ±0.2
266	412-425	KRFKQDGGASHASP	45 ± 0	1.0 ± 0
352	412-423	KRFKQDGGWSHW	82 ± 1	1.9 ±0.2
262	scrambled	KRFKQDGGWWSP	114 ± 6	2.1 ±0.2
401	412-419	KRFKQDGG	106 ± 10	2.1 ±0.1
402	412-415	KRFK	105 ± 10	2.0 ±0.1
	415-473	second type 1 repeat (lacking amino-terminal KRF)	45 ± 5	1.0 ± 0
		GST Vector control	48 ± 1	1.0 ± 0
	412-473	second type 1 repeat (contains KRF 412-414)	95 ± 7	2.0 ± 0

Small latent TGF-ß (2 nM) was incubated with sTSP (11 nM) or equimolar concentrations of peptides or the fusion constructs for 1 h at 37°C in a total volume of 0.5 ml PBS. Peptide concentrations are based on monomers of TSP. Samples were assayed for TGF-ß activity by NRK colony formation in soft agar in the presence of EGF. Results are expressed as the means of triplicate determinations, ± SD, from one assay and is representative of > 8 experiments. The increase in TGF-ß activity is expressed as fold-increase above baseline and is the average of data from > 8 different experiments.

TABLE III: TSP1 peptides increase TGF-ß immunoreactivity

Sample	Absorbance at 450 nm	TGF-ß Activity
LTGFß + PBS	0.174 ± .004	~ 0.1 ng
+ TSP	0.310 ± .009	~ 0.26ng
+ 246	0.270 ± .003	~ 0.24 ng
+ 402	0.272 ± .020	~ 0.24 ng
+ VTCGGGVQKRSRL	0.181 ± .008	~ 0.1 ng
TGFß [ng/well]		
0	0.094 ± .009	
0.1	0.189 ± .002	
0.26	0.304 ± .006	
0.64	0.532 ± .020	
1.6	1.001 ± .039	

Recombinant latent TGF-ß (30 nM, 1 μg/300 μl) was incubated with 56 nM sTSP (3 μg/300 μl) or equimolar concentrations of the peptides for 5 min at 37°C. Recombinant latent TGF-ß was also incubated with 1 N HCl for 1 h at 4°C as a positive control for activation, yielding > 4 ng of TGF-ß activity. Samples were then tested for increased TGF-ß activity by ELISA as described in materials and methods and absorbance was monitored at 450 nm. Peptide 246 corresponds to KRFKQDGGWSHWSPWSS (residues 412-428) and peptide 402 corresponds to KRFK (residues 412-415). Results are expressed as the means of triplicate determinations, ± SD, from one assay and are representative of four experiments.

ability of a Lys residue to substitute for Arg 413 without a loss of activity supports this finding (Table IV).

The minimal basic sequence required for activation is BFB. A similar motif, BBXB, has been shown to be a heparin binding motif (8). However, not all BBXB motifs are sufficient for activity, because a peptide from the heparin-binding domain of TSP1, which contains this motif (ASLRQMKKTRGTLLALERKDHS, residues 74-95; 21) and the peptide VTCGGGVQKRSRL, are unable to activate latent TGF-ß even at concentrations of 11 μM (data not shown). These data further support our observation that Phe 414 is critical for activity. Peptide 246 has been shown to bind to heparin, albeit with weak affinity (12,13). Therefore, we tested whether heparin could inhibit activation of latent TGF-ß by either peptide 246 or KRFK. Increasing concentrations of heparin, ranging from 0.01 μM - 100 μM, were preincubated with the peptides and then added to latent TGF-ß and tested for TGF-ß activity by colony formation. Heparin failed to inhibit activation by the peptides (Fig. 3). Heparin also had no effect on colony formation by active TGF-ß (data not shown). Another preparation of heparin, the low molecular weight form (3000 daltons), was also tested and found to be inactive (data not shown). These data suggest that the peptides have a higher affinity for latent TGF-ß than for heparin and activate latent TGF-ß even when potentially bound to heparin (at concentrations greater than 10 μM).

The activation of latent TGF-ß by KRFK-containing peptides is stable over the pH range 5.4 to 8.0. Activation also occurred at NaCl concentrations of 0.05 to 0.5 M (data not shown). This is consistent with our previous observations that TSP-TGF-ß remains complexed in the presence of 0.55 M NaCl (20). Although we were only

able to examine the effects of pH and salt over a limited range, these data are suggestive that electrostatic interactions are not the primary forces mediating interactions between the KRFK-containing peptides and latent TGF-ß. Consistent with this is the absolute requirement for the hydrophobic phenylalanine residue. Moreover, preliminary data suggest that RKFK may fit into a hydrophobic pocket within the latency associated peptide at the N-terminus of the latent molecule (pages 166-187).

Activation of latent TGF-ß by KRFK is specific for TSP1. Two common proteins that contain the sequence KRFK or HRFK, calcineurin (11,14,15,22) and BSA (7), respectively, fail to activate latent TGF-ß at concentrations up to 1 μM (data not shown). However, we can not rule out the possibility that this is due to inaccessibility of this sequence in these proteins.

Although the sequence (K)RFK is sufficient for activation of latent TGF-ß, Trps 420 and 423 in the longer peptides 246 (amino acids 412-428) and 263 (amino acids 412-425) are also important for activity (Table II and Fig. 4), because substitution of Trps 420 and 423 with Ala render the peptides inactive at concentrations otherwise effective for either TSP1 or the (K)RFK peptide (Table II). The EC_{50}s of the peptides also show that substituting Trps 420 and 423 with Ala or changing the spacing of WSXW (peptide 262, KRFKQDGGWWSP) results in a shift in the EC_{50} from 0.02 nM for peptide 246 to 0.2 nM for peptide 262 and 200 nM for peptides 388 (amino acids 412-428 with Trps 420,423, and 426 substituted for Ala) and 266 (amino acids 412-425 with Trps 420 and 423 substituted for Ala) (Table II

TABLE IV: The minimal sequence for activation of latent TGF-ß is Basic-Phe 414-Basic

Peptide #	Sequence	Colonies Formed TGF-ß Activity	Increase in
LTGFß	+ PBS	31 ± 3	1.0 ± 0
	sTSP	71 ± 1	2.0 ± 0
402	KRFK	71 ± 7	2.0 ± 0.2
408	KRAK	30 ± 2	1.0 ± 0
246	KRFKQDGGWSHWSPWSS	78 ± 2	2.0 ± 0.1
436	KRAKQDGGWSHWSPWSS	35 ± 3	1.0 ± 0
424	KRIK	35 ± 5	1.0 ± 0
422	KRYK	47 ± 13	1.0 ± 0
423	KRWK	33 ± 1	1.0 ± 0
428	TRIR	27 ± 1	1.0 ± 0
420	QRFK	71 ± 1	2.0 ± 0
421	HRFK	63 ± 6	2.0 ± 0
409	KQFK	25 ± 1	1.0 ± 0
414	KRFQ	40 ± 9	1.0 ± 0
413	KKFK	62 ± 4	2.2 ± 0.3
412	RFK	68 ± 7	2.5 ± 0.5

Small latent TGF-ß (2 nM) was incubated with sTSP or equimolar concentrations of the peptides (11 nM) for 1 h at 37°C in a total volume of 0.5 ml PBS. Samples were tested for TGF-ß activity in the NRK colony forming soft agar assay in the presence of EGF. Results are expressed as the means of triplicate determinations, ± SD, from one assay and is representative of > 8 experiments. The fold increase in TGF-ß activity is the average of data from > 8 different experiments.

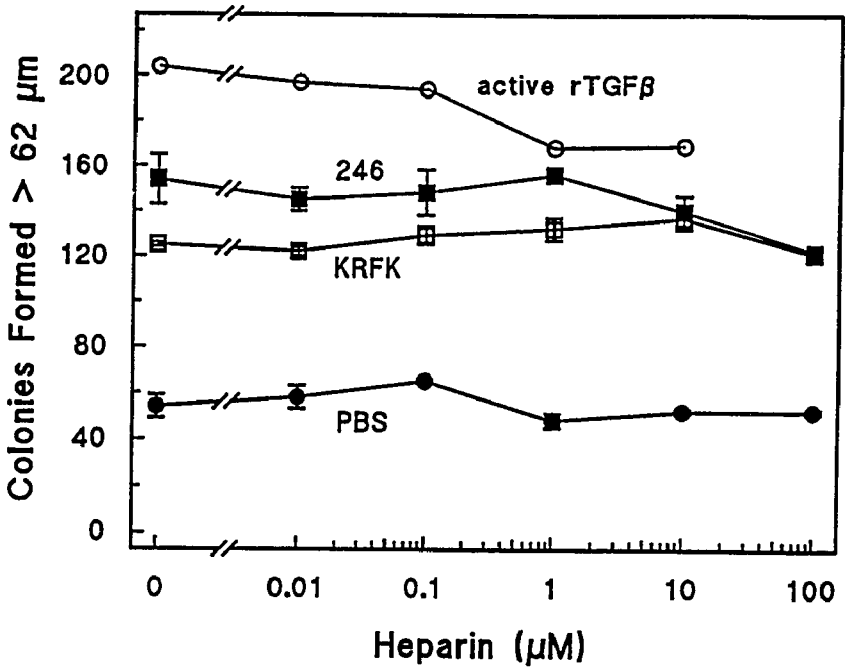


Figure 3. Activation of latent TGF-ß by TSP1 peptides is not inhibited with heparin. Latent TGF-ß (2 nM) was incubated with 11 nM peptides 246 or KRFK (peptide 402) and increasing concentrations of heparin (ranging from 0.01 μM - 100 μM) for 1 h at 37°C in a total volume of 0.5 ml 0.01 M phosphate buffer, pH 7.2, 0.005 M NaCl. Data include: heparin alone (closed circles), heparin + peptide 246 (closed squares), heparin + KRFK (open squares), or heparin + recombinant active TGF-ß (open circles). Samples were tested for TGF-ß activity using the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations $\pm$ SD.

and Fig. 4). In addition, peptides 263, 388, and 266 never reach the same levels of activity as those achieved using peptide 246 (Fig. 4).

In order to clarify the role of the WSXW motif in modulation of TGF-ß activity, the peptide GGWSHW (amino acids 418-423) was constructed and tested for activity. GGWSHW (11 nM - 11 μM) was unable to active latent TGF-ß (Fig. 5 *a*). In contrast, incubation of 100-fold molar excess (1.1 μM) GGWSHW with TSP completely inhibited activation of latent TGF-ß in the NRK assay (Fig. 5 *a*). In addition, GGWSHW (1 μM) partially (~60%) reversed the TSP-mediated inhibition of bovine aortic endothelial cell proliferation (data not shown). It does not appear likely that this inhibitory activity is due to GGWSHW blocking active TGF-ß from binding to TGF-ß receptors, because incubation of GGWSHW with active TGF-ß has no effect on TGF-ß biological activity (data not shown). The inhibitory activity of the GGWSHW sequence is specific based on the inability of many analogous sequences, including GGWSHY, GGWAHW, and GGWSKW, to activate latent TGF-ß (data not shown). In addition, ^{125}I-TGF-ß specifically bound to immobilized GGWSHW (Fig. 5 *b*). A 100-fold molar excess soluble GGWSHW and peptide 246 completely inhibit binding of ^{125}I-TGF-ß to the peptide. However, a 100-fold molar excess of peptide 402 (KRFK) does not inhibit binding of ^{125}I-TGF-ß to GGWSHW, indicating that binding of active TGF-ß to peptide 246 is mediated through the GGWSHW sequence. Substituting the first Trp residue of GGWSHW with a Tyr results in the inability to compete for binding (Fig. 5 *b*). Controls include the Hep I peptide (ELTGAARKGSGRRLVKGPD, residues 17-35; 27) and BSA, both of which are unable to inhbit the binding of ^{125}I-TGF-ß to GGWSHW. Soluble GGWSHW also

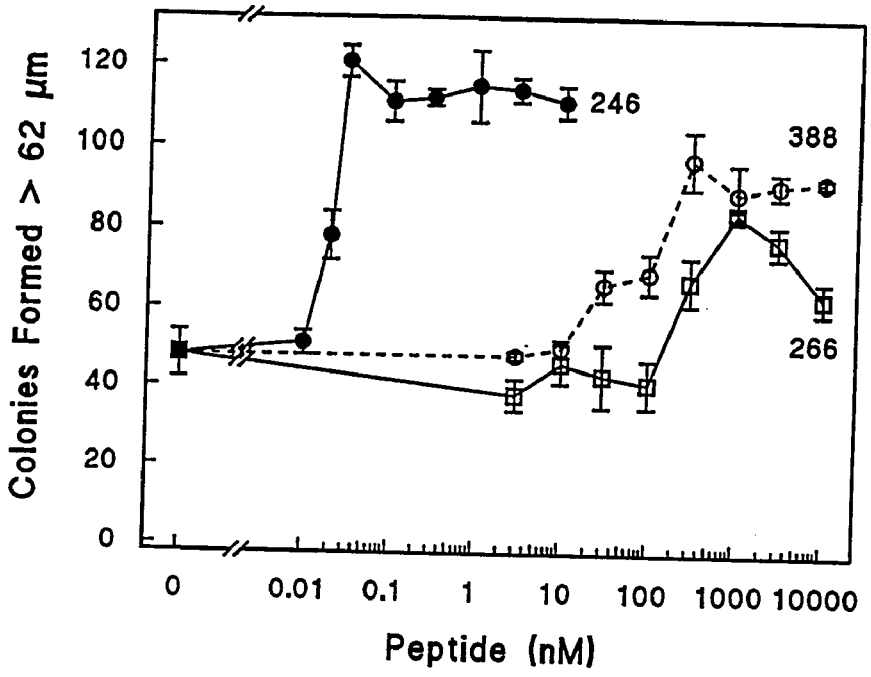
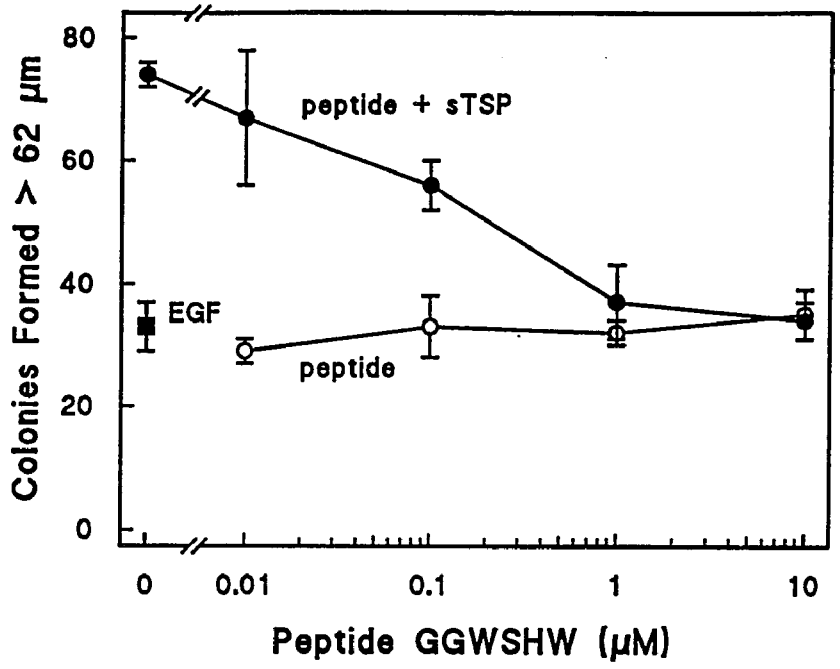


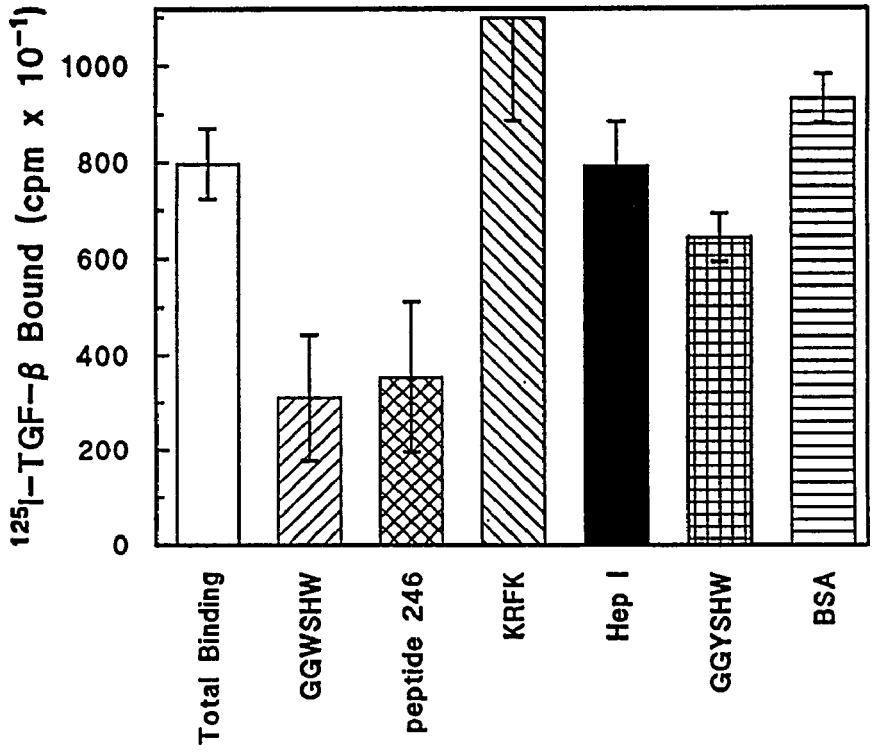
Figure 4. Substitution of Trps 420 and 423 with Ala result in a loss of activity. Recombinant latent TGF-ß (2 nM) was incubated with increasing concentrations of peptide 246 (KRFKQDGGWSHWSPWSS, closed circles), peptide 388 (KRFKQDGGASHASPASS, open circles), or peptide 266 (KRFKQDGGASHASP, open squares) for 1 h at 37°C in a total volume of 0.5 ml PBS. Samples were then tested for TGF-ß activity using the NRK colony forming soft agar assay. Results are expressed as the means of triplicate determinations ± SD.

inhibits the binding of ^{125}I-TGF-ß to immobilized TSP (data not shown). These data suggest that GGWSHW-containing peptides inhibit activation of latent TGF-ß by TSP through blocking the ability of TSP to bind to the latent TGF-ß complex and that this is the mechanism by which TSP2 inhibits activation of latent TGF-ß by TSP1. Although the peptide sequence used in these studies is from the second type 1 repeat of TSP1, the corresponding sequences from the first (DGWSPW) and third (GGWGPW) type 1 repeats of both TSP1 and TSP2 also inhibit TSP1-mediated activation of latent TGF-ß (data not shown). This implies that any of the three type 1 repeat WSXW motifs is potentially capable of binding to the active TGF-ß molecule.

These results show that the minimal sequence effective for activation of latent TGF-ß is RFK. Within the type 1 repeats of TSP1, KRFK is followed by the conserved motif WSHW. We have shown that peptides containing GGWSHW inhibit TSP1-mediated activation of latent TGF-ß, perhaps through inhibiting the binding of TSP to TGF-ß. The activity of the fusion construct consisting of the intron-exon boundaries of the first and second type 1 repeats with the N-terminal KRF suggests that the KRFK sequence is exposed in the TSP1 protein. Based on the results of these studies, we propose that TSP1 utilizes a two-step mechanism for the activation of latent TGF-ß. The GGWSHW sequence in TSP1 and TSP2 mediates TSP-TGF-ß interactions, potentially through the mature portion of TGF-ß. Interactions between GGWSHW and TGF-ß may orient the TSP1 molecule so that the (K)RFK sequence is in a favorable configuration for interacting with latent TGF-ß. We are currently investigating where KRFK binds in the latent TGF-ß complex. Initial data suggest that KRFK may activate latent TGF-ß by binding to the latency associated peptide

Figure 5. Peptide GGWSHW inhibits sTSP-mediated activation of latent TGF-ß. A, Latent TGF-ß (2 nM) was incubated with 11 nM sTSP and increasing concentrations of peptide GGWSHW for 1 h at 37°C in a total volume of 0.5 ml PBS. BSA (0.1%) was added to all of the samples to reduce non-specific binding to the tubes. Samples were tested for TGF-ß activity in the NRK colony forming soft agar assay in the presence of EGF. Results are expressed as the means of triplicate determinations ± SD. B, Peptide GGWSHW (0.1 μg) was immobilized on a PVC-coated 96-well plate for 2 h at 37°C in a total volume of 100 μl PBS. Non-specific binding sites were blocked with 1% BSA/PBS for 30 minutes at 37°C. ^{125}I-TGF-ß (0.125 ng) was added to the wells in the presence of 100-fold molar excess unlabelled peptide GGWSHW, or equimolar amounts of peptide 246, KRFK, Hep I (ASLRQMKKTRGTLLALERKDHS), GGYSHW, or BSA and incubated for 1.5 h at room temperature. Wells were then washed four times in wash buffer containing 0.1% Triton X-100. Results are expressed as the total binding (cpm/well) of the means of triplicate determinations ± SD.


Colonies Formed > 62 μm
80
60
40
20
0
peptide + sTSP
EGF
peptide
0
0.01
0.1
1
10
Peptide GGWSHW (μM)


125I–TGF–β Bound (cpm x 10−1)
1000
800
600
400
200
0
Total Binding
GGWSHW
peptide 246
KRFK
Hep I
GGYSHW
BSA

(unpublished results). The interaction of the (K)RFK sequence with latent TGF-ß potentially induces a conformational change in the latent complex enabling the complex to recognize its cellular receptors. This hypothesis is supported by data that show that TSP2, which has the GGWSHW sequence but which lacks the activation sequence, binds ^{125}I-active TGF-ß, but does not activate latent TGF-ß. Furthermore, TSP2 inhibits activation of latent TGF-ß by TSP1, presumably by TSP2 competing with TSP1 for binding to latent TGF-ß. An anti-peptide 246 polyclonal antibody, which recognizes the GGWSHW sequence, also inhibits TSP1 and peptide 246-mediated activation of latent TGF-ß by ~70% (data not shown). This work is significant in that it confirms that activation of latent TGF-ß by TSP results from TSP interactions with multiple binding sites within the latent TGF-ß complex.

The (K)RFK and GGWSHW peptides have potential therapeutic uses in situations where TGF-ß activity requires modulation, for example, during wound healing or in fibrotic scar formation. Furthermore, these data suggest that regulation of the relative expression levels of TSP1 and TSP2 are important determinants controlling TGF-ß activity.

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ABBREVIATIONS

BAE: bovine aortic endothelial cells, NRK: normal rat kidney, sTSP: thrombospondin free of TGF-ß activity, TBS-C: tris buffered saline containing calcium, TGFß: transforming growth factor-ß, TSP: thrombospondin

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THE TGF-ß-ACTIVATING SEQUENCE OF TSP1 ACTS VIA BINDING INTERACTIONS WITH A SEQUENCE IN THE LATENCY ASSOCIATED PEPTIDE

STACEY SCHULTZ-CHERRY, SOLANGE M. F. RIBEIRO, AND JOANNE E. MURPHY-ULLRICH

Department of Pathology, Division of Molecular and Cellular Pathology, University of Alabama at Birmingham, Birmingham, AL 35294

ABSTRACT

The transforming growth factor-ßs (TGF-ß) are a family of multifunctional proteins that play a pivotal role in the control of cellular growth. TGF-ß is secreted by virtually all cells as a latent molecule. In previous studies, we showed that active TGF-ß is complexed to the glycoprotein thrombospondin (TSP) in platelet releasates and that TSP activates latent TGF-ß secreted by cells in vitro. Activation by TSP occurs as the result of binding of two discrete sequences unique to TSP1 to the latent TGF-ß complex. This two-site binding interaction involves the binding of the WSXW motif within the type 1 repeats of TSP1 to the mature domain of TGF-ß and the subsequent interaction of the RFK sequence of TSP1 with a portion of the latent complex. In these studies, we show that the (K)RFK sequence activates latent TGF-ß by binding to the latency associated peptide (LAP) at a site near the amino terminus of the latent complex. Specifically, the RFK sequence in TSP1 interacts with the sequence LSKL (amino acids 54-57) in the LAP. The LSKL sequence is highly conserved in TGF-$ß_{1-3}$, suggesting that TSP can potentially activate other isoforms of TGF-ß. Indeed, we observe that TSP can also activate a recombinant form of human latent TGF-$ß_2$. Furthermore, both the LAP and mature TGF-ß domains can remain associated with TSP after activation.

INTRODUCTION

Transforming growth factor-ß (TGF-ß) is a small polypeptide growth factor that is involved in diverse biological activities, including wound healing, cancer progression, and angiogenesis (8). TGF-ß is secreted by cells and circulates in the serum and plasma as a biologically latent molecule that must be activated in order to

bind to cellular receptors and elicit a given biological response (18). We showed that the extracellular matrix protein thrombospondin (TSP) activates latent TGF-ß via binding interactions (14,15). The active site of TSP was localized to the sequence RFK, located within the type 1 repeats of TSP1 and unique to TSP1 (15,16). In order to better understand the mechanism of TSP-mediated activation of latent TGF-ß, we sought to determine the region of the latent TGF-ß complex that interacts with the TSP activating sequence RFK.

The small latent TGF-ß molecule is the product of a single gene and consists of an amino terminal 278 amino acid latency associated peptide (LAP) that is cleaved intracellularly from the 112 amino acid carboxy terminus comprising the mature domain. Although the LAP and mature regions of TGF-ß are physically dissociated, they are secreted by cells as a latent molecule that is complexed through electrostatic forces. Interruption of these forces by chaotrophic agents or extremes of pH results in the activation of latent TGF-ß (4). Mutagenesis studies showed that amino acids 40-80 in the LAP are important for maintaining the latent complex (17). Disruption of these amino acids results in the secretion of active TGF-ß by the cells. Additionally, the LAP must be dimeric to inactivate TGF-ß (9,17). Modifying the cysteine residues responsible for LAP dimerization results in little to no secretion of TGF-ß by the cells (9). These studies suggest that the tertiary structure of the LAP may be important in latent TGF-ß complex formation.

In vitro, isolated LAP can bind the mature TGF-ß protein, rendering the molecule biologically inactive (9,12). The binding of the LAP to the active TGF-ß molecule occurs in a 1:1 ratio (9). However, it appears that once the LAP is

dissociated from active TGF-ß reassociation is not favored (9). The metabolism of free LAP by cells in vivo is not understood.

Previously, we showed that activation of latent TGF-ß by TSP could be inhibited by a polyclonal antibody raised against a peptide from the amino terminus of the LAP (amino acids 81-94, antibody 1125, 14). These data suggested that a site in/or near this region of the LAP was involved in TSP-mediated activation of latent TGF-ß. In this work, we now report that the active sequence of TSP1 (KRFK) binds to a sequence located at the amino terminus of the LAP (LSKL, amino acids 54-57), resulting in the activation of latent TGF-ß. The LAP and mature TGF-ß domains remain bound to TSP, after activation consistent with our previous observation of TGF-ß biological activity associated with TSP. These studies further our understanding of latent TGF-ß activation by TSP.

MATERIALS AND METHODS

Purification of Thrombospondin

sTSP was purified as described, performing gel filtration chromatography at pH 11 in order to dissociate TGF-ß (11). In addition, TSP was purified from the α-granules of human platelets associated with TGF-ß activity (native TSP, nTSP) by performing gel permeation chromatography at neutral pH. TSP purity was assessed by SDS-PAGE and Coomassie blue staining.

Peptide Synthesis

Synthetic peptides were prepared as described using standard Merrifield solid-phase synthesis protocols and *t*-Boc chemistry (5,6). The LSKL peptide (amino acids

54-57) was synthesized and was a generous gift of Dr. David Roberts (National Cancer Institute, National Institutes of Health).

Peptide Iodination

Peptide 437 (KRFKQDGGWSHWSPWSSYS) and the LSKL peptide were iodinated using the lactoperoxidase method as described (10). Briefly, the peptides (1 mg/ml) were dissolved in 0.1 M sodium phosphate pH 7, and 100 μg of the peptide solution were added to 1 mCi $Na^{125}I$ (DuPont-NEN, Wilmington, DE). Lactoperoxidase (4 μg, Sigma Chemicals, St. Louis, MO) and 30% H_2O_2 (4 μl of 1:20,000 dilution) were added to this mixture and incubated for 10 min at room temperature. The reaction mixture was applied to a Sep-pak C18 cartridge (Millipore, Arlington Heights, IL) to separate free iodine from the peptide. The peptide was eluted with 50% acetonitrile in water.

Formation of Peptide-Protein Complexes as Monitored by SDS-Page Analysis

Iodinated peptides were incubated with the samples for 1 h at 37°C. Assays using TSP were chemically cross-linked using 0.25 mM DSS (dissuccinimidyl suberate; Pierce, Rockford, IL) and incubated for an additional 15 min at 4°C as described (11). Samples were then analyzed by SDS-PAGE and autoradiography for complex formation.

Cells

Bovine aortic endothelial cells (BAE) were isolated from aortas obtained at a local abattoir and characterized by Dil-AcLDL uptake and staining for Factor VIII antigen. Stocks were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM glutamine, and 20% FBS. NRK-49F cells

were purchased from the ATCC and stocks were maintained in DMEM supplemented with 4.5 g/l glucose, 2 mM glutamine, and 10% CS. All cells tested negative for mycoplasma.

NRK Colony Formation in Soft Agar

TGF-ß activity was assayed by determining the colony formation of NRK cells in soft agar as described (11). The number of colonies greater than 62 μm (~8-10 cells) in diameter were counted after a 7 day incubation. Experiments were performed in triplicate.

Activation of Recombinant Latent TGF-ß by TSP or the Peptides

Equimolar concentrations (11 nM) of sTSP or the peptides were incubated with recombinant latent TGF-ß (2 nM) in a final volume of 0.5 ml PBS for 1 h at 37°C. Samples were then tested for TGF-ß activity in the NRK colony forming soft agar assay. Experiments were performed in triplicate.

Goat Anti-LAP Affinity Column

A cyanogen bromide (CNBr) activated Sepharose-4B (Sigma Immunochemicals, St. Louis, MO) polyclonal goat anti-LAP affinity column was prepared. CNBr activated Sepharose-4B (0.15 g) was hydrated in 1 mM HCl for 15 min and then centrifuged at 1000 rpm for 5 min. The gel was washed five times with 1 mM HCl and then centrifuged at 1000 rpm. A goat polyclonal antibody specific for human LAP (0.5 mg, R&D Systems, Minneapolis, MN) was added to the Sepharose gel in 2 volumes of carbonate buffer (0.1 M sodium bicarbonate, 0.5 M NaCl, pH 8.3) and incubated for 2 h with mixing. The mixture was centrifuged and the supernatant was removed. Glycine buffer (0.2 M glycine, pH 8) was incubated with

the antibody/Sepharose mixture for 2 h. The mixture was added to a column and washed with carbonate buffer, followed by 0.1 M acetate buffer, pH 4, and TBS-C, pH 7.4. Platelet-derived nTSP in TBS-C, pH 7.4, was applied to the column and the flow through was collected and reapplied three times. Bound protein was eluted with TBS-C, pH 11, and fractions were analyzed for the presence of TSP and LAP by Western blotting using a mouse monoclonal anti-TSP antibody 133 (13) and a goat polyclonal anti-LAP. Proteins were detected using an enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) following manufacturers instructions.

Purification of Recombinant Latent TGF-$ß_2$

Recombinant human latent TGF-$ß_2$ was expressed in Chinese Hamster ovary cells (CHO) as described (7). Latent TGF-$ß_2$ in cellular conditioned medium was purified by precipitating the conditioned medium with 80% (volume/volume) ammonium sulfate for 2 h at 4°C. The conditioned medium was then dialyzed in 3500 molecular weight cutoff dialysis tubing against two liters of PBS, pH 7.4, overnight at 4°C. The conditioned medium was concentrated with polyethylene glycol and centrifuged to remove precipitate. The purified protein was analyzed for purity by Western blot analysis against an antibody specific for TGF-$ß_2$ (a generous gift of Dr. David Danielpour, National Institutes of Health).

Additional Materials

Recombinant simian latent TGF-$ß^1$ was a generous gift of Ms. Jane Ranchalis, Bristol-Myers Squibb (Seattle, WA). Purified LAP from human TGF-$ß_1$ was purchased from R&D Systems (Minneapolis, MN). Recombinant human latent TGF-

$ß_2$ in CHO cell conditioned medium was a generous gift of Dr. Patricia Segarini (Celtrix, San Francisco, CA).

RESULTS

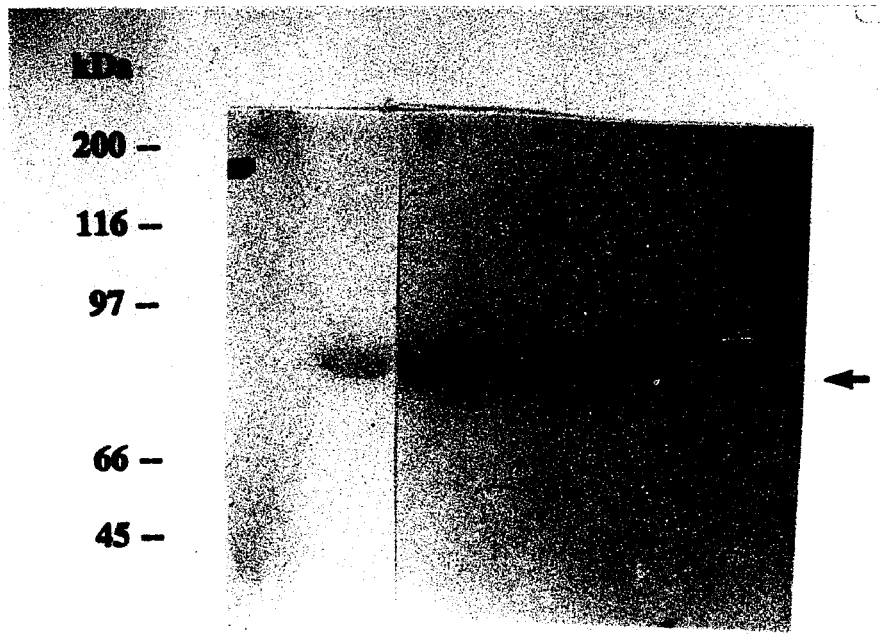
Activation of latent TGF-ß by TSP involves a two-step mechanism. Initially, WSXW (in the type 1 repeats of TSP 1 and TSP2) interacts with the active portion of TGF-ß, leading to the interaction of the RFK site with a second site in the latent TGF-ß complex, resulting in activation (16). Therefore, we wanted to determine where KRFK bound in the latent TGF-ß complex resulting in activation. Previous results showed that a polyclonal antibody raised against a peptide from the amino terminus of the LAP (amino acids 81-94) inhibited TSP-mediated activation of latent TGF-ß (14), thus we investigated whether KRFK binds to the LAP. Peptide 246 (residues 412-428, KRFKQDGGWSHWSPWSS) was synthesized with Tyr and Ser residues at the carboxy terminus (KRFKQDGGWSHWSPWSSYS, peptide 437) and iodinated. Peptide 437 activates latent TGF-ß indicating that the additional Tyr and Ser residues do not stearically hinder interactions of the RFK sequence with latent TGF-ß (data not shown). ^{125}I-peptide 437 was incubated with purified LAP and complex formation was monitored by SDS-PAGE and autoradiography. A radioactive band migrating at ~75 kDa under non-reducing conditions was observed, indicating that ^{125}I-peptide 437 binds to purified LAP under these conditions (data not shown).

The binding of peptide 437 to the LAP is mediated through the KRFK site. Increasing concentrations of unlabeled peptide 402 (KRFK) compete for the binding of peptide 437 to the LAP (Fig. 1 *a*). Incubation of ^{125}I-peptide 437 (3 μM) and LAP

(0.3 μM) with 1.2 mM unlabeled KRFK (400-fold molar excess to peptide 437) results in complete inhibition of binding. In addition, the binding of peptide 437 to the LAP is apparently specific for the KRFK sequence because when 400 μM (150-fold molar excess) of peptides GGWSHW, and VTCGGGKRSRL were incubated with peptide 437 and the LAP, they failed to compete for the peptide binding to the LAP (Fig. 1 *b*). These results suggest that peptide 437 binds specifically to the LAP through the RFK site.

To identify the KRFK-binding site within the LAP, we used an approach based on the hydropathic complementarity of amino acids. It has been shown that there is an inverse correlation between the hydropathic score of amino acids encoded by complementary DNA strands (1,2,3). Using the codons for the KRFK sequence within TSP1, we deduced that the non-coding strand on the LAP would contain the sequence LSKL. The sequence LSKL is located at the amino terminus of the LAP (residues 54-57) and is conserved throughout TGF-$\beta_{1\text{-}5}$ (Table I). If KRFK is binding to the LSKL site in the LAP resulting in activation, exogenous LSKL should inhibit TSP- and KRFK-mediated activation of latent TGF-ß. Therefore, the peptide LSKL was synthesized and tested for its ability to inhibit the activation of latent TGF-ß by the peptides and TSP. Increasing concentrations of LSKL (1 nM - 10 μM) were preincubated with equimolar amounts of TSP1 (11 nM), peptides 246, KRFK, or RFK and then added to latent TGF-ß. TGF-ß activity was monitored by NRK colony formation. LSKL inhibited the activation of latent TGF-ß by peptides 246, KRFK, RFK, and TSP1 (Fig. 2). Peptides 246, KRFK, and RFK were maximally inhibited by 0.1 μM LSKL. The EC_{50} for inhibition using the peptides was 11 nM.

Figure 1. Peptide 437 specifically binds to the LAP. A, ^{125}I-peptide 437 (0.3 μg, 3 μM, lane 1) was incubated with purified LAP (0.3 μM, lane 2) or LAP and increasing concentrations of unlabeled KRFK for 1 h at 37°C (lanes 3-6). Concentrations of KRFK peptides are: 3 μM (lane 3), 30 μM (lane 4), 300 μM (lane 5), and 1.5 mM (lane 6). Samples were run non-reduced on a 5-20% SDS-PAGE gel and exposed to x-ray film for 1 week. B, ^{125}I-peptide 437 (2 μM, lane 1) was incubated with purified LAP (0.3 μM, lane 2) in the presence of 400 μM peptides KRFK (lane 3), GGWSHW (lane 4), VTCGGGKRSRL (lane 5), or RFK (lane 6), for 1 h at 37°C. Samples were run on a 5-20% SDS-PAGE gel under non-reducing conditions and exposed to x-ray film for 1 week.


kDa
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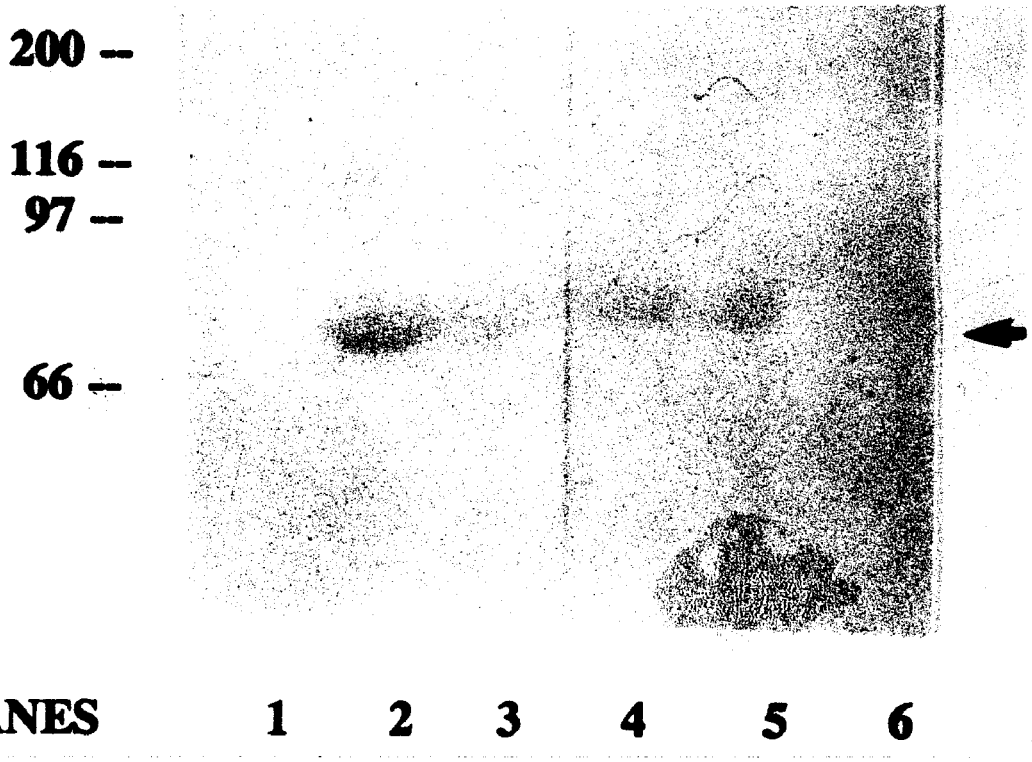
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TSP1 requires 1-10 μM for maximal inhibition of activation with an EC_{50} of 0.5 μM (Fig. 2). LSKL alone was unable to activate latent TGF-ß. The ability of LSKL to inhibit TGF-ß activation by RFK suggests that LSKL may be competitively blocking RFK-LAP interactions. In order to examine this, we performed chemical cross-linking studies using TSP and iodinated LSKL. These studies show that LSKL binds to TSP and that binding can be inhibited with excess unlabeled KRFK (Fig. 3). A 50-fold molar excess of KRFK (350 μM) completely inhibits the binding of TSP1 to LSKL (Fig. 3). These results suggest that KRFK binds to the LSKL site within the LAP.

The conserved nature of LSKL throughout the TGF-ß family suggests that all of the TGF-ß family members may be capable of being activated by TSP. Therefore, we tested whether recombinant human latent TGF-$ß_2$ expressed by Chinese Hamster ovary cells (CHO) could be activated by TSP1. Latent TGF-$ß_2$ was purified from the CHO cell conditioned media as described in Materials and Methods and incubated with equimolar concentrations (11 nM) of either TSP, peptide 246, or KRFK and tested for TGF-ß activity by NRK colony formation in soft agar (Fig. 4). Treatment of latent TGF-$ß_2$ with TSP or the peptides increased colony formation five-fold as compared to untreated latent TGF-$ß_2$. These levels of colony formation were similar to those obtained by HCl-activation (Fig. 4 *a*). TSP and peptide-mediated activation of latent TGF-$ß_2$ were also inhibited with the LSKL peptide (Fig. 4 *b*). These data show that TSP can activate other TGF-ß isoforms. Furthermore, these data are consistent with the hypothesis that RFK-LSKL interactions mediate activation of latent

TABLE I: The LSKL site is conserved in TGF-$ß_{1-5}$

	51
TGF-$ß_1$-HU	GQILSKLRLA
TGF-$ß_2$-HU	GQILSKLKLT
TGF-$ß_3$-HU	GQILSKLRLT
TGF-$ß_4$-AV	GQILSKLRLT
TGF-$ß_5$-XE	GQILSKLKLD

Amino acid sequences of TGF-$ß_{1-5}$. Sources of TGF-ß sequences are as follows: TGF-$ß_1$-HU: human; TGF-$ß_2$-HU: human; TGF-$ß_3$-HU: human; TGF-$ß_4$-AV: avian; and TGF-$ß_5$-XE: xenopus laevis.

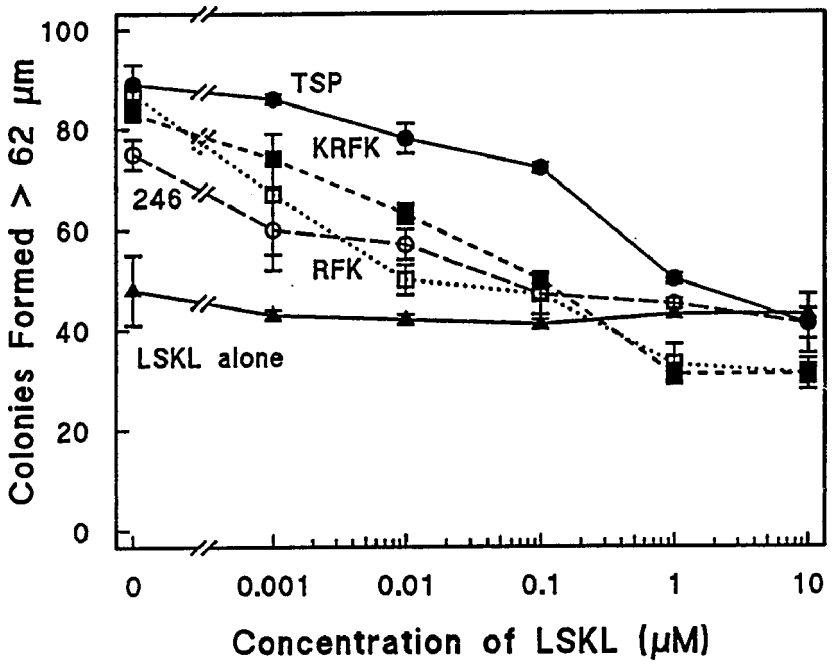


Figure 2. Peptide LSKL inhibits the activation of latent TGF-ß by peptides 246, KRFK, RFK, and TSP1. TSP1 (11 nM, closed circles) and equimolar concentrations of peptide 246 (open circles, long-hatched line), KRFK (closed squares, hatched line), or RFK (open squares, dotted line) were incubated with recombinant latent TGF-ß (2 nM) in the presence of increasing concentrations of peptide LSKL for 1 h at 37°C. Samples were tested for TGF-ß activity in the NRK colony forming soft agar assay. LSKL alone is represented by the solid line, triangles, and is considered background. Results are expressed as the means of triplicate determinations ± SD.

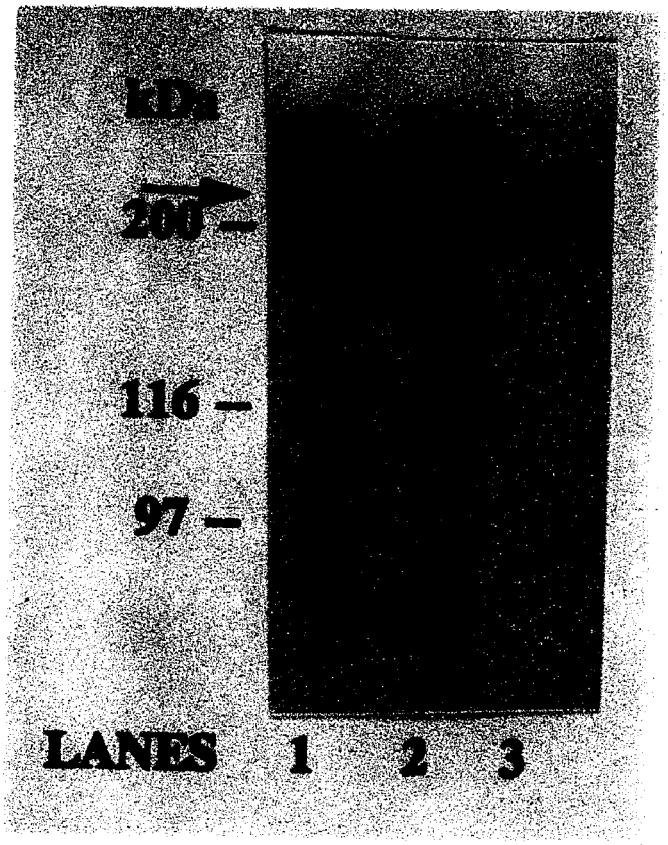


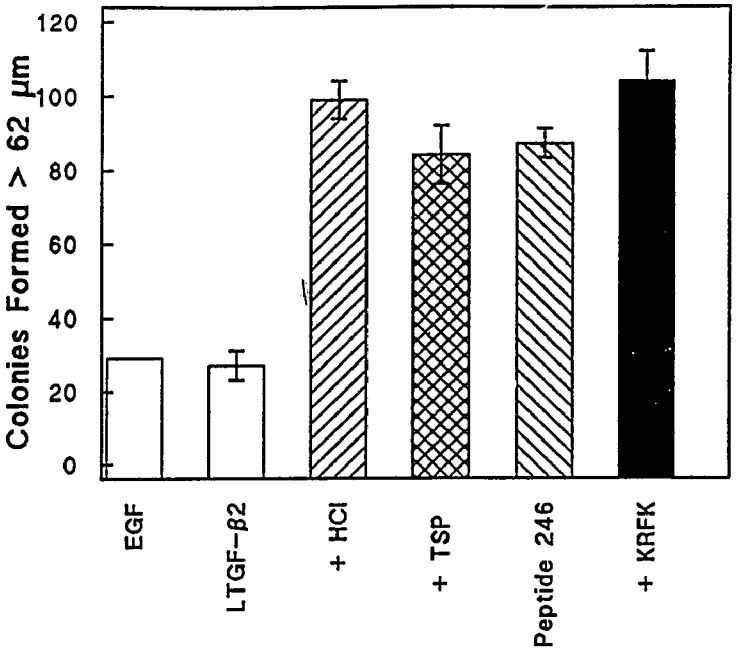
Figure 3. Peptide LSKL binds TSP1. ^{125}I-peptide LSKL (0.1 μg, 7 μM) was incubated with TSP1 (0.2 μM, lane 1) or TSP1 in the presence of peptide 246 (70 μM, lane 2; and 350 μM, lane 3) and incubated for 1 h at 37°C. The chemical crosslinker DSS, 0.25 mM, was added to the samples and incubated for 15 min at 4°C. Complex formation was monitored by a 6% SDS-PAGE electrophoresis run under reducing conditions and autoradiography.

TGF-ßs. The TGF-ß isoforms have different temporal and spatial expression in vivo, thus it is possible that the expression of TSP in a given system may be the regulatory mechanism in the TSP-TGF-ß interaction.

The LSKL site is not present in the non-TGF-ß members of this superfamily. A Genebank search of other TGF-ß superfamily members, including Drosophila decapentaplegic protein (DPP), bone morphogenetic protein (BMP), activins/inhibins, VGR-1, and dorsalin, indicate that the LSKL site is unique to the TGF-ßs. This suggests that TSP1 may not regulate activation of other TGF-ß superfamily members. However, this possibility remains to be explored.

Activation of latent TGF-ß by plasmin results in the dissociation of mature TGF-ß from the LAP (8). Previous studies showed that TGF-ß activity is associated with TSP purified from platelets (12) and with endothelial cell-derived TSP (data not shown). However, it was not known whether the LAP remained associated with or was released from the TSP/TGF-ß complex after latent TGF-ß activation. Therefore, we examined platelet-derived TSP (nTSP) for the presence of the LAP.
Goat polyclonal anti-LAP affinity column was prepared, and nTSP, sTSP (stripped TSP, free of associated TGF-ß activity), and recombinant latent TGF-ß were applied to the column. Bound protein was eluted with an alkaline buffer and the fractions were analyzed by Western blot for the presence of TSP and LAP. Recombinant latent TGF-ß bound to the LAP column and was eluted with pH 11 buffer as expected (data not shown). In contrast, non-aggregated sTSP did not bind to the column (data not shown). However, nTSP eluted in both the column flow through and the fractions (Figure 5), although TGF-ß activity is only found in the fractions bound to the anti-

Figure 4. TSP activates recombinant latent TGF-$ß_2$. A, Equimolar concentrations (11 nM) of either TSP, peptide 246, or KRFK were incubated with 100 μl recombinant latent TGF-$ß_2$ for 1 h at 37°C. Recombinant latent TGF-$ß_2$ (100 μl) incubated with 1 M HCl was used as a positive control for activation. Samples were tested for TGF-ß activity by NRK colony formation in soft agar. Results are expressed as the means of triplicate determinations $\pm$ SD. B, Equimolar concentrations (11 nM) of either TSP, peptide 246, or KRFK were incubated with 100 μl recombinant latent TGF-$ß_2$ in the presence or absence of LSKL (10 μM) for 1 h at 37°C. Samples were tested for TGF-ß activity by NRK colony formation in soft agar. Results are expressed as the means of triplicate determinations $\pm$ SD.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100
80
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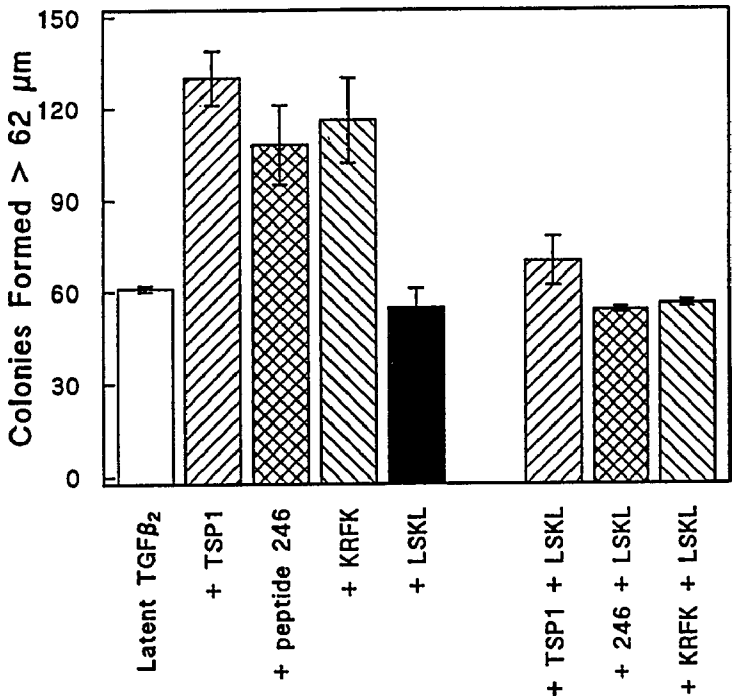
EGF
LTGF-β2
+ HCl
+ TSP
Peptide 246
+ KRFK


Colonies Formed > 62 µm
150
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Latent TGFβ2
+ TSP1
+ peptide 246
+ KRFK
+ LSKL
+ TSP1 + LSKL
+ 246 + LSKL
+ KRFK + LSKL

LAP column, even after activating the flow through fractions with pH 11 buffer. These results suggest that the nTSP present in the flow through does not contain bound LAP or active TGF-ß. This finding is not unexpected because stochiometry studies suggest that there is a ratio of one TGF-ß molecule for every 500-2000 TSP molecules. In addition, if the TSP in the flow through is aggregated associated LAP may be unable to bind to the affinity column. Similar results were obtained when endothelial cell-derived TSP was immunoprecipitated with antibodies specific for TSP and the LAP (data not shown). These results suggest that the LAP remains associated with nTSP.

DISCUSSION

We have shown in these studies that the sequence LSKL binds to the KRFK sequence in TSP1 leading to the activation of latent TGF-ß. LSKL is present at the extreme amino terminus of the LAP (amino acids 54-57). Amino acids 1-30 are the signal sequence for secretion and are cleaved intracellularly. In models of latent TGF-ß, the amino acids surrounding the LSKL site are shown to be responsible for the non-covalent forces that complex the LAP to mature TGF-ß (8). Additionally, Sha et al. showed that the deletion of residues 50-85 in TGF-$ß_1$ LAP affects latent TGF-ß complex formation (17). However, the mutagenesis of residues 50-85 does not affect LAP dimer formation, which is required to confer latency (9,17). Residues 40-60 are rich in basic amino acids and may be responsible for the electrostatic interactions stabilizing the latent complex. The binding of (K)RFK/TSP to LSKL may result in the interruption of these forces and the exposure, or unfolding, of mature TGF-ß.

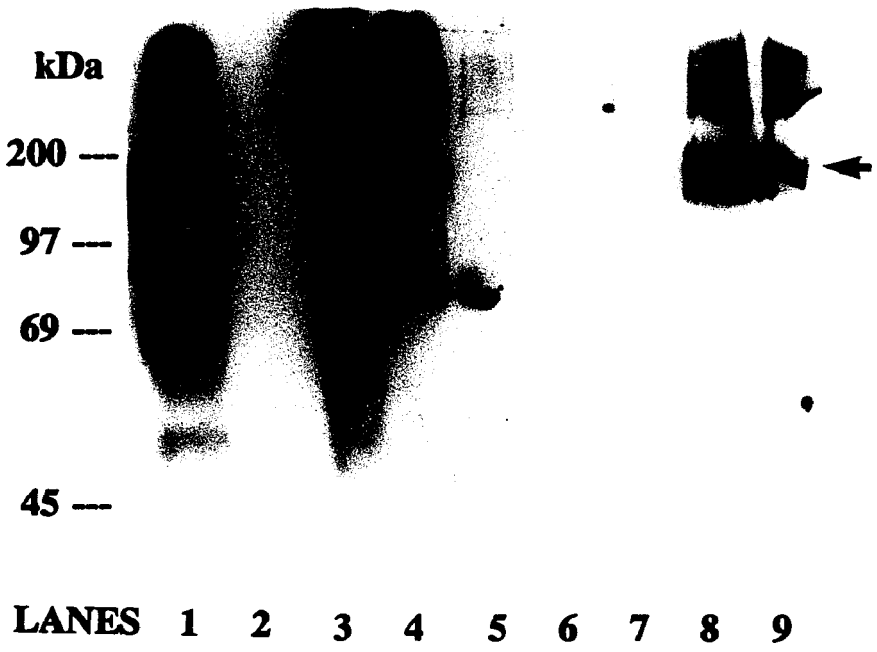

Figure 5. The LAP remains associated with TSP after activation. nTSP (25 μg) in a total volume of 500 μl TBS-C, pH 7.4, was loaded on a goat polyclonal anti-human LAP affinity column and fractions (2 ml) were collected. Bound material was eluted with TBS-C, pH 11, and 50 μl of the fractions were run on an 8% SDS-PAGE gel in the presence of reducing agent. The proteins were transferred to nitrocellulose and Western blotted using a mouse monoclonal antibody specific for TSP1 (antibody 133, 1:5000 dilution in Tris-buffered saline containing 0.1% Tween 20). Proteins were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL). Lanes correspond to: TSP standard (1 μg, lane 1), non-bound fractions (lanes 3-8), and fractions eluted at pH 11 (lanes 9 and 10).

Activation of latent TGF-ß by TSP and (K)RFK can not be inhibited with high salt or extremes of pH (16). This suggests that binding of TSP to latent TGF-ß is not mediated by electrostatic interactions. The binding of KRFK to LSKL may involve hydrophobic interactions. We will test this possibility by preparing an affinity column with either LSKL or peptide 246 (KRFKQDGGWSHWSPWSS) and determining if binding interactions can be disrupted with hydrophobic interactions. In addition, we will test a scrambled LSKL peptide, changing the hydropathicity of the peptide, for the ability to inhibit the activation of latent TGF-ß by TSP1. If this peptide is inactive, it will support our theory that hydrophobic interactions between TSP and latent TGF-ß are important for activation.

The LAP region of TGF-$ßs_{1\text{-}5}$ are not well conserved. However, residues 40-60 are the largest conserved continuous sequence of the pro domain in all three TGF-ßs. The ability of TSP to activate recombinant latent TGF-$ß_2$ suggests that TSP may be involved in the activation of all latent TGF-ß isoforms. The expression of the three mammalian TGF-ß isoforms, thus TSP expression, may be a regulatory mechanism.

The localization of the TSP1 binding site to the sequence LSKL may have important therapeutic applications. Both the LSKL peptide and the GGWSHW peptide inhibit the activation of latent TGF-ß by TSP1, although through different sites. Perhaps these peptides could be used concurrently <u>in vivo</u> to inhibit TGF-ß activation in situations where TGF-ß activity is detrimental. These studies will be important to pursue in the future.

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DISCUSSION

TGF-ß is a small polypeptide growth factor involved in such diverse biological functions as wound healing, angiogenesis, and cancer progression (9,88,89,129). The active form of TGF-ß is a 25 kDa homodimer that is capable of binding to cellular receptors and eliciting numerous responses depending on the cell type and extracellular milleau. However, TGF-ß is secreted by virtually all cells in culture and is found circulating in the serum and plasma as a biologically latent molecule (93,120,167,168). Latent TGF-ß must be activated before it can bind to receptors. Because the expression of TGF-ß receptors is constitutive, activation of latent TGF-ß is a primary means of regulation.

The paradigm for in vivo activation of latent TGF-ß revolved around a co-culture model of endothelial cells and smooth muscle cells or pericytes. This system requires the direct contact of the different cell types, components of the cell surface and the serine protease plasmin. However, this system did not explain the ability of certain tumor cells, whether endogenously or in response to anti-estrogens, to secrete increased levels of active TGF-ß in the absence of increased synthesis. We have shown that there is another regulator of TGF-ß activity, namely the platelet α-granule and extracellular matrix protein TSP.

These studies have served to define the mechanism of latent TGF-ß activation by TSP. We have shown: 1) TSP activates latent TGF-ß secreted by endothelial cells

by a novel mechanism, independent of interactions with the cell surface and proteases; 2) TSP activates both the small and large forms of latent TGF-ß via binding interactions; 3) the activation of latent TGF-ß involves a two-step mechanism, in which the WSXW motif present in the type 1 repeats of TSP binds to the active domain of TGF-ß, thereby orienting the RFK sequence in TSP1 to enable it to interact with the sequence LSKL in the LAP; and 4) that the components of the latent TGF-ß complex, the LAP and the mature growth factor, remain bound to TSP after activation, although the TGF-ß is now biologically active.

In the first study we showed that exogenous TSP specifically activates latent TGF-ß secreted by endothelial cells. TSP-mediated activation of latent TGF-ß is not specific for endothelial cell-secreted latent TGF-ß, as epithelial cell and fibroblast TGF-ß can also be activated by TSP. Potentially, the latent TGF-ß secreted by many different cell types, including neuronal cells, monocytes/macrophages, and neutrophils, may also be activated by exogenous TSP. These cell types also secrete TSP. We observed that endothelial cell-derived TSP has associated TGF-ß activity (unpublished observations). It is not known if the TSP containing TGF-ß activity also has associated LAP. The TGF-ß bound to the cell-derived TSP is not obtained from the serum, because these studies were performed under serum-free conditions. Additionally, we showed that TGF-ß in fetal bovine serum can not be activated by TSP. TGF-ß circulating in serum is possibly complexed to another serum component (such as α_2-macroglobulin) blocking the ability of TSP to bind/activate latent TGF-ß. Hence, the TGF-ß bound to TSP must be derived from the cells. Although we have

only tested endothelial cell-derived-TSP for TGF-ß activity, the possibility exists that most cell-derived TSP is associated with TGF-ß.

We do not know when endogenous TSP and latent TGF-ß interact within the cellular environment. Interactions could occur intracellularly or within minutes after the secretion of TSP. Kinetic experiments indicate that activation occurs as quickly as 2 min after the addition of TSP to latent TGF-ß in a chemically-defined system. In the endothelial cell environment, activation is observed within 30 min after the addition of TSP. Therefore, it is reasonable to suggest that TSP activates latent TGF-ß almost immediately after secretion.

Plasmin is also present within the cellular environment and may potentially activate latent TGF-ß. This system does not appear to be as efficient as compared to TSP-mediated activation (126,133,134). Rifkin et al. reported that plasmin activates ~20% of the total latent TGF-ß secreted by cells (133). We have observed that TSP activates ~80% of endothelial cell-secreted latent TGF-ß, suggesting that TSP is a more efficient activator of cell secreted-latent TGF-ß. It is probable that both the TSP and plasmin systems are involved in many pathophysiological systems. Although, in our in vitro endothelial cell system, the TSP-mediated activation of latent TGF-ß is independent of plasmin activity. The primary difference between our studies of activation of BAE cell-secreted latent TGF-ß by TSP and those examining TGF-ß activation by plasmin in a BAE system is the starting cell density. Our studies utilize subconfluent cells, while activation by plasmin is most efficient in confluent cells (126). TSP synthesis is inversely proportional to cell density, hence plasmin may activate latent TGF-ß once TSP synthesis is down-regulated. Additionally, TSP

has been shown to bind and inactivate plasmin (54), potentially leading to less plasmin available for activation of latent TGF-ß. The plasmin system of TGF-ß activation is very complex, requiring numerous components, including transglutaminase, bFGF, and cell-surface components (126). In comparison, TSP activates latent TGF-ß independently of other factors. Presently, it would be difficult to compare the efficiency of latent TGF-ß activation by plasmin to TSP-mediated activation because the stoichiometries are unknown. However, latent TGF-ß activation by TSP may be more specific as compared to plasmin activation leading to a more regulated system. Thus, the TSP may be easier to regulate as compared to activation by plasmin.

Most cell types contain both the large and the small latent TGF-ß complexes (88,89). Both the plasmin system and TSP activate the large and small latent TGF-ß complexes. The covalent linkage of the latent TGF-ß binding protein (LTBP) to the amino terminus of the LAP does not interfere with the RFK site interacting with LSKL, the large latent complex. Although we showed that purified LTBP does not bind to TSP by chemical cross-linking studies, it does not eliminate the possibility that TSP and the LTBP interact, although independent of latent TGF-ß activation. Studies by Taiple et al. showed that large latent TGF-ß binds to the extracellular matrix through the LTBP (155). One could speculate that the LTBP is binding to matrix associated-TSP. Large latent TGF-ß bound to matrix-associated TSP could be activated by proteases present in the matrix. Additionally, the proteases may cleave the LTBP from the latent complex releasing latent TGF-ß from the matrix. Circulating TSP could then activate the latent TGF-ß freed from the matrix, in

addition to the large latent TGF-ß associated with the matrix. These possibilites remain unknown.

The components of the small latent TGF-ß complex remain associated with TSP after activation. This was shown by the ability of platelet-derived TSP containing TGF-ß activity to bind to an anti-LAP affinity column. However, platelet-derived large latent TGF-ß is not available in large quantities, therefore, we were unable to examine whether TSP dissociates active TGF-ß from the large latent complex. I would predict that activation is the same for the small and large complexes. This assumption is based on the ability of the RFK peptide to activate both forms of latent TGF-ß in a similar manner. However, the LAP-LTBP complex may not remain associated with TSP after activation. The affinity of the LAP-LTBP complex for TSP may differ from the LAP alone, resulting in the dissociation of LAP-LTBP from TSP after activation. One could speculate that the LAP-LTBP complex may bind either to an integrin (both the LAP and LTBP contain buried RGD sites) or to the mannose-6-phosphate receptor leading to internalization. These questions remain unanswered.

In vivo, TSP is a transient component of the extracellular matrix. In order for TSP to associate with the matrix in vitro, a pre-formed collagen/fibronectin matrix is required (71). Therefore, we can only speculate on the role of matrix-bound TSP in the activation of latent TGF-ß in vivo. TSP incorporated in the matrix may have a different conformation than circulating TSP. This conformation change may result in TSP that is sensitive to proteases sequestered in the matrix, which could cleave TSP into proteolytic fragments. The 50 kDa fragment (at an equimolar concentration) is a

more efficient activator of latent TGF-ß as compared to TSP. This could result in more active TGF-ß present at the cell-surface. Latent TGF-ß and active TGF-ß may also be sequestered in the matrix via binding to TSP, resulting in more efficient presentation of TGF-ß to cellular receptors. Immunofluorescence studies would indicate if TSP and latent/active TGF-ß are co-localized in the extracellular matrix. The turnover of matrix-bound TSP is a potential regulatory mechanism in the activation of latent TGF-ß.

The TSP-TGF-ß complex binds to TGF-ß and TSP cellular receptors. We do not know when or if TGF-ß is dissociated from TSP. Upon the binding of TGF-ß to its receptors, a conformation change in active TGF-ß may result in its dissociation from TSP. The internalization and degradation of TSP in the cell may also result in the dissociation of the LAP and mature TGF-ß. Perhaps they would then reform a latent complex. An additional possibility is that a protein, such as α_2-macroglobulin, which binds active TGF-ß, binds mature TGF-ß and dissociates it from TSP. Regardless, it is important to remember that TSP-TGF-ß complexes can bind to both TSP and TGF-ß receptors, leading to numerous cellular responses.

Within TSP there is a requirement for both the WSXW and the RFK sites. We have speculated that TSP-mediated activation requires two steps, namely the binding of WSXW to the active portion of TGF-ß, facilitating the interaction of RFK with the LSKL sequence in the LAP. Studies indicate that WSXW binding to active TGF-ß is the first step in the interaction. WSXW inhibits the activation of latent TGF-ß by peptide 246 (KRFKQDGGWSHWSPWSS) but not by peptide 402 (KRFK). If the interaction of RFK with LSKL was the initial step then WSXW should not

affect the ability of peptide 246 to activate latent TGF-ß. The WSXW motif is found in all three of the type 1 repeats of TSP1; therefore, any of the repeats may be mediating the initial interaction. We do not know if the interaction of WSXW with latent TGF-ß results in a conformation change within TGF-ß or TSP leading to activation by the RFK site. Only sophisticated biochemical studies can answer this question. The stoichiometry of the latent TGF-ß-TSP interaction may also contribute some insight into the interaction. However, an accurate ratio could not be obtained using recombinant latent TGF-ß due to the high percentage of anomalous disulfide bonds resulting in only 10% of the mass being functional (D. Twardzak, personal communication).

Our knowledge of the number of TSP family members is rapidly expanding. However, we showed that only TSP1 activates latent TGF-ß. As future studies identify more TSP family members, those containing type 1 repeats (specifically the RFK sequence) should be tested for the ability to activate latent TGF-ß. The complement component properdin and the malarial circumsporozoite protein have similar tertiary structures as the type 1 repeats of TSP1 and TSP2 (48,130). However, I do not know if they contain the seqeunce RFK. It would be interesting to test these proteins for activity.

Although TSP2 does not activate latent TGF-ß, we showed that it inhibits TSP1-mediated activation. In a system where both TSP1 and TSP2 are present, TSP2 could compete for the binding of latent TGF-ß to TSP1 through the WSXW sequence blocking activation. Beccause TSP1 and TSP2 are subject to differential transcriptional regulation and exhibit distinct patterns of temporal and spatial

expression, this is one potential regulatory mechanism within a complex cellular system.

The localization of the activation site to the sequence RFK may identify an important family of TGF-ß activators. Although we showed that calcineurin and albumin, both of which contain an RFK site, do not activate latent TGF-ß, there are numerous proteins that contain this sequence. Many of the proteins are viral in origin and may lead to studies showing that viruses activate latent TGF-ß. TGF-ß may be a causative agent in viral pathogenesis. The cadherin family also contains an RFK motif. One could test whether the cadherins are responsible for the cell-surface dependent activation of latent TGF-ß.

The sequence LSKL is conserved in the LAPs of TGF-$ß_{1-5}$. This is significant because the sequence homology in the LAP is only ~30%, suggesting that the LSKL site is highly conserved. TSP1 can activate both recombinant latent TGF-$ß_1$ and TGF-$ß_2$, suggesting that it is possible that TSP could activate all of the TGF-ß isoforms (88,89). This sequence is only found in the TGF-ß isoforms and is not a common sequence in the TGF-ß superfamily. Although the sequence LSKL is not present in the other family members, a sequence with a similar hydropathic profile may be present that could imitate the LSKL site leading to activation by TSP. This would be interesting to test in other systems.

We do not know what regulates the TSP-TGF-ß interaction. There are many possibilities. One involves the rapid internalization and degradation of TSP leading to inaccessibility. Another possibility may be the binding of TGF-ß or TSP to other proteins blocking the binding sites. Finally, the interaction may be regulated by other

proteins that turn off the synthesis of TSP or latent TGF-ß. The regulation of the interaction is one of the most important questions left unanswered and unfortunately one of the most difficult to resolve.

These studies identified peptides that potentially can be used in vivo to regulate TGF-ß activity. However, because all of the studies have been carried out in vitro, the question remains whether what is observed in a controlled system may hold true in an in vivo environment. There are many situations in which excess TGF-ß activity leads to health complications, including tumorigenesis and fibrosis. These conditions, and those in which TGF-ß activity would be beneficial, would be important models to determine the usefulness of the TSP antagonists and activators as therapeutic agents.

A physiological system where TSP-TGF-ß interactions may be important is wound healing. There are four major components in wound repair: migration and proliferation of fibroblasts, increased extracellular matrix deposition, angiogenesis, and remodeling. TGF-ß is involved in all of these steps, thus promoting wound repair. TGF-ß is primarily growth inhibitory. However, in low concentrations it induces the synthesis of PDGF and is thus indirectly mitogenic (88,89,129). Increased matrix deposition and angiogenesis are hallmarks of TGF-ß activity. Platelets and the wounded cells secrete TGF-ß in a latent form. However, there is a high concentration of TGF-ß activity at the wound site. It is not known how the TGF-ß becomes activated. TSP is also secreted by platelets and wounded cells. Therefore, I propose that TSP is involved in activating latent TGF-ß during wound healing.

When a wound occurs, platelets are recruited to the wound site, bind to the subendothelial matrix components, and become activated releasing the α-granule components. Previously, we showed that TGF-ß activity co-purifies with TSP from the α-granules of stimulated platelets (106). These results contradict previous studies showing that the TGF-ß present in the α-granules is primarily in a latent form (103,120,167). We speculate that the TGF-ß-TSP interaction occurs within the platelet and is not a result of thrombin-activation. This is a result of studies showing that TSP-TGF-ß complexes are present in the platelet releasates from Ca^{2+} ionophore-stimulated platelets. TSP is most likely bound to large latent TGF-ß, the primary form in human platelets (103,165). TSP may serve to localize the TGF-ß to wounded tissue and may also increase its half-life in vivo. TSP and latent TGF-ß are both present in high concentrations within platelets, thus more TGF-ß should be released from the α-granule in an active form. The reason why we do not see this is unknown. However we have observed that TSP is associated with other α-granule components, including platelet derived growth factor and platelet factor-4 (unpublished results). This binding of other proteins may lead to the masking of the KRFK/GGWSHW site within TSP, resulting in the inability to activate the majority of the latent TGF-ß. Additionally, the activation of latent TGF-ß by TSP is not enzymatic; thus, once TSP binds/activates latent TGF-ß, it may be incapable of interacting with another latent molecule. Alternatively, latent TGF-ß may be complexed to other proteins and inaccessible.

Once the platelet degranulates, latent TGF-ß and the TSP-TGF-ß complex are released into the wound. The TSP-TGF-ß complex may be targeted to TGF-ß cell

surface receptors resulting in the increased synthesis of TSP and TGF-ß. This would serve to increase local TSP concentrations. Indeed, TSP is found on the surface of cells at the injured site within 1 h after injury (71). The level of cell-associated TSP continues to increase, and then TSP becomes transiently incorporated into the matrix or disappears completely depending on the tissue type (71). The removal of TSP most likely occurs through receptor-mediated endocytosis and degradation (103). Latent TGF-ß secreted by the wounded cells and by the degranulated platelets could either be bound to the cell surface, associated with the extracellular matrix, or found circulating within the wound environment. Latent TGF-ß has a relatively long half-life in vivo, therefore, one could speculate that latent TGF-ß would still be present within the wound during TSP secretion. Cell-secreted TSP could activate the latent TGF-ß present at the cell surface, as well as that found in the circulation, thus increasing the concentration of active TGF-ß at the injured site. Our previous studies showed that TSP activates latent TGF-ß in the presence of high concentrations of serum (20%). Once activated, the dissociated active TGF-ß binds to cellular receptors or to proteins such as α_2-macroglobulin or decorin, which inhibit TGF-ß activity and also increase its half-life. The rate-limiting factor within this environment appears to be the TSP concentration. Although TSP is secreted by proliferating cells in vitro in nM concentrations, the levels of latent TGF-ß circulating in the serum and secreted by the cells may be much higher ($\sim$20 μg/g platelets and $\sim$1.5 ng/ml from non-platelet sources, 166 and 168). Thus, the interaction is dependent on the amount of available TSP.

Within this system there are many questions left unanswered. It is not known what the fate of the TSP-LAP complex is. We know that TSP can bind to its receptors even in the presence of LAP. An additional factor is that the wounded cells may secrete other TGF-ß isoforms. This is an important consideration because TGF-$ß_1$ and TGF-$ß_2$ have different roles in wound healing. Both forms enhance wound healing, but TGF-$ß_1$ causes excessive granulation tissue deposition, while TGF-$ß_2$ does not. It is not known whether other active TGF-ß isoforms bind TSP, specifically through the GGWSHW site. This would be important to determine before using the peptides in a therapeutic manner. The KRFK peptides could be used to increase TGF-ß activity, thus enhancing wound healing in situations such as diabetic ulceration where scarring is not a concern. After healing is initiated, the GGWSHW or LSKL peptides could be used during cosmetic surgery to reduce the potential scarring caused by TGF-$ß_1$. In vivo wound healing studies with the TSP peptides are currently in progress. If these studies in the rat prove promising, more extensive studies will be undertaken.

In these studies, we have described a potentially important physiologic activator of latent TGF-ß. Future studies include: determining the binding constant and stoichiometry of the latent TGF-ß-TSP interaction, elucidating the regulation of the interaction, and examining the conformation change leading to activation in more detail. I feel that more questions have been generated than answered through my studies. Unfortunately, I am leaving at an exciting time in the project. In the future, I would like to see the KRFK-containing peptides applied to a Sesame Street band-aid in order to enhance wound healing.

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Dissertation Committee:

Joanne E. Murphy-Ullrich, Chairman

Tom Wenokur

John R. Couchman

Thomas N. Wight

Ann Wells

Director of Graduate Program Ronald M. Lynch

Dean, UAB Graduate School John R. Roberts

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