The Tryptophan-rich Motifs of the Thrombospondin Type 1 Repeats Bind VLAL Motifs in the Latent Transforming Growth Factor-β Complex

Received for publication, May 3, 2004, and in revised form, August 11, 2004
Published, JBC Papers in Press, September 1, 2004, DOI 10.1074/jbc.M404918200

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Transforming growth factor-β (TGF-β) is secreted as a latent complex of the latency-associated peptide (LAP) and the mature domain, which must be activated for TGF-β to signal. We previously identified thrombospondin 1 (TSP1) as a physiologic activator of TGF-β in vitro and in vivo. The WSXW sequences in the type 1 repeats of TSP1 interact with the mature domain of TGF-β, and WSXW peptides inhibit TSP1-mediated activation by blocking TSP1 binding to the TGF-β latent complex. However, the binding site for the WSXW sequence was not identified. In this report, we show that the WSXW sequences bind the 61VLAL sequence in mature TGF-β and also bind 77VLAL in LAP. A glutathione S-transferase (GST) fusion protein of the second TSP1 type 1 repeat (GST-TSR2) binds immobilized VLAL peptide. VLAL peptides inhibit binding of LAP and mature TGF-β to soluble GST-TSR2 and immobilized WSXW peptide. VLAL peptide inhibits TSP1-mediated activation of recombinant and endothelial cell-derived latent TGF-β. Furthermore, TGF-β or LAP deleted in the VLAL sequence fails to bind immobilized WSXW or soluble GST-TSR2, indicating that binding to both VLAL sequences is important for association of TSP1 and the latent complex. Additionally, TSP1 is unable to activate latent TGF-β when VLAL is deleted from the mature domain. These data show that the WSXW motif binds VLAL on both LAP and mature TGF-β, and these interactions are critical for TSP1-mediated activation of the TGF-β latent complex.

TGF-β is a cytokine involved in diverse processes, including development, angiogenesis, wound healing, inflammation, neoplasia, and fibrosis (1–4). TGF-β is a member of a large family of related peptide growth factors, including activins, inhibins, and bone morphogenetic proteins (1). There are five isoforms of TGF-β, including the three mammalian isoforms, TGF-β1, -2, and -3. Mature TGF-β is secreted as part of the small latent complex with latency-associated peptide (LAP) (5). The small latent complex can also be bound to latent TGF-β-binding protein to form the large latent complex (6). Mature TGF-β and LAP are the products of a single gene that undergoes extensive post-translational modification, including cleavage of the LAP from the mature domain by furin convertase (7). Despite this cleavage, LAP and TGF-β remain noncovalently associated in a manner that confers latency.

Mechanisms that involve conversion of the latent complex to a physiologically active form represent a major point of regulation of appropriate TGF-β activity levels (8, 9). Since LAP association with the mature domain is critical for latency, agents that activate the latent complex disrupt the association of LAP with the mature domain either by cleaving or denaturing LAP or by altering the folding of LAP (8). Mechanisms of in vivo TGF-β activation include proteolysis, integrin binding, and interactions with the matricellular protein, thrombospondin 1 (TSP1) (8, 10, 11).

TSPs are a family of secreted glycoproteins present in the α-granules of platelets and wound fluids (12–14). TSP1, the best characterized family member, has diverse effects on cell behavior (15). We previously identified that TSP1 binds the TGF-β latent complex in both the mature domain and the LAP (16, 17). As a result of TSP1 binding to the latent complex, the latent complex becomes activated, both in vitro and in vivo (11, 16, 17). These interactions are complex and involve multiple sequences in TSP1 (18). The KRFK sequence in the type 1 repeats (TSRs) of TSP1 binds LAP through the LSKL sequence, resulting in biologically active TGF-β (17). We also showed that tryptophan-rich (WSXW) sequences present in each of the TSRs bind to the mature domain (16). Unlike the KRFK sequence, the WSXW sequence is not sufficient to activate latent TGF-β. However, the WSXW sequence is important for activation by TSP1, since the WSXW sequence, when expressed as a peptide, competitively blocks both TSP1 binding to the mature domain and activation of the latent TGF-β complex by TSP1 (16, 17). Furthermore, mutation of the WSXW sequence in peptides containing the KRFK activation sequence renders the KRFK sequence significantly less effective at activating latent TGF-β (16).

The WSXW sequences are present in all of the TSRs of both TSP1 and TSP2. In fact, TSP2, which lacks the KRFK activation sequence, can inhibit the activation of TGF-β by TSP1 in vitro, presumably due to TSP2 binding to latent TGF-β through the tryptophan-rich motifs (16). The crystal structure of the second and third TSR has recently been solved; the structure of the TSRs suggests that the WSXW sequences are on exposed surfaces and potentially available for binding interactions (19). This is consistent with other published data showing that the
tryptophan-rich motifs bind heparin and sulfated proteoglycans (20, 21) and inhibit the interaction of TSP with the gelatin binding domain of fibronectin (22). Interestingly, the WSXX motif is common to the cytokine receptor superfamilies (23).

Despite the importance of the WSXX sequence for TSP1-dependent TGF-β activation and the utility of the WSXX peptides in inhibiting activation, WSXX interactions with the TGF-β latent complex are poorly understood. In this work, we now identify the binding site for the WSXX sequence in mature TGF-β as the VLAL sequence. Unexpectedly, this sequence is also present in the LAP, and we now show that LAP also binds to the TSRs through the WSXX sequence. VLAL peptides and VLAL mutations inhibit binding of the latent complex to WSXX and the type 1 repeats. Also, VLAL peptides and deletion of VLAL in the mature domain inhibit TSP1-mediated activation of the latent TGF-β complex. These data support the model that VLAL/WSXX interactions are necessary for proper association of TSP1 and the latent complex and are critical for TSP1-mediated TGF-β activation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cells were cultured in Dulbecco’s modified Eagle’s medium containing 4.5 g/liter glucose, 2 mM glutamine, and 10% fetal bovine serum. Bovine aortic endothelial (BAE) cells were isolated and cultured as described (17). COS-1 cells were obtained from the ATCC (CRL-1650). TGF-β1 (−/−) mouse embryonic fibroblasts were a generous gift of Dr. John Munger (New York University). Mink lung epithelial cells (Mv1Lu) transfected with the TGF-β response element of the human plasminogen activator inhibitor-1 gene promoter fused to the firefly luciferase reporter gene were a generous gift from Dr. Daniel Rifkind (New York University) (24) and were cultured in the above medium supplemented with 200 μg/ml G-418 (Invitrogen).

**Peptides**—Peptides, purified (>95% purity) and analyzed by mass spectrometry, were purchased from AnaSpec, Inc. (Palo Alto, CA).

**Thrombospondin Purification**—TSP1 stripped of TGF-β activity (sTSP) was purified as described from human platelets purchased from the American Red Cross (25). sTSP purity was assessed by SDS-PAGE and Coomassie Blue staining. Depletion of TGF-β activity was confirmed by a plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay, which showed less than 1 pM TGF-β activity in 10 nM sTSP.

**Fusion Protein Preparation**—Proteins containing GST fused to the second type 1 repeat of thrombospondin 1 were a generous gift of Dr. Jack Lawler (Harvard University) and were prepared exactly as described (26). The resulting protein, GST-TSR2, migrated as a 37-kDa band in SDS-PAGE under nonreducing conditions.

**Production of the FLAG-TGF-β Latent Complex**—Full-length cDNA for human TGF-β1 latent complex (TGF-β and LAP) was a gift from Dr. Lalage Wakefield (NCI, National Institutes of Health) (27). Using techniques similar to those previously reported, a FLAG epitope was introduced at the N terminal of the mature cytokine after a 5-amino acid spacer, separating it from the LAP cleavage site (28). The purified PCR product was then inserted, according to the manufacturer’s instructions, into the mammalian expression vector pEF6/V5-His-TOPO (Invitrogen). Resulting plasmids were verified by DNA sequencing at the University of Alabama Birmingham Center for Aids Research DNA sequencing facility. Placement of the FLAG epitope at this site has been shown not to affect expression and function of active TGF-β1 (28). Using similar protocols, we expressed the construct in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the PAIL assay was used to verify latency and activation by heat, acid, and TSP1.

**Production of LAP-FLAG**—Using the human TGF-β1 latent complex as a template, a FLAG epitope/termination codon was introduced following the LAP coding sequence. The resulting PCR product was cloned into pEF6/V5-His-TOPO (Invitrogen) as described above and sequenced for verification. LAP-FLAG protein expression in transfected COS-1 cells was confirmed by immunoblot, and the ability to confer latency to active TGF-β was verified by the PAIL assay.

**Site-directed Mutagenesis and DNA Purification**—Using FLAG-TGF-β and LAP-FLAG vectors as templates, primers were designed to produce site-specific mutations or deletions using the QuikChange kit (Stratagene). All mutant clones were verified by sequencing. Before transfection, all plasmids were produced, purified free of endotoxin, and concentrated according to the manufacturer’s instructions (Qiagen).

**Transfection and Conditioned Medium Collection**—Upon reaching 70% confluence in 100-mm dishes, COS-1 cells were washed in serum-free medium and transfected with 2 μg of DNA and 6 μl of Fugene-6 (Roche Applied Science) per the manufacturer’s instructions in a final volume of 5 ml of serum-free Dulbecco’s modified Eagle’s medium with ITS (insulin, transferrin, selenium) supplement (Sigma). After 48 h, the

**Fig. 1.** TGF-β1 latent complex binds WSXX. Binding of 25 pM purified mature FLAG-TGF-β1 (A) or LAP-FLAG in TGF-β1 (−/−) conditioned media (B) to immobilized WSXX peptides (biotin-GGWSHW). Data (absorbance at 450 nm) are expressed as the means of triplicate determinations ± S.D. Binding to blank wells, soluble WSXX peptide (GGWSHW) and an immobilized modified sequence (biotin-GGWASHA) are included as controls (*, P < 0.0002). Additional controls for nonspecific binding of WSXX (side panels) include 5 μM FLAG peptide, 0.5 μM recombinant LAP, or 0.1 μM recombinant TGF-β1 substituted for the FLAG-tagged proteins (n = 3).

**Table I**

| VLXX motifs in the mature domain of vertebrate TGF-β isoforms |
|---------------------------------------------------------------|
| Homo sapiens | TGF-β1 | TGF-β2 | TGF-β3 |
| Sus scrofa   | 60KVLALYN | 60RVLALYN | 60TVLGLYN |
| Mus musculus | 60KVLALYN | 60RVLALYN | 60TVLGLYN |
| Rattus norvegicus | 60KVLALYN | 60RVLALYN | 60TVLGLYN |
| Gallus gallus | 60KVLALYN | 60RVLALYN | 60TVLGLYN |
| Consensus    | VLAL   | VLSL   | VLGL   |
conditioned media were collected, centrifuged free of cell debris, and stored for analyses. TGF-β1 (+/−/+), 70% confluent on 6-well plates, were transfected overnight with 1 μg of DNA using LipofectAMINE/Plus and Opti-MEM as per instructions (Invitrogen). Wells were then washed with serum-free media, followed by the addition of 1 ml/well serum-free media containing ITS. After 48 h, media were collected, centrifuged free of cell debris, and stored. BAE cells were seeded in 100-mm dishes, grown to 70% confluence, and washed with serum-free media. A final volume of 5 ml of serum-free media was placed on the cells for 48 h, collected, centrifuged free of cell debris, and stored at 80 °C until analysis.

**FLAG-TGF-β Purification**—Recombinant FLAG-TGF-β or FLAG-TGF-β2VLAL was purified from conditioned media by anti-FLAG M2 affinity gel according to the manufacturer’s instructions (Sigma). Briefly, 15 ml of conditioned media was concentrated to 5 ml using 6-ml Vivaspin columns (Vivascience). The resulting concentrated medium was incubated overnight at 4 °C with 200 μl of prewashed anti-FLAG M2 resin. The following morning, the resin was washed three times in TBS. Bound FLAG-TGF-β was released by incubation in 200 μl of 0.1 M glycine HCl, pH 3.5. These acidic conditions also denature any associated LAP. 22 μl of 10× TBS was added, and the purified protein was stored at −80 °C until use. Purity was determined by SDS-PAGE and immunoblot with antibodies against TGF-β and FLAG. Protein concentration and activity were verified by the PAIL assay.

**Immunoblotting**—Conditioned media were added to 6× Laemmli sample buffer, and a 50-μl final volume was separated by SDS-PAGE (4–20% gradient) under nonreducing conditions. Proteins were electrophoretically transferred to nitrocellulose membranes and then blocked for 1 h in 5% nonfat dry milk in Tris-buffered saline with 0.05% Tween 20 (TBST). Membranes were incubated with primary antibody, 12.5 μg of mouse anti-TGF-β (R&D Systems), or 2 μg of goat anti-human LAP (R&D Systems), overnight in 10 ml of TBST plus 2.5% nonfat dry milk at 4 °C. Membranes were washed (four 10-min washes in TBST) and then incubated for 45 min with an appropriate horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (Jackson Immunoresearch) diluted 1:10,000 in 10 ml of TBST plus 0.05% Tween 20 (TBST). Membranes were incubated with primary antibody, 12.5 μg of mouse anti-TGF-β (R&D Systems), or 2 μg of goat anti-human LAP (R&D Systems), overnight in 10 ml of TBST plus 2.5% nonfat dry milk at 4 °C. Membranes were washed (four 10-min washes in TBST) and then incubated for 45 min with an appropriate horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (Jackson Immunoresearch) diluted 1:10,000 in 10 ml of TBST plus 2.5% nonfat dry milk. For anti-FLAG blots and anti-GST blots, the membrane was blocked overnight, washed, and incubated with either a 1:2000 dilution of anti-FLAG-M2-peroxidase conjugate (Sigma) or a 1:5000 dilution of anti-GST peroxidase conjugate (Amersham Biosciences) in 10 ml of

**Table II**

|              | β1 LAP | β2 LAP | β3 LAP |
|--------------|--------|--------|--------|
| H. sapiens   | AVLALYN| EVDISYN| AVLALYN|
| S. scrofa    | AVLALYN| EVDISYN| AVLALYN|
| M. musculus  | AVLALYN| EVDISYN| AVLALYN|
| R. norvegicus| AVLALYN| EVDISYN| AVLALYN|
| G. gallus    | DVLALYN| EVDISYN| I I LALYN|
| Consensus    | VLAL   | VISI   | VLAL   |

**Fig. 2.** The TSP1 type 1 repeats bind VLAL. A, wire diagrams from the published structures of TGF-β1 and the second and third type 1 repeats of TSP. The arrows indicate the helical VLAL motifs of TGF-β and the tryptophan-rich motifs (WSXW) of the type 1 repeats. Reprinted with permission from the authors (19, 29). B, binding of 100 nM GST-TSR2 to the immobilized VLAL sequence from mature TGF-β1 (biotin-KVLALYN). Data (absorbance at 450 nm) are expressed as the means of triplicate determinations ± S.D. Binding to blank wells, soluble peptide (KVLALYNK), and an immobilized modified sequence (biotin-KVLAGYNYK) are included as controls (*p < 0.001). As an additional control for nonspecific binding of VLAL (side panel), 1 μl GST was substituted for GST-TSR2 (n = 3).
TBST for 45 min. Immunoblots were developed by enhanced chemiluminescence (PerkinElmer Life Sciences). Blots probed with more than one antibody were stripped between developments with Re-blot Plus (Mild) Solution according to the manufacturer’s instructions (Chemicon). Densitometry was performed using the one-dimensional gel analysis function of ImageJ (National Institutes of Health).

**PAIL Assay for TGF-β Activity**—TGF-β activity in conditioned media was determined by an established bioassay using mink lung epithelial cells expressing the TGF-β response element of the plasminogen activator inhibitor-1 promoter fused to luciferase as a reporter (24). Human TGF-β1 (R&D Systems) was used to establish the standard curve. Total TGF-β levels were assayed in conditioned media that had been heat-activated at 100 °C for 3 min and then diluted 1:10 in serum-free media. Levels of active TGF-β were determined in media diluted 1:5 that received no heat treatment. Recombinant latent TGF-β or recombinant active TGF-β (R&D Systems) was diluted in serum-free Dulbecco’s modified Eagle’s medium to the indicated concentrations. VLAL and control peptides were incubated with purified stripped TSP1 or KRKF peptide for 30 min. The preceding mixtures were then incubated with conditioned media or latent TGF-β for 30 min to assess activation of TGF-β by TSP1 or KRKF.

**Immunoprecipitation**—In order to detect soluble protein-protein interactions, either 25 pm purified FLAG-TGF-β or FLAG-TGF-β/VLAL in 500 µl of Dulbecco’s modified Eagle’s medium or 500 µl of TGF-β1 (-/-) conditioned medium expressing LAP-FLAG or ∆VLAL LAP-FLAG was incubated for 1 h at room temperature with 100 nm GST-TSR2. 20 µl (final volume) of prewashed anti-FLAG M2 affinity gel was used to pull-down the indicated protein. Resin was centrifuged and washed in TBS three times. The washed resin was incubated with 20 µl of 0.1 M glycine, pH 3.5, to release any bound complexes. The eluate was combined with 2.2 µl of 10× TBS and 6× SDS Laemmli sample buffer and analyzed by immunoblotting. In some experiments, peptides were added and incubated for 30 min at room temperature prior to the addition of the gel loading buffer. Equal pull-down of FLAG-TGF-β or LAP-FLAG was verified by FLAG immunoblot/densitometry. Negative controls included resin alone and resin incubated with 100 nm GST-TSR2. 500 ng of recombinant GST-TSR2 was used as a positive control.

**Modified Enzyme-linked Immunosorbent Assay**—In order to detect immobilized protein-protein interactions, we developed a novel modified enzyme-linked immunosorbent assay for these studies. Briefly, biotin-tagged peptides (10 µg/ml) were incubated on preblocked neutravidin-coated plates overnight at 4 °C (Pierce). After three washes with TBST, conditioned media containing either 25 pm purified FLAG-TGF-β or FLAG-TGF-β ∆VLAL in TBS, TGF-β1 (-/-) conditioned medium expressing LAP-FLAG or ∆VLAL LAP-FLAG, or 100 nm GST-TSR2 in TBS was incubated for 3 h at 4 °C. Following three washes with TBST, wells were incubated in 1:1000 anti-FLAG M2 peroxidase conjugate or 1:5000 anti-GST peroxidase conjugate in TBST. After five washes in TBST, wells were incubated for 30 min in OPD substrate (Sigma). The reaction was stopped with 1 N HCl, and absorbance was determined using a spectrophotometric plate reader (Bio-Tek). After background subtraction, the mean absorbance at 450 nm of triplicate wells was reported. In some experiments, conditioned media were incubated with peptides for 45 min at 4 °C prior to the addition to the wells. Biotin-tagged peptides were analyzed for equal binding to the plate by detection with Streptavidin-horseradish peroxidase. Controls included wells incubated with each reagent alone or wells coated with non-biotin-tagged peptides. To assess any nonspecific binding, 5 µM ovalbumin, 0.5 µM recombinant LAP (R&D Systems), 0.1 µM recombinant TGF-β1 (R&D Systems), 5 µM FLAG peptide (Sigma), or 1 µM GST was incubated with immobilized biotin-tagged peptides.

**Statistics**—Significance was determined by using the paired, two-tailed Student’s t test. All error bars shown represent S.D.
**Tryptophan-rich Motifs of TSP Bind VLAL of Latent TGF-β**

**RESULTS**

Both Mature TGF-β and LAP Bind WSXW—In order to detect the association of TGF-β and WSXW, as has been previously reported (16), a modified enzyme-linked immunosorbent assay was employed. Biotin-tagged peptides containing the sequence, GGWSHW, from the tryptophan-rich motif of the TSRs, were immobilized to neutravidin-coated plates. Purified FLAG-tagged mature TGF-β1 (FLAG-TGF-β1) was then added to each well and detected with an anti-FLAG antibody conjugated to peroxidase. Binding, as indicated by absorbance at 450 nm, was highest between TGF-β and the immobilized tryptophan motif peptide (biotin-GGWSHW) (Fig. 1A). Binding to peptides in which the tryptophan was substituted with alanine (biotin-GGASHA) was significantly reduced (p < 0.002). There was no significant binding to soluble tryptophan motif peptide (GGWSHW) or uncoated wells. Finally, to demonstrate that the detection of FLAG-TGF-β bound to immobilized GGWSHW is specific, FLAG peptide or unlabeled recombinant TGF-β1 was substituted for FLAG-TGF-β, and binding levels were similar to uncoated wells. There was also no detectable binding of an irrelevant protein (ovalbumin) to wells coated with biotin-GGWSHW (data not shown).

TSP1 is able to form a ternary complex with both LAP and mature TGF-β (17). Therefore, the ability of LAP to bind the tryptophan motif was also tested. Utilizing a similar assay, recombinant LAP with a C-terminal FLAG-tag (LAP-FLAG) was produced in TGF-β1 (-/-) cells to avoid complications of endogenous TGF-β1. Conditioned medium obtained from these cells was heat-activated and tested in the PAIL assay to verify lack of endogenous TGF-β (data not shown). The modified enzyme-linked immunosorbent assay was used to test the ability of LAP-FLAG to bind the immobilized tryptophan motif. LAP-FLAG binding to the immobilized tryptophan peptide (biotin-GGWSHW) was greater than binding to the control peptide (biotin-GGASHA) (Fig. 1B). As with FLAG-TGF-β, no significant binding of LAP-FLAG to soluble peptide (GGWSHW) or uncoated wells was observed. There was no detectable binding between immobilized GGWSHW and unlabelled recombinant LAP or FLAG peptide in this assay. These data show that both FLAG-TGF-β and LAP-FLAG bind the immobilized WSXW motif.

**Analysis of the TGF-β Sequence for Potential WSXW Binding Sequences**—Since the WSXW sequence binds to both the LAP and the mature domain, the sequence of the latent complex was scanned to determine whether there might be a sequence common to both proteins. The sequence VLAL is present in both the LAP (amino acids 77–80) and the mature domain (amino acids 61–64). The VLXL motif is conserved in the mature domain of vertebrate TGF-β1 to -3 isoforms (Table I). Each VLXL sequence begins at amino acid 61 of the mature domain and has only a conserved variation (Ala, Ser, or Gly) in the third position. The LAP VLXL motifs (Table II) are conserved in vertebrate β1 and β3 Laps. The β2 LAP sequence (VISI) contains conservative isoleucine for leucine substitutions. The VLAL sequence in the mature TGF-β1 dimer is part of a three-turn a-helix (Fig. 2A), which is in an exposed region of the molecule where the subunits of the dimer interface (29).

According to Tan et al. (19), the WSXW sequences of the second and third TSRs are exposed and oriented so that the polar atoms of the tryptophans are available for potential ligand binding (Fig. 2A). As mentioned previously, these tryptophan-rich regions are common to all three of the TSRS of TSP1 (Table III) and TSP2 (Table IV). The sequence WSHWSWPW in the second TSR of both TSP1 and TSP2 is the most conserved across species. Together, these observations suggest that the VLAL sequences in TGF-β might constitute a binding site for the WSXW sequence of TSP.

**The Type 1 Repeats of TSP1 Bind VLAL**—In order to determine whether the TSRS bind VLAL, biotin-tagged peptides containing the proposed binding region of mature TGF-β (KV-LALYN) synthesized with a C-terminal lysine to increase peptide solubility were immobilized on neutravidin-coated plates. Binding of a GST fusion protein expressing the second TSR of TSP1 (GST-TSR2) to the immobilized VLAL-containing peptide was tested. GST-TSR2 contains the sequence GGWSHW, which is identical to the sequence in the second type 1 repeat of TSP2. Constructs of the TSRS had previously been shown to bind to the TGF-β latent complex (16). Bound GST-TSR2 was detected by anti-GST antibody conjugated to peroxidase. Binding was observed between GST-TSR2 and the immobilized VLAL-containing sequence (biotin-KV-LALYNK) (Fig. 2B). This association appears specific, since there is only background binding to a control peptide with leucine to glycine substitutions (biotin-KV-QA-LYNK). This substitution was chosen because glycine residues have no bulky side chains and are less hydrophobic than leucine. Furthermore, there was no significant binding of GST-TSR2 to soluble peptide (KV-LALYNK) or uncoated wells. To assess the effects of GST alone, immobilized biotin-KV-LALYNK was incubated with GST protein, and no significant binding was observed. Thus, these data show that the second thrombospondin type 1 repeat is able to bind immobilized VLAL sequences.
VLAL Peptide Inhibits the Association of Mature TGF-β/H9252 with GST-TSR2 and WSXW Peptides—In order to determine whether soluble VLAL peptides inhibit the association of the TSP1 type 1 repeats and mature TGF-β/H9252, purified FLAG-tagged mature TGF-β/H9252 (FLAG-TGF-β/H9252) was incubated with GST-TSR2 in the presence or absence of VLAL peptide, complexes were isolated by immunoprecipitation with anti-FLAG resin, and associated GST-TSR2 was detected by immunoblot with anti-GST. Total FLAG-TGF-β/H9252 pulled down was detected with anti-FLAG antibody. Under these conditions, there was association between mature TGF-β/H9252 and soluble TSR2 (Fig. 3A). Increasing concentrations of VLAL peptides incubated with TGF-β/H9252 and TSR2 prevented complex formation in a dose-dependent manner (Fig. 3A). Inhibition of TGF-β/H9252-TSR2 complex formation is specific to the VLAL peptide, since there was no inhibition with the VGAL control peptide. These data show that TSR2 binds to the VLAL sequence and that soluble VLAL inhibits association of mature TGF-β/H9252 and TSR2.

VLAL Peptide Inhibits the Association of Mature TGF-β with GST-TSR2 and WSXW Peptides—In order to determine whether soluble VLAL peptides inhibit the association of the TSP1 type 1 repeats and mature TGF-β, purified FLAG-tagged mature TGF-β (FLAG-TGF-β) was incubated with GST-TSR2 in the presence or absence of VLAL peptide, complexes were isolated by immunoprecipitation with anti-FLAG resin, and associated GST-TSR2 was detected by immunoblot with anti-GST. Total FLAG-TGF-β pulled down was detected with anti-FLAG antibody. Under these conditions, there was association between mature TGF-β and soluble TSR2 (Fig. 3A). Increasing concentrations of VLAL peptides incubated with TGF-β and TSR2 prevented complex formation in a dose-dependent manner (Fig. 3A). Inhibition of TGF-β-TSR2 complex formation is specific to the VLAL peptide, since there was no inhibition with the VGAL control peptide. These data show that TSR2 binds to the VLAL sequence and that soluble VLAL inhibits association of mature TGF-β and TSR2.
We next sought to determine whether VLAL can inhibit association of mature TGF-β with the WSXW sequence itself. Immobilized GGWSHW was incubated with purified FLAG-TGF-β in the presence or absence of increasing concentrations of VLAL peptide (Fig. 3B). The VLAL peptide, but not the control VGAL peptide, inhibited binding of mature FLAG-TGF-β to immobilized WSXW. These data suggest that the VLAL peptide is able to inhibit association of mature TGF-β with the tryptophan-rich motif.

VLAL Inhibits the Association of LAP with the Tryptophan-rich Motifs and the Type 1 Repeats of TSP1

VLAL and FLAG-TGF-βΔVLAL were incubated with GST-TSR2. LAP-GST-TSR2 complexes were isolated by immunoprecipitation with anti-FLAG resin, and bound GST-TSR2 was detected by immunoblot with anti-GST antibody (Fig. 4A). Total LAP-pulled down was detected with anti-FLAG antibody. LAP-TSR2 forms complexes; however, complex formation was inhibited by the VLAL peptide. Total LAP-FLAG pulled down was detected with anti-FLAG antibody. LAP-TSR2 forms complexes; however, complex formation was inhibited in the presence of increasing concentrations of VLAL peptide (Fig. 4A). Inhibition was specific to the VLAL peptide, since VGAL control peptide did not prevent LAP-TSR2 complex formation. These data show that the VLAL peptide blocks association of TSR2 with mature TGF-β and LAP.

To determine whether the VLAL peptide inhibited LAP binding to the WSXW peptide, immobilized GGWSHW was incubated in the absence or presence of increasing concentrations of VLAL peptide and then examined for the ability to bind LAP-FLAG in the conditioned media of TGF-β(+/−/+) cells (Fig. 4B). The VLAL peptide, but not the control peptide (VGAL), inhibited binding to immobilized GGWSHW peptide (Fig. 4B). These data suggest that the VLAL sequence in the LAP is important for LAP-TSP interactions through WSXW sequences of the TSRs.

VLAL Peptides Inhibit TSP1-mediated Activation of Recombinant and Endogenous Latent TGF-β

Peptides containing both WSHW and the activation sequence (KRFK) are significantly less efficient at activation if the WSXW motif is substituted (16). Since the VLAL peptide blocks association of TSR2 with mature TGF-β and LAP, one would predict that the VLAL peptide would inhibit activation of latent TGF-β by TSP1. Increasing concentrations of VLAL peptides inhibit activation of recombinant latent TGF-β by sTSP1 (Fig. 5A). The IC50 for this dose-dependent response is ∼0.1 μM. Inhibition is specific to the VLAL sequence, since the VGAL control peptide did not inhibit activation. In addition, VLAL peptides were unable to inhibit activation of latent TGF-β by KR1 peptides (Fig. 5B). This result was expected, since KR1 peptides do not require VLAL/WSXW interactions for activation.
orientation as does TSP1. To determine whether the inhibitory ability of the VLAL peptide was specific to TSP1-mediated activation, the latent complex was activated with plasmin in the presence of increasing concentrations of VLAL; VLAL peptide had no effect (Fig. 5C). Finally, VLAL peptides did not interfere with TGF-β receptor binding or signaling, since there was no inhibition of TGF-β activity when VLAL was incubated with active TGF-β (Fig. 5D).

To test the effects of VLAL on TSP1-mediated activation in a cell culture system, the ability of VLAL peptides to block TSP1-dependent activation of BAE cell-secreted latent TGF-β was assayed. αTSP-activated BAE cell-derived latent TGF-β in the conditioned media (Fig. 6A) and VLAL, but not control VGAL peptide, inhibited TSP1-mediated activation in a dose-dependent manner with an approximate IC₅₀ of 0.5 μM. Concentrations of VLAL peptide above 20 nM did not further suppress TGF-β activity. VLAL peptides also had no effect on basal levels of TGF-β activity in BAE conditioned media (Fig. 6B). In addition, the VLAL peptide in serum-free media had no effect on the Mv1Lu reporter cells used in the PAIL assay (not shown). These data indicate that VLAL peptides are able to inhibit TSP1-mediated activation of recombinant and endothelial cell-derived latent TGF-β.

**Mature TGF-β Mutated in VLAL Has Impaired Binding to WSXW Peptides and TSR2**—LAP-FLAG and LAP-FLAG deleted in the VLAL sequence (ΔVLAL LAP-FLAG) were expressed in TGF-β1 (-/-) cells, and binding to the WSXW peptide and TSR2 fusion protein was assayed. Conditioned media containing either LAP-FLAG or ΔVLAL LAP-FLAG were incubated with immobilized WSXW (biotin-GGWSHW) or soluble WSXW (GWSHW) (Fig. 8A). The binding observed between LAP-FLAG and immobilized WSXW is completely abrogated when VLAL is deleted from the mature domain (p < 0.006). In addition, we were unable to detect association of GST-TSR2 with purified FLAG-TGF-β deleted in VLAL (Fig. 7B). Thus, these data show that the VLAL sequence in the mature domain is important for interactions with WSXW and the TSR2.

**LAP Mutated in VLAL Has Impaired Binding to WSXW Peptides and TSR2**—LAP-FLAG and LAP-FLAG deleted in the VLAL sequence (ΔVLAL LAP-FLAG) were expressed in TGF-β1 (-/-) cells to immobilized WSXW (biotin-GGWSHW) or soluble WSXW (GGWSHW) (Fig. 7A). The binding observed between FLAG-TGF-β and immobilized WSXW is completely abrogated when VLAL is deleted from the mature domain (p < 0.006). In addition, we were unable to detect association of soluble GST-TSR2 with ΔVLAL LAP-FLAG in conditioned media from TGF-β1 (-/-) cells (Fig. 7B).
Tryptophan-rich motifs of TSP Bind VLAL of Latent TGF-β

8B). These data therefore indicate that the VLAL sequence of LAP is necessary for interactions with the WSXXW sequences of the type 1 repeats of TSP1.

The Latent TGF-β Complex Deleted in VLAL Cannot Be Activated by TSP1—FLAG-tagged TGF-β latent complex (FLAG-TGF-β LC) and FLAG-tagged TGF-β latent complex deleted in the VLAL sequence of the mature domain (FLAG-TGF-β ΔVLAL LC) were expressed in COS-1 cells (Fig. 9A), and activation by TSP1 and KRFK peptides was assessed. Deletion of VLAL from the mature domain of the latent complex resulted in the loss of ability to be activated by TSP1 (Fig. 9B). sTSP does not increase TGF-β activity above background (empty vector, EV) in the conditioned media of cells expressing FLAG-TGF-β ΔVLAL latent complex. In contrast, sTSP increased TGF-β activity 2.6 fold above background in cells expressing wild type FLAG-TGF-β latent complex. The lack of activation is not due to a lack of recombinant TGF-β secretion as indicated by the increase in total (heat-activated) levels of TGF-β in the VLAL mutant relative to the empty vector. To show that the VLAL deletion did not render the latent complex unable to be activated, activation with KRFPK peptides was tested. KRFK peptides do not require WSXXW/VLAL interactions for activation and thus are predicted to be able to activate the latent complex deleted in VLAL. Data show that, whereas TSP1-mediated activation of the small latent complex deleted in VLAL is impaired, KRFK is able to effectively activate this modified complex (Fig. 9B). In contrast, both KRFK and TSP1 are similarly effective at activating the wild type latent complex. Thus, these data show that the intact VLAL sequence of the mature domain is necessary for TSP1 to activate the TGF-β latent complex.

DISCUSSION

These data show that VLAL sequences on LAP and mature TGF-β are important for association of the latent TGF-β complex with the tryptophan-rich motifs of the TSP TSRs. TSR2 binds immobilized VLAL-containing peptides, and association of the repeat with either TGF-β or LAP was dose-dependently inhibited by soluble VLAL peptides. VLAL binds the tryptophan-rich regions of TSR2 specifically, since VLAL peptides or deletions of the VLAL sequence inhibited binding of LAP and mature TGF-β to immobilized WSXXW sequences. Finally, competition with VLAL peptides or deletion of the VLAL sequence from the mature domain of the TGF-β latent complex inhibited TSP1-mediated activation of latent TGF-β.

The WSXXW domains are common to a superfamily of TSP-containing proteins that have recently been subgrouped based on positioning of conserved cysteines and hydrophobic and basic residues (30). The TSRs of TSP cluster into a subgroup with peropadin and ADAMTS proteins, and the structure of the TSRs is defined by alternate stacking of the tryptophan and arginine residues, which create a continuously positively charged domain and a backbone for the recognition face of the molecule (19). The tryptophans are also oriented so that their polar atoms are exposed and available for potential ligand binding (19). The solvent accessibility and the stacking interaction among the tryptophans in this unique structure could account for some of the observed properties of peptides that contain the three conserved tryptophans (30). WSXXW sequences can also be found in members of the cytokine receptor superfamily (23). The oncostatin-M receptor contains a WSXWSXW sequence, and the cytokine oncostatin-M has an N-terminal VLAL motif (amino acids 14–17). However, not all the binding partners of WSXXW containing proteins have VLXL motifs. In fact, the structures of the human growth hormone and erythropoietin receptors show different molecular arrangements of the WSXXW motifs as compared with the unique structure of the TSR, which might account for differences in the ability of WSXXW containing proteins to associate with VLAL motifs. Structures of complexes between WSXXW-containing proteins, such as TSP, and VLAL-containing proteins, such as the TGF-β latent complex, will be necessary to confirm the specific molecular interactions involved in VLAL/WSXXW binding.

The VLAL sequence of mature TGF-β is part of a three-turn α-helix (29). In fact, this specific α-helix is an element that structurally differentiates the TGF-βs from other family members, including the bone morphogenic proteins (17, 29). Interestingly, the crystal structure of TGF-β predicts that this helical motif is involved in a hydrophobic dimer interface (29). Our data corroborate this, since deletion of the VLAL sequence from mature TGF-β results in a decrease of dimer with a concomitant increase in monomer on Western blot.

In an attempt to determine the importance of the VLAL sequence of LAP in TSP1-mediated TGF-β activation, we produced the FLAG-tagged latent complex deleted in the LAP VLAL sequence. Unfortunately, this deletion resulted in an impaired ability to confer latency and in impaired secretion of mature TGF-β as determined by PAIL assay and immuno-blot (not shown) and could not be tested for activation by TSP1. Although the VLAL sequence is in a 35-amino acid region of the LAP previously shown to be important for binding to mature domain and for latency (31), it is not known if the VLAL sequence itself is important for latency. VLAL could be involved in dimerization of LAP, as appears to be the case with the mature domain. However, although dimerization of LAP is necessary for latency, it is not necessary for secretion of the
mature domain (31, 32), suggesting that the VLAL motif might play an additional role in proper assembly of the latent complex. This idea is supported by evidence that a histidine substitution for tyrosine at amino acid position 81 (immediately following the VLAL sequence of LAP) results in impaired latent complex formation and is found in patients with Camurati-Engelmann disease, a rare bone disorder thought to result from abnormal TGF-β1 activity (33). Although these data suggest that an intact VLAL sequence is critical for LAP function, LAP structural data are needed to accurately determine the role of the motif.

The data shown in this study have added to our understanding of TSP1-mediated TGF-β activation. As reported previously, peptides containing the WSXXW sequence C-terminal to the KRFK activation sequence have an enhanced molar effectiveness of TGF-β activation when compared with peptides with the tryptophanophans substituted for alanines (16). This led to the conclusion that the WSXXW sequences act as “docking sites,” providing for proper orientation of TSP1 with the latent complex. This is consistent with data now showing that the small latent domain deleted in the mature domain VLAL sequence cannot be activated by TSP1. The binding of the VLAL sequence in the LAP to additional tryptophan-rich regions in TSP1 might be important in docking and orientation or possibly stabilization of the activation complex.

We have identified that interactions between TSP1 and latent TGF-β represent a major point of regulation of appropriate TGF-β activity levels both in vitro and in vivo (18). Recent studies have shown that peptides that specifically inhibit TSP1-mediated activation ameliorate TGF-β-induced fibrosis in renal and hepatic model systems (34, 35). In neoplasia, determined that mimic their structure and/or interactions, remain to be

Acknowledgments—We thank Dr. John Munger and Dr. Daniel Rifkin for supplying TGF-β(−/−) and Mv1Lu reporter cells, Dr. Lalage Wakefield for providing TGF-β and Dr. Jack Lawler for providing the TSP type 1 repeat fusion protein. We additionally acknowledge Dr. Dennis Torchia (National Institutes of Health) and Dr. Jia-huai Wang (Harvard University) for allowing us to print ribbon diagrams based on their structures of TGF-β and the TSP type 1 repeats, respectively. We also thank Manuel Antonio Pallero for management of general laboratory operations.

REFERENCES

1. Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
2. Lawrence, D. A. (1996) Eur. Cytokine Netw. 7, 363–374
3. Koch, R. M., Roche, N. S., Parks, W. T., Ashcroft, G. S., Letterio, J. J., and Roberts, A. B. (2000) Wound Repair Regen. 8, 179–191
4. Dumont, N., and Arteaga, C. L. (2002) Differentiation 70, 574–582
5. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) Mol. Cell. Biol. 8, 4162–4168
6. Miyazono, K., Hellman, U., Wernerstedt, C., and Heldin, C. H. (1988) J. Biol. Chem. 263, 6407–6415
7. Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. (1995) J. Biol. Chem. 270, 10618–10624
8. Munger, J. S., Harpel, J. G., Gleizes, P. E., Mazzieri, R., Nunes, I., and Rifkin, D. B. (1997) Kidney Int. 51, 1376–1382
9. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2005) J. Cell Sci. 116, 217–224
10. Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J., Dalton, S. L., Wu, J., Pittel, J. F., Kaminski, N., Garat, C., Matthyau, M. A., Rifkin, D. B., and Sheppard, D. (1999) Cell 96, 319–328
11. Crawford, S. E., Steilmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynea, R. O., Buivin, G. P., and Bouck, N. (1996) Cell 93, 1159–1170
12. Muthuramalingam, T. (1996) Annu. Rev. Med. 47, 469–477
13. Bier, F. M., Poczatek, M., Schultz-Cherry, S., Villain, M., and Murphy-Ullrich, J. J. (1999) J. Biol. Chem. 274, 13586–13593
14. Murphy-Ullrich, J. E., and Poczatek, M. (2000) Cytokine Growth Factor Rev. 11, 59–69
15. Tan, K., Duquette, M., Liu, J. H., Dong, Y., Zhang, R., Joachimiak, A., Lawler, J., and Wang, J. H. (2002) J. Biol. Chem. 277, 1375–1382
16. Guo, N. H., Krutzsch, H. C., Negre, E., Vogel, T., Blake, D. A., and Roberts, D. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3040–3044
17. Guo, N. H., Krutzsch, H. C., Negre, E., Zahrenetzkaya, V. S., and Roberts, D. D. (1992) J. Biol. Chem. 267, 19549–19555
18. Seljes, J. M., Guo, N., Negre, E., Vogel, T., Krutzsch, H. C., and Roberts, D. D. (1993) J. Cell Biol. 121, 469–477
19. Bazan, J. F. (1990) Annu. Rev. Med. 41, 85–117
20. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., and Kusakoff, D. J., and Rifkin, D. B. (1994) Annu. Rev. Cell Biol. 10, 217–248
21. Murphy-Ullrich, J. E., Schultz-Cherry, S., and Hook, M. (1992) Mol. Biol. Cell 3, 181–188
22. Adams, J. C., and Lawler, J. J. (1994) Mol. Biol. Cell 5, 423–437
23. Wolfgram, L., Alkemade, G. M., Allen, B., Sharpe, S., Parks, W. T., and Letterio, J. J. (2002) J. Immunol. Methods 266, 7–18
24. Hinck, A. P., Archer, S. J., Qian, S. W., Roberts, A. B., Sporn, M. B., Wathenbee, J. A., Tsang, M. L., Lucas, R., Zhang, B. L., Wenker, J., and Torchia, D. A. (1996) Biochemistry 35, 8517–8534
25. Huwiler, K. G., Vestling, M. M., Annes, J. D., and Mosher, D. F. (2002) Biochemistry 41, 14329–14339
26. Sha, X., Yang, L., and Gentry, L. E. (1991) J. Cell Biol. 114, 827–839
27. Brunicard, A. M., Marquardt, H., Malacca, A. R., Lioubin, M. N., and Puchio, A. F. (1999) J. Biol. Chem. 274, 13660–13664
28. Janssen, E., ten Dijke, P., Paloya, S., and Van Hul, W. (2005) J. Biol. Chem. 278, 7718–7724
29. Hugo, C. P., Pichler, R. P., Schultz-Lohoff, E., Prols, F., Adler, S., Krutsch, H. C., Murphy-Ullrich, J. E., Ceaser, W. G., Roberts, D. D., and Johnson, R. J. (1999) Kidney Int. 55, 2236–2249
30. Kondou, H., Mushiake, S., Etani, Y., Miyoshi, Y., Michigami, T., and Ozono, K. (2000) J. Biol. Chem. 275, 742–749
31. Albo, D., Berger, D. H., Vogel, J., and Tususynski, G. P. (1999) J. Gastron. Surg. 3, 411–417
32. Albo, D., Berger, D. H., and Tususynski, G. P. (1996) J. Surg. Res. 66, 86–90
33. Kawasaki, T., Naganuma, H., Sasaki, A., Yoshikawa, H., Takaoka, K., and Nukui, H. (2000) Neuroapoth. 20, 161–169
34. Sasaki, A., Naganuma, H., Satoh, E., Kawataki, T., Amagasaki, K., and Nukui, H. (2001) Nervol. Med. Chir. (Tokyo) 41, 253–259
