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 (KRFK) 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 (KRFF) and a sequence (LSKL) near the amino terminus of LAP that is conserved in TGF-β1,2. The interactions of LAP with thrombospondin-1 through the LSKL and KRFF sequences are important for thrombospondin-mediated activation of latent TGF-β since LAP peptides can competitively inhibit latent TGF-β activation by thrombospondin or KRFF-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 KRFF 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 I repeats (14): specifically, the KRFF sequence located between the first and second type I 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 KRFF 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 co-workers (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

*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.

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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 (KRFFK) 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 KRFFK binds LAP through interactions that involve a specific sequence at the amino terminus of β₁-LAP (I²SLK²). The KRFFK 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 KRFFK-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 was assayed based on its latency in the presence of reduced serum concentrations since thrombospondin-1 can also activate latent TGF-β (13). Alternatively, stripped thrombospondin was loaded in the presence of antibody 133 against thrombospondin or with goat polyclonal anti-LAP antibodies. For dose-response inhibition assays, LAP protein mixture was 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 ng) and recombinant human β₁-LAP (28 ng) 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 µmol) or peptide LSKL, SSLK, or RGGHSLLKR (28 µmol). 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 ml 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. 17-0885-01, Amersham Biosciences). A mouse monoclonal anti-mouse IgG and peroxidase-conjugated rabbit anti-goat IgG were used as 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 KRFKQDGCC or TRIRQDGCC (5 mg/1 ml in 50 mM Tris and 5 mM EDTA, pH 8.5) was coupled to Sulfolink (1 ml; Pierce) according to the manufacturer's instructions and equilibrated in PBS. 2.4 µmol of peptide KRFKQDGCC or 3.3 µmol of peptide TRIRQDGCC were coupled to the Sulfolink resin. Recombinant human β₁-LAP (10 µg/mg) 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 SLLK, 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 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.

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 human TGF-β₁, TGF-β₂, and TGF-β₃ were obtained from Celtrix Corp. (Palo Alto, CA), and was purified as described (24). Recombinant human β₁-LAP (catalogue no. 246-10/LCF) and mouse monoclonal and goat polyclonal anti-LAP antibodies (catalogue no. AB-246-NA) were purchased from R&D Systems (Minneapolis, MN). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell culture—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 concentrations 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-β₂ (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 mg/ml; Life Technologies, Inc.) and in the presence or absence of TGF-β₂ 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-β₂ (2.5 mg/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). Nonspecific protein-binding sites present in the membranes were blocked by incubation with 5% bovine serum albumin in Tris-buffered saline (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 0.05% 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.
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 hydropathic 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 raised 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 sam-

**Fig. 1. Native thrombospondin-1 contains associated LAP.** A, native TSP1 was subjected to SDS-polyacrylamide gel electrophoresis on a 5–15% gradient gel under reducing conditions, transferred to nitrocellulose membranes, and analyzed by Western blotting (WB) with mouse monoclonal anti-LAP antibodies (2 μg/ml, 1 h, room temperature). First lane, recombinant human latent TGF-β (LTGF-β; 1.64 μg); second lane, native TSP1 (nTSP; 18.6 μg). B, proteins present in media conditioned by BAE cells were immunoprecipitated (IP) with antibodies to LAP and analyzed by Western blotting with a monoclonal antibody to thrombospondin. Lane 1, conditioned media prior to immunoprecipitation; lane 2, supernatant after immunoprecipitation; lane 3, goat polyclonal anti-LAP antibodies loaded directly onto the gel; lane 4, media incubated with beads that were not coupled to antibodies; lane 5, proteins immunoprecipitated from conditioned media by incubation with anti-LAP antibodies coupled to beads.
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 KRRAK, 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 these two proteins, even when present at a 1000-fold molar excess (2.5 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).

As an alternative approach, we assessed the ability of recombinant human β1-LAP to bind to an affinity column made by coupling the KRFK peptide to the matrix through a C-terminal cysteine residue on the peptide. The aim of this experiment was to directly assess whether LAP binds to the KRFK sequence in thrombospondin-1 and whether this binding could be disrupted by the LSKL peptide. Recombinant human β1-LAP bound to the KRFK column with no detectable LAP in the flow-through fractions, and LAP bound to the KRFK column was specifically eluted with the LSKL peptide (Fig. 5A), suggesting that the LSKL sequence bound to the KRFK affinity matrix. This interaction is apparently specific since a scrambled version of the LSKL sequence, peptide SLLK, did not elute bound LAP from the KRFK column. The failure of LAP to bind to the TRIR affinity matrix provides further evidence for the specificity of the LAP-KRFK interaction (Fig. 5B).

Based on these results, we conclude that the binding between thrombospondin-1 and LAP is mediated by interactions involving the KRFK sequence in the thrombospondin-1 molecule and the LSKL sequence present in LAP. The role of the LSKL sequence appears to be dependent on this specific amino acid sequence and is not simply charge-based since the scrambled peptide SLLK is unable to prevent LAP-KRFK interaction.
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 (KRFKQDGWWSHSPWSS), 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-
Figs. 5 and 6. LAP binds to a KRFK affinity column and is specifically eluted by LSKL. LAP (10 μg/0.5 ml of PBS) was applied to a KRFK-Sulfolink column (A) or a TRIR-Sulfolink column (B) as described under “Experimental Procedures.” After extensive washing of unbound LAP with PBS, the columns were sequentially treated with a 150-fold molar excess of peptide LSKL, followed by a 150-fold molar excess of peptide SLLK. In B, elution with LSKL was followed by a wash with a 150-fold molar excess of TRIR. Fractions (0.25 ml each fraction, 64 μl/fraction loaded onto the gel) were collected and analyzed for the presence of LAP by Western blotting (WB) with goat polyclonal antibodies against LAP (1 μg/ml, 1 h, room temperature). The secondary antibody used was rabbit anti-goat IgG (0.08 μg/ml, 1 h, room temperature). Fractions 3–5 were eluted with peptide SLLK, LSKL, or TRIR, as indicated. Pre, starting material; FT, flow-through fraction.

FIG. 6. Peptide LSKL inhibits recombinant latent TGF-β activation by thrombospondin-1 and thrombospondin-1-derived peptides. Stripped TSP1 (closed circles), peptide 246 (open circles), or KRFK (closed squares), all used at 11 nM, was incubated with recombinant human latent TGF-β1 (2 nM) in the absence or presence of increasing concentrations of peptide LSKL for 1 h at 37 °C. Samples were tested for TGF-β activity in the NRK colony formation assay. LSKL by itself (closed triangles) is representative of the negative control (i.e. assay performed in the presence of epidermal growth factor and in the absence of TGF-β). Results are expressed as means ± S.D. of triplicate determinations.

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 (KRKFQDGWGHSPWSS), 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 motif 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, K+/4RFK+/15, 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. 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-ACTR receptor (27), fibronectin-integrin (37, 38), and interleukin-1β-type 1 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-β2 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 morphogenetic 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 constraints that preclude complex formation, as Bailly 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-conformationally 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 activation 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 TGF-β activity in vitro, is also important for the regulation of TGF-β activity in vivo.

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REFERENCES
1. Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
2. Roberts, A. B. (1995) Wound Rep. Reg. 3, 408–418
3. Lawrence, D. A. (1996) Eur. Cytokine Netw. 7, 363–374
4. Munger, J. S., Harpel, J. G., Gleizes, P. E., Mazieri, R., Nunes, I., and Rifkin, D. B. (1997) Kidney Int. 51, 1376–1382
5. Adams, J., and Laveler, J. (1993) Curr. Biol. 3, 188–190
6. Bernstein, P., and Sage, E. H. (1994) Methods Enzymol. 245, 62–84
7. Mosher, D. F. (1990) Annu. Rev. Med. 41, 85–97
8. Frazier, W. A. (1991) Curr. Opin. Cell Biol. 3, 792–799
9. Bernstein, P. (1992) FASEB J. 6, 3290–3299
10. Lahav, J. (1993) Biochim. Biophys. Acta 1182, 1–14
11. Murphy-Ullrich, J. E., Schultz-Cherry, S., and Hook, M. (1992) Mol. Biol. Cell 3, 181–188
12. Schultz-Cherry, S., and Murphy-Ullrich, J. E. (1993) J. Cell Biol. 122, 923–932
13. Schultz-Cherry, S., Ribeiro, S. M. F., Gentry, L., and Murphy-Ullrich, J. E. (1994) J. Biol. Chem. 269, 26775–26782
14. Schultz-Cherry, S., Lawler, J., and Murphy-Ullrich, J. E. (1994) J. Biol. Chem. 269, 26783–26788
15. Schultz-Cherry, S., Chen, H., Mosher, D. F., Misheineimer, T. M., Krutzsch, H. C., Roberts, D. D., and Murphy-Ullrich, J. E. (1995) J. Biol. Chem. 270, 7004–7010
16. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) Mol. Cell. Biol. 8, 4162–4168
17. Brown, P. D., Wakefield, L. M., Levison, A. D., and Sporn, M. B. (1990) Growth Factors 3, 35–43
18. Miyazono, K., Hellman, U., Wernstedt, C., and Heldin, C.-H. (1988) J. Biol. Chem. 263, 6407–6415
19. Wakefield, L. M., Smith, D. M., Flanders, K. C., and Sporn, M. B. (1988) J. Biol. Chem. 263, 7646–7654
20. Flammenga, R., Abe, M., Sato, Y., Miyazono, K., Harpel, J. G., Heldin, C.-H., and Rifkin, D. (1995) J. Cell. Biol. 120, 995–1002
21. Sha, X., Yang, L., and Gentry, L. E. (1991) J. Cell Biol. 114, 827–839
22. Miller, D. M., Ogawa, Y., Iwata, K. K., ten Dijke, P., Purchio, A. F., Soloff, M. S., and Gentry, L. E. (1992) Mol. Endocrinol. 6, 694–702
23. McMahon, G. A., Dignam, J. D., and Gentry, L. E. (1996) Biochem. J. 313, 343–351
24. Lioubin, M. N., Madisen, L., Marquardt, H., Roth, R., Kovacina, K. S., and Purchio, A. F. (1991) J. Cell Biochem. 45, 112–121
25. Maier, C. C., Moseley, H. N. B., Zhou, S.-R., Whitaker, J. N., and Blalock, J. E. (1994) Immunomethods 5, 107–113
26. Blalock, J. E., and Smith, E. M. (1984) Biochem. Biophys. Res. Commun. 121, 203–207
27. Bost, K. L., Smith, E. M., and Blalock, J. E. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1772–1775
28. Blalock, J. E. (1985) Nat. Med. 1, 876–878
29. Derynck, R., Rhee, L., Chen, Y. E., and Van Tilburg, A. (1997) Nucleic Acids Res. 15, 3188–3199
30. Qian, S. W., Kondaiah, P., Roberts, A. B., and Sporn, M. B. (1990) Nucleic Acids Res. 18, 3059
31. Miller, D. A., Lee, A., Pelton, R. W., Chen, E. Y., Moses, H. L., and Derynck, R. (1989) Mol. Endocrinol. 3, 1108–1114
32. Derynck, R., Linquist, P. B., Lee, A., Wen, D., Tamm, J., Graycar, J. L., Rhee, L., Mason, A. J., Miller, D. A., Coffey, R. J., Moses, H. L., and Chen, E. Y. (1988) EMBO J. 7, 3737–3743
33. Burt, D. W., and Jalowwe, S. B. (1992) Mol. Endocrinol. 6, 989–992
34. Kondaiah, P., Sands, M. J., Smith, J. M., Fields, A., Roberts, A. B., Sporn, M. B., and Melton, D. A. (1990) J. Biol. Chem. 265, 1089–1095
35. Baily, S., Brand, C., Chambaz, E. M., and Feige, J.-J. (1997) J. Biol. Chem. 272, 16329–16334
36. Hennessy, S. W., Frazier, B. A., Kim, D. D., Deckwerth, T. L., Baumgartel, D. M., Rotwein, P., and Frazier, W. A. (1989) J. Biol. Chem. 264, 729–736
37. Brentani, R. R., Ribeiro, S. M. F., Potenjak, P., Pasqualini, R., Lopes, J. D., and Nakaie, C. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 364–367
38. Pasqualini, R., Chomone, D. F., and Brentani, R. R. (1989) J. Biol. Chem. 264, 14566–14570
39. Fasina, G., Verdoliva, A., Cassani, G., and Melli, M. (1994) Growth Factors 10, 99–106
40. Miano, J. M., Vlastic, N., Tota, R. R., and Stemerman, M. B. (1993) Arterioscler. Thromb. 13, 211–219
41. Slater, M., Patava, J., and Mason, R. S. (1995) Experientia (Basel) 51, 235–244
42. Hugo, C., Shankland, S. J., Pichler, R. H., Couser, W. G., and Johnson, R. J. (1998) Kidney Int. 53, 302–311
43. Lau, L. P., and Nathans, D. (1991) Mol. Aspects Cell. Regul. 6, 257–293
44. Dameron, K. M., Volpert, O. V., Tainsky, M. A., and Bouck, N. (1994) Science 265, 1582–1583
45. Yang, Y., Dignam, J. D., and Gentry, L. (1997) Biochemistry 36, 11923–11932
46. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M. F., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. (1998) Cell 93, 1159–1170
47. Munger, J. S., Huang, X., Kawakatsu, H., Griffiths, M. J. D., Dulton, S. L., Wu, J., Pittet, J.-P., Kaminiski, N., Garat, C., Matthey, M. A., Rifkin, D. B., and Sheppard, D. (1999) Cell 96, 319–328