The Activation Sequence of Thrombospondin-1 Interacts with the Latency-associated Peptide to Regulate Activation of Latent Transforming Growth Factor-β*

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One of the primary points of regulation of transforming growth factor-β (TGF-β) activity is control of its conversion from the latent precursor to the biologically active form. We have identified thrombospondin-1 as a major physiological regulator of latent TGF-β activation. Activation is dependent on the interaction of a specific sequence in thrombospondin-1 (K415RFK418) with the latent TGF-β complex. Platelet thrombospondin-1 has TGF-β activity and immunoreactive mature TGF-β associated with it. We now report that the latency-associated peptide (LAP) of the latent TGF-β complex also interacts with thrombospondin-1 as part of a biologically active complex. Thrombospondin-LAP complex formation involves the activation sequence of thrombospondin-1 (KRFK) and a sequence (LSKL) near the amino terminus of LAP that is conserved in TGF-β1,3. The interactions of LAP with thrombospondin-1 through the LSKL and KRFK sequences are important for thrombospondin-mediated activation of latent TGF-β since LSKL peptides can competitively inhibit latent TGF-β activation by thrombospondin or KRFK-containing peptides. In addition, the association of LAP with thrombospondin-1 may function to prevent the reformation of an inactive LAP-TGF-β complex since thrombospondin-bound LAP no longer confers latency on active TGF-β. The mechanism of TGF-β activation by thrombospondin-1 appears to be conserved among TGF-β isoforms as latent TGF-β2 can also be activated by thrombospondin-1 or KRFK peptides in a manner that is sensitive to inhibition by LSKL peptides.

Transforming growth factors-β are a family of small peptide growth factors (25 kDa) involved in the regulation of a variety of cellular functions (1–3). Processes regulated by TGF-β1 include angiogenesis, embryogenesis, wound healing, and inflammation. There are five isoforms of TGF-β, three of which are expressed in mammals. TGF-β is synthesized by virtually all cell types in a latent form that must be activated in order to be recognized by cell-surface receptors and to trigger biological responses (1–4). Mechanisms controlling conversion of the latent complex to the active state are key regulators of TGF-β activity (1–4). Physiological mechanisms of activation are not well understood, although proteolytic processing by plasmin, exposure to reactive oxygen species, and binding to integrins may participate in this process (4, 47). Our laboratory has shown that interaction of latent TGF-β with the multifunctional platelet and matrix protein thrombospondin-1 (5–10) results in activation of latent TGF-β (12–15). Thrombospondin purified from human platelets (thrombospondin-1) is associated with TGF-β activity (11). The site in thrombospondin responsible for latent TGF-β activation has been localized to the type 1 repeats (14): specifically, the KRFK sequence located between the first and second type 1 repeats of thrombospondin-1 (15). To better understand the mechanism of thrombospondin-mediated activation of latent TGF-β, we sought to determine the region of the latent TGF-β complex recognized by the TGF-β-activating sequence KRFK in thrombospondin.

Small latent TGF-β (reviewed in Refs. 1–4) is a dimeric complex of ~100 kDa, composed of two identical chains in which an amino-terminal 278-amino acid latency-associated peptide (LAP) is noncovalently associated with the carboxy-terminal 112-amino acid active peptide. This latent complex is the product of a single gene. Prior to secretion, LAP is enzymatically cleaved from the active peptide (16), and the integrity and latency of the secreted complex are presumably maintained via electrostatic interactions (17). Although latent TGF-β can also exist as a large complex in which small latent TGF-β is associated with a latent TGF-β-binding protein, the presence of the latent TGF-β-binding protein is neither necessary nor sufficient to confer latency on the active peptide (18–20). On the other hand, latency is dependent on the presence of LAP, and modification of the cysteine residues responsible for LAP dimerization results in altered TGF-β secretion (21–23), suggesting that the tertiary structure of LAP is important for the formation of the latent TGF-β complex. Gentry and coworkers (21) showed through mutagenesis studies that amino acids 40–80 in LAP are important for maintenance of the latency of the complex. In a previous study, we observed that

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1The abbreviations used are: TGF-β, transforming growth factor-β; LAP, latency-associated peptide; NRK, normal rat kidney; BAE, bovine aortic endothelial; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; TSP, thrombospondin; ACTH, adrenocorticotropic hormone.
antibodies raised against a sequence present in the amino terminus of LAP (residues 81–94) inhibited activation of latent TGF-β by thrombospondin (13). These observations led us to propose that thrombospondin-mediated activation of latent TGF-β involves interactions between the thrombospondin activation sequence (KRFK) and a site present in the amino-terminal region of LAP.

We now show that LAP is complexed with thrombospondin-1 in association with biologically active TGF-β and that the thrombospondin-1 sequence KRFK binds LAP through interactions that involve a specific sequence at the amino terminus of β1-LAP (L14SKL37). The KRFK sequence in thrombospondin-1 and the LSKL sequence in LAP are apparently critical for activation of latent TGF-β by thrombospondin-1 since soluble LSKL peptides can competitively block activation of latent TGF-β by either thrombospondin-1 or KRFK-containing peptides. LAP binding to thrombospondin may play a role in preventing re-formation of the latent complex. In addition, the mechanism of thrombospondin-mediated activation of latent TGF-β appears to be conserved in the mammalian isoforms of TGF-β since thrombospondin-1 can also activate latent TGF-β2 in an LSKL-sensitive manner.

**EXPERIMENTAL PROCEDURES**

Thrombospondin Purification—Thrombospondin-1, native or stripped of TGF-β activity, was purified as described (11) from human platelets obtained from the Birmingham American Red Cross. Thrombospondin purity was assessed by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining. The depletion of TGF-β activity in preparations of stripped thrombospondin was confirmed in the NRK colony formation assay (11).

Peptides, Antibodies, and Other Reagents—The peptides used in this work were synthesized by the University of Alabama at Birmingham Comprehensive Cancer Center/Peptide Synthesis and Analysis shared facility. Initial peptides and peptide 246 used in this study were a gift from Dr. David Roberts (NCI, National Institutes of Health). Recombinant latent TGF-β2 was a generous gift from Dr. Patricia Segarini and Celtrix Corp. (Palo Alto, CA), and was purified as described (24). Recombinant latent TGF-β1 was a gift from Jane Ranchels (Bristol-Myers Squibb, Seattle, WA). Monoclonal antibody 133 against stripped thrombospondin-1 was developed in a joint effort between our laboratory and the University of Alabama at Birmingham Hybriddoma core facility (12). Recombinant human β1-LAP (catalogue no. 246-150/CPF) and mouse monoclonal and goat polyclonal anti-LAP antibodies (catalogue no. AB246-NA) were purchased from R&D Systems (Minneapolis, MN). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Cells of endothelial (BAE) cells were isolated in our laboratory from aortas obtained at a local abattoir and were characterized by Dil-AcLDL uptake and staining for factor VIII antigen, according to established protocols. Stocks were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 4.5 g/liter glucose and supplemented with 20% fetal bovine serum. Conditioned media experiments were performed in the presence of reduced serum concentration, as described in the figure legends. NRK-49F cells (CRL-1570) were purchased from the American Type Culture Collection (Rockville, MD) and were kept in DMEM supplemented with 10% calf serum. All cells were routinely tested for mycoplasma.

**Activation of TGF-β by Thrombospondin or Peptides—** Equimolar concentrations of stripped thrombospondin-1 or peptides (11 nm) were incubated with recombinant latent TGF-β (2 nm) in a final volume of 0.5 ml of PBS for 1 h at 37 °C (13). Alternatively, stripped thrombospondin-1 or peptides were incubated with BAE cell-conditioned media as described (11). A positive control for latent TGF-β activation consisted of heat treatment of the latent complex at 80 °C for 5 min.

**Assay of TGF-β Activity—** TGF-β activity was assayed based on its ability to stimulate growth of NRK fibroblasts in suspension as described (11). In brief, 1–3 × 105 NRK cells were plated in a 0.3% agar suspension in the presence of epidermal growth factor (2.5 ng/ml; Life Technologies, Inc.) and in the presence or absence of TGF-β and incubated at 37 °C for 7 days. At the end of this incubation period, colonies containing 8–10 cells (i.e. colonies larger than 62 μm) were counted. Active TGF-β (2.5 ng/ml) was used as a positive control. Experiments were performed in triplicate at least twice.

**Western Blots—** Samples were separated by SDS-polyacrylamide gel electrophoresis (% acrylamide indicated in the figure legends) and transferred to nitrocellulose membranes (2 h, 100 V). Non-specific protein-binding sites present in the membranes were blocked by incubation with 6% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (Tris-buffered saline/Tween). Membranes were then incubated with primary antibodies diluted in Tris-buffered saline/Tween (antibody 133 used at 0.05 μg/ml, goat polyclonal anti-LAP at 1 μg/ml, and other antibodies and dilutions specified in the figure legends) followed by extensive washes in Tris-buffered saline/Tween with 0.1% Tween 20. After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (peroxidase-conjugated goat anti-mouse IgG used at 0.1 μg/ml for 1 h at room temperature, peroxidase-conjugated rabbit anti-goat IgG at 0.08 μg/ml, and dilutions and incubation times for other antibodies as indicated in the figure legends) and developed by enhanced chemiluminescence (Pierce) according to the manufacturer’s instructions. Multiple exposures were obtained to assure linearity of the response.

**Peptide Affinity Column—** Peptide KRFKQDGGC or TRIRQDGGC (5 mg/1 ml in 50 mm Tris and 5 mm EDTA, pH 8.5) was coupled to Sulforlink (1 ml; Pierce) according to the manufacturer’s instructions and equilibrated in PBS: 2.4 μmol of peptide KRFKQDGGC or 3.3 μmol of peptide TRIRQDGGC were coupled to the Sulforlink resin. Recombinant human β1-LAP (10 μg/ml) was loaded and incubated with the affinity matrix (bed volume = 1 ml) for 20 min at room temperature and then circulated through the column five times. Prior to elution, the column was washed with 25 ml of PBS. Proteins bound to the affinity matrix were then eluted stepwise, first with 4 ml of peptide SLK, followed by 10 ml of peptide LSKL and, for the TRIR affinity column, peptide TRIR (all peptides at 86 μM, a 150-fold molar excess to LAP). Fraction size was 0.25 ml, and all LAP protein eluted and incubated between fractions 3 and 5 (0.75–1.25 ml). Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with anti-LAP antibodies.

**Immunoprecipitation—** Stripped thrombospondin (11 nm) and recombinant human β1-LAP (28 nm) were incubated together in a total volume of 0.5 ml of PBS in the presence or absence of peptide KRFK, TRIR, or KRAK (11 μM) or peptide LSKL, SLK, or RGHL/SLKL (28 μM). Peptides were used at a 1000-fold molar excess to either TSP or LAP, respectively. When peptides were present, each protein was preincubated with the appropriate inhibitory peptide for 30 min at 4 °C (TSP preincubated with LSKL and LAP preincubated with KRFK). The second protein was then added to the peptide/protein mixture and incubated for 1 h at 4 °C. The protein mixture was incubated for 1 h at 4 °C with goat polyclonal antibodies raised against recombinant human LAP (0.5 μg of antibody/0.5 ml of sample), followed by a 30-min incubation with protein G-Sepharose 4B beads (Sigma) in 10 mm Tris, pH 7.4, containing 150 mm NaCl, 1% Triton X-100, and 0.5% Nonidet P-40. Alternatively, the protein mixture was incubated for 1 h at 4 °C with GammaBind G-Sepharose (catalogue no. 17-0085-01, Amersham Pharmacia Biotech) conjugated to monoclonal antibodies in wash buffer (PBS containing 1 g/liter ovalbumin and 5 ml/liter Tween 20; 10 μg of antibody/0.5 ml of matrix). Immune complexes were washed with wash buffer, resuspended in reducing Laemmli buffer, and analyzed by Western blotting with monoclonal antibody 133 against thrombospondin or with goat polyclonal anti-LAP antibodies. For dose-response inhibition assays, LAP was preincubated with 0.11–110 μg/ml peptide KRFK, whereas thrombospondin was incubated with 0.28–280 μg/ml peptide LSKL.

**For immunoprecipitation of proteins from media conditioned by BAE cells, ~3 × 10^6 cells were incubated for 24 h in 3 ml of DMEM containing insulin-transferrin-sodium selenite media supplement (Sigma) at the concentration recommended by the manufacturer. Conditioned media were harvested and immediately incubated with goat polyclonal antibodies against recombinant human LAP (10 μg/ml) for 1 h at 4 °C, followed by incubation with protein G-Sepharose 4B beads for 1 h at 4 °C. Immune complexes were washed and analyzed by Western blotting with monoclonal anti-TSP antibodies as described above.

**LAP Immunodepletion Experiments—** To deplete native thrombospondin of LAP, 20 μg of thrombospondin in 25 μl of PBS was incubated three times with 50 μl of goat polyclonal anti-LAP coupled to CNBr-activated Sepharose (coupling per manufacturer’s instructions) for 20 min each time. Following each incubation, samples were centrifuged, and the supernatant was transferred to another tube containing antibodies coupled to resin. Prior to the first incubation and following the last incubation, protein concentration in the sample was measured by A_280 using a molar extinction coefficient of 1.37. Sample volumes
to be tested for TGF-β activity and Western-blotted for LAP were adjusted so that the same amount of protein (6.25 μg) was used in all cases.

Analysis of TGF-β Activity in Complexes—Assay conditions were those previously described as ideal for re-formation of the latent TGF-β complex (22). In brief, thrombospondin (9 μg; amount chosen based on our previous studies of latent TGF-β activation by thrombospondin) was incubated with LAP (28 ng) in 100 μl of serum-free DMEM at 1 h at room temperature. TGF-β (2 ng in 2 μl) was then added to the appropriate samples, followed by an additional incubation for 1 h at room temperature. Samples to which no TGF-β was added, samples containing TGF-β alone, and samples in which LAP and thrombospondin were not incubated together prior to addition of TGF-β were incubated at the same temperature for the same extent of time to minimize variations due to loss of protein to the tube or loss of TGF-β activity over time. Immediately following the second incubation, samples were tested for TGF-β activity as described above.

Hydropathic Complementarity Analysis—The search for a sequence in LAP complementary to the thrombospondin-1 sequence KRFK was performed through computer analysis utilizing a computer program designed to identify patterns of inverted hydropathy (25). The parameter settings used were as follows: 1) search a window size of five amino acids (hits are searched for in a window of five residues, sliding the window down the sequence one amino acid at a time); 2) average chain complementarity set at 1.2 (this value represents the average of the differences in the hydrophobic scores of aligned amino acids for the window size selected; the closer to 0, the better the complementarity); and 3) the cutoff point for considering if two amino acids are opposite set to 2.0 (the absolute value of the two aligned residues added together is denoted as the cutoff).

RESULTS

LAP Co-purifies with Thrombospondin-1 Secreted by Human Platelets and by BAE Cells—Previous results indicated that an antibody raising against the amino terminus of LAP could block thrombospondin-mediated activation of latent TGF-β (13), suggesting a possible interaction of thrombospondin with the LAP portion of the latent complex. During the course of our studies, Yang et al. (45) reported that recombinant dimeric LAP binds to immobilized thrombospondin. Since the presence of LAP is both necessary and sufficient to confer latency on TGF-β and since TGF-β associated with thrombospondin-1 is in its active state, one would predict that LAP would not be present in biologically active thrombospondin-1-TGF-β complexes. However, human platelet thrombospondin-1 that has TGF-β bioactivity (11) also contains detectable LAP, suggesting that LAP may potentially be associated with thrombospondin-1-TGF-β complexes (Fig. 1A). Furthermore, LAP isolated by immunoprecipitation from media conditioned by BAE cells in culture co-purifies with thrombospondin-1, as detected by Western blotting (Fig. 1B). These observations show that in biological fluids, thrombospondin and LAP can exist in complexes. Furthermore, these data suggest the possibility that active TGF-β can form a ternary complex with thrombospondin-1 and LAP.

LAP, Thrombospondin, and TGF-β Form Ternary Complexes That Retain TGF-β Activity—Since previous observations showed that thrombospondin-1 contains associated TGF-β activity, we hypothesized that LAP, thrombospondin, and TGF-β may form ternary complexes that maintain bioactivity. To investigate this hypothesis, it was determined whether removal of thrombospondin molecules that had associated LAP resulted in depletion of TGF-β activity present in the thrombospondin-1 preparation. The thrombospondin-associated TGF-β activity was measured prior to and following immunodepletion of LAP-associated thrombospondin-1 with anti-LAP antibodies coupled to Sepharose beads. Equal concentrations of protein in both the starting and immunodepleted materials were evaluated for TGF-β activity. As shown in Fig. 2A, thrombospondin-1 that had been depleted of LAP by immunoprecipitation with anti-LAP antibodies was correspondingly depleted of TGF-β activity. Immunodepletion of LAP from the thrombospondin-1 samples was confirmed by our inability to detect LAP on Western blots of treated samples (Fig. 2A).

To further investigate the hypothesis that ternary complexes containing thrombospondin, TGF-β, and LAP retain TGF-β activity, these proteins were incubated under conditions that allow them to form binary and/or ternary complexes, and the resulting TGF-β activity was measured (Fig. 2B). As expected, incubation of active TGF-β with LAP resulted in inactivation of the growth factor, indicating that the latent TGF-β complex was re-formed under these conditions. However, when TGF-β was incubated with preformed complexes of thrombospondin and LAP (Fig. 2B), LAP failed to inactivate TGF-β.

These observations show that LAP complexes with thrombospondin in biological fluids and that active TGF-β, thrombospondin, and LAP can form ternary complexes. These data also show that interactions of thrombospondin with LAP alter the ability of this precursor portion of TGF-β to confer latency on active TGF-β.

LAP Interacts with the KRFK Sequence in Thrombospondin-1, but Not with the Thrombospondin-2 Sequence TRIR—Polyclonal antibodies raised against a peptide from the LAP amino terminus (amino acids 81–94) inhibit latent TGF-β activation by thrombospondin-1 (10). This observation and the presence of LAP in biologically active thrombospondin-1-TGF-β complexes suggest that the thrombospondin-1 sequence (KRFK) responsible for activation of latent TGF-β might interact with LAP.

To test this hypothesis, we chose an approach based on co-immunoprecipitation. When thrombospondin-1 and LAP were incubated together for 1 h at room temperature, they formed complexes that were immunoprecipitated by both polyclonal antibodies against LAP (Fig. 3, A and B, third lanes) and
monoclonal antibodies against thrombospondin (Fig. 3, C and D, third lanes). This association between thrombospondin and LAP was competitively inhibited by preincubation of LAP with the thrombospondin-derived peptide KRFK. Partial inhibition occurred when the peptide was present at a 10–100-fold molar excess relative to the thrombospondin concentration, whereas complete inhibition was observed when the peptide was present at a 1000-fold molar excess (Fig. 3, A and C, fourth through seventh lanes). These data suggest a role for the thrombospondin-1 activation sequence KRFK in LAP binding.

The inability of the related sequence KRAK, which does not activate latent TGF-β (15), to inhibit complex formation between the two proteins (Fig. 3, B and D, sixth lane) provides evidence that this interaction between the thrombospondin-1 sequence KRFK and LAP is specific. Furthermore, data from this experiment also suggest that the interaction between the KRFK sequence and LAP is relevant for the ability of thrombospondin to activate latent TGF-β since the inactive KRFK homologue in thrombospondin-2 (TRIR) had no inhibitory effect on complex formation between thrombospondin-1 and LAP (Fig. 3, B and D, fifth lanes).

These data show that thrombospondin-1 binds to the LAP portion of the latent TGF-β molecule. This binding is apparently mediated by the TGF-β-activating sequence KRFK in thrombospondin-1 and may be part of the mechanism by which thrombospondin-1 activates latent TGF-β.

The LSKL Sequence in LAP Interacts with the Thrombospondin-1-derived Peptide KRFK—To identify the sequence on LAP responsible for binding the thrombospondin-1 sequence KRFK, we used the molecular recognition theory as a strategy to identify sequences in LAP complementary to the KRFK sequence in thrombospondin-1 that could potentially form a binding site (25–28). A search based on this theory, utilizing a computer program developed by Blalock and co-workers (25) (parameters used described under “Experimental Procedures”), identified only one sequence in LAP complementary to the thrombospondin-1 sequence KRFK. This sequence (LSKL) is present in the amino terminus of β1-LAP (positions 54–57). As a control, this program was also used to identify sequences complementary to the TRIIR sequence in thrombospondin-2, which is homologous to the thrombospondin-1 sequence KRFK, but does not activate latent TGF-β; there were no sequences in LAP predicted to match the thrombospondin-2 sequence TRIIR. This finding is consistent with our hypothesis that the interaction between KRFK and a specific sequence in LAP is important for the ability of thrombospondin-1 to activate latent TGF-β.

To test the hypothesis that thrombospondin-1 binds to LAP through interactions involving the thrombospondin-1 sequence KRFK and the LAP sequence LSKL, we used two different approaches: immunoprecipitation and affinity chromatography. Initially, we investigated the ability of LSKL peptides to inhibit complex formation between thrombospondin-1 and LAP. Thrombospondin-LAP complexes were isolated by immunoprecipitation with anti-LAP or anti-thrombospondin antibodies and detected by Western blotting with anti-thrombospondin and anti-LAP antibodies, respectively. Fig. 4 shows that preincubation of thrombospondin with the LAP sequence LSKL prevented complex formation between thrombospondin-1 and LAP. Although some inhibition could be observed when the molar concentration of the peptide exceeded that of LAP by 10-fold (Fig. 4A), significant inhibition was consistently seen when the peptide was used at a 100-fold excess, with 1000-fold excess resulting in complete inhibition of binding between LAP and thrombospondin (Fig. 4, B and C). Furthermore, the inhibition of LAP-thrombospondin association was specific for the LAP-derived peptide LSKL, as indicated by the inability of the scrambled peptide SLLK to inhibit complex formation between these two proteins, even when present at a 1000-fold molar excess (2.8 μM) (Fig. 4, B and D, fourth lanes). Also, as shown in Fig. 4 (B and D, fifth and sixth lanes), inhibition occurred whether or not the LSKL sequence was accompanied by its flanking sequences (RGQILSKLRL).
tions. Furthermore, the observation that LAP fails to bind to a TRIR affinity matrix is consistent with the hypothesis that interactions of the KRFK sequence with LAP are important to the mechanism of latent TGF-β activation by thrombospondin-1.

Activation of Recombinant or Endothelial Cell-secreted Latent TGF-β by Thrombospondin Is Inhibited by Peptide LSKL—To test the hypothesis that interactions involving the KRFK sequence in the thrombospondin type 1 repeats and the LSKL sequence in LAP are important for activation of latent TGF-β by thrombospondin, we tested the ability of peptide LSKL to competitively inhibit activation. In these experiments, thrombospondin-1, peptide 246 (KRFKQDGWSHSPWSS), or peptide KRFK (all at 11 nM) was preincubated with increasing concentrations of LSKL (from 1 nM to 10 μM) prior to incubation with latent TGF-β. Activation of latent TGF-β by either thrombospondin or KRFK-containing peptides was inhibited by peptide LSKL in a concentration-dependent manner (Fig. 6). LSKL alone did not activate latent TGF-β, and it did not affect the ability of NRK cells to respond to active TGF-β (data not shown). In experiments examining thrombospondin-dependent activation of latent TGF-β secreted into the conditioned medium of BAE cells, peptide LSKL, but not a control scrambled peptide (SLLK), similarly inhibited thrombospondin-
dependent activation of latent TGF-β (Fig. 7). These data support the hypothesis that interactions involving the KRFK sequence in thrombospondin-1 and the LSKL sequence in LAP play an essential role in activation of latent TGF-β by thrombospondin.

**Thrombospondin-1 Activates TGF-β2 in an LSKL-dependent Manner**—The LSKL sequence is conserved in all TGF-β isoforms (29–34). This suggests that if thrombospondin-1 activation of latent TGF-β is mediated via interactions with LAP that involve the LSKL sequence, all isoforms of TGF-β should be subject to activation by thrombospondin-1. Therefore, the ability of thrombospondin-1 to activate recombinant latent TGF-β2 expressed by Chinese hamster ovary cells was tested. Purified latent TGF-β2 was incubated with equimolar concentrations (11 nM) of stripped thrombospondin-1, peptide 246 (KRKFQDGGWSHWSPWSS), or KRFK and then tested for TGF-β activity (Fig. 8). Treatment of latent TGF-β2 with either stripped thrombospondin-1 or thrombospondin-1 peptides resulted in a 5-fold increase in TGF-β activity as compared with untreated latent TGF-β2, which was similar to the activation obtained by acid treatment of the latent complex (data not shown). When latent TGF-β2 was incubated with stripped thrombospondin-1 or thrombospondin-1 peptides in the presence of 10 μM peptide LSKL, however, activation was totally inhibited. These data show that thrombospondin-1 activates latent TGF-β2 and that this activation, like that of latent TGF-β1, involves both the KRFK sequence in thrombospondin and the LSKL sequence in LAP.

**Discussion**

The mechanism previously proposed for activation of latent TGF-β by thrombospondin-1 involves multiple interactions between the two molecules (15). The WXXW sequence present in type 1 repeats of thrombospondin-1 enhances the molar effectiveness of activation mediated by peptides containing this sequence C-terminal to the KRFK activation sequence. Peptides containing the WXXW sequence can competitively inhibit binding of the active portion of TGF-β to thrombospondin, although the WXXW sequence in itself does not activate TGF-β. The function of this interaction has not yet been clearly defined; however, it is felt that the WXXW motifs in the type 1 repeats may act as “docking sites” to facilitate interactions of thrombospondin with the latent TGF-β complex. The second interaction involves a sequence unique to the thrombospondin-1 isoform, K142RFK145, which is responsible for activation of the latent TGF-β complex (15). We now show that LAP in the latent TGF-β complex is recognized by the KRFK sequence in thrombospondin-1 and the LSKL sequence in LAP, which play an essential role in activation of latent TGF-β by thrombospondin.

**Fig. 7.** Peptide LSKL blocks TSP-mediated activation of latent TGF-β secreted by BAE cells. Approximately 24 h after seeding, BAE cells (1 × 107 cells/25 cm² flask) were washed and incubated for 24 h in the presence of DMEM containing 2% fetal bovine serum (2 ml/flask). Conditioned media from five flasks were harvested, pooled together, and incubated with or without 11 nM TSP1 in the presence or absence of increasing concentrations of peptide LSKL or control peptide SLLK (1.5 h, 37 °C). After incubation, samples were tested for TGF-β activity in an NRK colony formation assay. The solid bar represents the number of colonies in control wells containing conditioned medium that was not treated with TSP1. Results from controls in which conditioned media were incubated with the peptides in the absence of TSP1 did not differ from those obtained with conditioned medium by itself (data not shown). Results are expressed as means ± S.D. of triplicate determinations.
sequence in thrombospondin-1. Furthermore, we have identified a sequence (LSKL) at the amino terminus of LAP that is important for LAP-KRFK interactions and modulation of latent TGF-β activation by thrombospondin-1. Formation of the latent TGF-β complex involves structural changes in both LAP and the mature peptide (23). Based on our previous work, we postulated that activation of latent TGF-β by thrombospondin also involves a change in the conformation of the inactive complex (13–15). The role of LAP in the activation process and its fate following activation were, however, unknown. These data now show that LAP can remain associated with the thrombospondin-TGF-β complex without inhibiting the activity of thrombospondin-associated TGF-β. The physiological significance of the continued association of LAP with thrombospondin-1 following activation is not entirely clear. However, based on our observation that LAP associated with thrombospondin is unable to confer latency on active TGF-β (Fig. 2B), it is reasonable that the LAP-thrombospondin association following activation modulates the persistence of TGF-β activity by preventing refolding of the complex and inactivation of TGF-β. It remains to be determined whether LAP-thrombospondin-active TGF-β complexes are deposited in the extracellular matrix or processed differently by TGF-β receptors.

We took advantage of the fact that the thrombospondin-1 gene has been sequenced to deduce the putative site for thrombospondin-1 binding in the LAP molecule by utilizing the molecular recognition theory (28, 36). According to this concept, translation of complementary DNA strands predicts sequences that have exactly complementary hydrophobic profiles and that could thus function as complementary binding sites. Examples of protein–protein interactions in which this theory has been useful include ACTH-ACTH receptor (27), fibronectin-integrin (37, 38), and interleukin-1β-type I receptor (39), among others. Applying this principle to the interaction between the thrombospondin-1 sequence KRFK and the LAP molecule, we identified the LSKL sequence in LAP of TGF-β1,5 as the putative binding site for thrombospondin-1. These data show that the LSKL sequence in LAP is indeed involved in the interaction of LAP with thrombospondin-1 and that the LSKL peptide inhibits activation of both latent TGF-β1 and TGF-β3 by thrombospondin-1. More direct approaches are currently being investigated to determine whether the LSKL sequence is indeed the actual binding site for thrombospondin.

Although the overall degree of conservation among the pro-regions of the various TGF-β isoforms is only 30–45%, the LSKL sequence is conserved in all five TGF-β isoforms (29–34). Conversely, this sequence is absent in other members of the TGF-β superfamily, including Drosophila decapentaplegic protein, bone morphogenic proteins, activins/inhibins, VGR-1, and dorsalin (GenBank™ Data Bank search). The biological relevance of the conserved nature of the LSKL sequence among the different LAPs is made even more apparent by the fact that different TGF-β isoforms are products of distinct genes, located on different chromosomes (1). It remains to be determined whether thrombospondin-1 can indeed activate latent TGF-β1,5 or whether there are additional determinants in LAP that regulate thrombospondin’s ability to activate latent TGF-β. Nevertheless, the conservation of LSKL suggests that this is an important sequence for regulation of activation of all TGF-β isoforms by thrombospondin-1. This interpretation is supported by the observation that in vivo, increased thrombospondin expression is frequently associated with increased TGF-β activity (40–42). Since thrombospondin-1 is an early response gene that is rapidly up-regulated in response to a number of serum factors (7, 43, 44), it is possible that regulation of thrombospondin-1 expression by these factors also results in regulation of the activity of all mammalian forms of TGF-β.

The interaction between KRFK and LAP does not appear to be dependent solely on electrostatic forces. Peptide LSKL effectively prevents LAP from binding to thrombospondin-1 (Fig. 4), disrupts the binding of LAP to KRFK (Fig. 5), and inhibits latent TGF-β activation by thrombospondin-1 (Figs. 6 and 7), whereas the scrambled peptide SLLK, which retains the same overall charge, has no effect on LAP-thrombospondin-1 or LAP-KRFK binding or the ability of thrombospondin-1 to activate latent TGF-β (Figs. 4, 5, and 7). Furthermore, activation of latent TGF-β by thrombospondin-1 is not inhibited by the presence of 0.5 M NaCl (15). The regions surrounding the LSKL sequence in all latent TGF-β isoforms are, however, considerably charged (29–34) and may be responsible for electrostatic interactions involved in the stabilization of the latent TGF-β complex, consistent with the hypothesis that electrostatic interactions are important for maintenance of TGF-β latency. Although other approaches will be needed to determine whether the LSKL and KRFK sequences form the actual binding site between LAP and thrombospondin, we believe that the data presented here are sufficient to allow us to propose that there is minimally a sequence-specific interaction between thrombospondin-1 and LAP, involving KRFK and LSKL. Additional conformational factors may also be important for the interaction of these two proteins. This is consistent with recent work of Gentry and co-workers (45), who showed that only dimeric, but not monomeric, β1-LAP binds to immobilized thrombospondin-1. It should be noted that there may be certain conformational restraints that preclude complex formation, as Baily et al. (35) reported that LAP and thrombospondin failed to bind each other when assayed using a plasmon resonance approach. We therefore suggest that sequence- and conformation-dependent interactions between thrombospondin-1 and LAP cause a rearrangement of LAP, which disrupts the electrostatic interactions between LAP and the active domain, thus converting the latent complex into a biologically active molecule.
These new findings are significant in that they further our understanding of the mechanisms involved in activation of latent TGF-β. In doing so, they provide us with new tools (LSKL-containing peptides) to modulate in vivo TGF-β activity in situations such as fibrosis, in which regulation of TGF-β activity would be beneficial. Supporting evidence for this suggestion is offered by our recent observation that thrombospondin and its derived peptides play a significant role in the regulation of latent TGF-β activity in vivo (46). In that study, we showed that TGF-β1 and thrombospondin-1 knockout mice have similar histological abnormalities in nine organ systems and that treatment of thrombospondin-1 knockout pups with KRKF peptides resulted in reversion of the lung and pancreatic phenotypes and detection of active TGF-β in situ. Furthermore, treatment of wild-type pups with peptide LSKL in vivo resulted in phenotypic alterations similar to those observed in the TGF-β null animals. These observations indicate that the interaction between the KRKF sequence in thrombospondin and the LSKL sequence in LAP, described here as important for the regulation of latent TGF-β in situ, is also important for the regulation of TGF-β activity in vivo.

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