Molecular Interactions That Confer Latency to Transforming Growth Factor-β*

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A major point of regulation of transforming growth factor-β (TGF-β) function is through control of activation of the latent TGF-β complex, which consists of the latency associated peptide (LAP) secreted in non-covalent association with mature TGF-β. Activation involves proteolysis, dissociation, or altered binding of LAP. However, the mechanism by which LAP confers latency to TGF-β is poorly understood. Previously, we identified a conserved sequence near the N terminus of LAP as a site of thrombospondin-1 (TSP1) binding to the latent complex. Now we show that expression of the TGF-β1-latent complex deleted in the TSP1 binding site (ΔLSKL) of LAP results in secretion of LAP, but not of mature TGF-β. ΔLSKL LAP also fails to bind soluble or immobilized TGF-β1. Consistent with an inability to bind LAP, mature TGF-β1(−)ΔLSKL LAP is unable to confer latency to TGF-β, suggesting that the LSKL sequence is important, not only for TSP1 binding and activation, but also for binding to the mature domain. We identified the sequence RPKP in the receptor-binding region of mature TGF-β1 as the binding site for LAP. Peptides of the RPKP sequence bind LAP and inhibit LAP/TGF-β association. RPKP peptides also activate latent TGF-β1, presumably by disrupting LAP-mature TGF-β1 interactions. These studies provide a molecular basis for both latency and activation by TSP1 through the LSKL sequence of LAP binding to the RPKP sequence of mature TGF-β1.

Transforming growth factor β (TGF-β) is a widely expressed regulatory growth factor that is important for cell cycle progression, apoptosis, differentiation, and extracellular matrix accumulation, with well documented roles in wound healing, angiogenesis, fibrosis, and tumor progression (1–3). Tight regulation of appropriate levels of TGF-β activity is critical for homeostasis as excessive TGF-β activity is a primary cause of fibrotic disease and TGF-β1 deficiencies are implicated in neoplasia and inflammation (4, 5). The major point of control of TGF-β activity is at the level of conversion of the latent pre-cursor to the biologically active growth factor, a process termed activation (6).

TGF-β is secreted as part of a latent complex consisting of an N-terminal latency-associated peptide (LAP) and the mature domain. LAP and TGF-β are the products of a single gene, which (after post-translational modifications and cleavage by furin convertase) remains non-covalently associated, forming the small latent complex (SLC) (7). The SLC is secreted by cells as an inactive complex, and in some cases it is disulfide-bonded to the latent TGF-β-binding protein to form the large latent complex.

LAP association with the mature domain is critical for latency, and agents that activate the latent complex disrupt the association of LAP with the mature domain either by proteolysis or denaturing the LAP or by altering the folding of LAP (7). It is known that LAP association is critical for expression of the mature domain as mutations that disrupt LAP-mature domain binding prevent secretion of the mature domain (8). However, the molecular mechanism by which LAP confers latency to mature TGF-β is largely unknown.

Previously, we showed that thrombospondin 1 (TSP1) can activate latent TGF-β both in vitro and in vivo through specific molecular interactions (9, 10). The KRFK sequence in the type 1 repeats of TSP1 binds to a conserved sequence (ΔLSKL) near the N terminus of LAP. Binding of TSP1 to LAP renders the LAP unable to confer latency (11). This LSKL sequence (amino acids 54–57) lies within a region of the LAP (amino acids 50–85) previously shown to be necessary for latency (8). To determine the importance of the LSKL sequence of LAP in latency and TGF-β activation, we examined the effect of LSKL mutations on the secretion of the SLC, LAP/TGF-β association, and the ability of LAP to confer latency to mature TGF-β. We now report that the LSKL sequence mediates LAP binding to the mature domain, and we have identified a sequence in the mature domain that binds the LAP through LSKL. These studies establish a molecular basis for both LAP induction of latency and TSP1-mediated 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 cells were isolated and cultured as described (11). 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 transfected with the TGF-β response element of the human plasminogen activator inhibitor-1 gene promoter fused to firefly luciferase reporter gene were a generous gift from Dr. Daniel Rifkin, New York University (12), 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). 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.
Fig. 1. Secretion of TGF-β latent complex. A, FLAG-tagged TGF-β SLC constructs. β, immunoblots of conditioned medium and cell lysates from COS-1 cells expressing various latent TGF-β SLC constructs. Anti-TGF-β β detects both endogenous and FLAG-tagged TGF-β. Anti-FLAG blots detect only FLAG-tagged TGF-β secreted in the conditioned medium. Anti-LAP blots detect levels of LAP secreted into the medium. Anti-FLAG blots of cell lysates show production of recombinant FLAG-tagged precursors. The bar graph (bottom) indicates total TGF-β activity (heat-activated) in the COS-1 conditioned medium expressing SLC constructs (above) as measured by the PAIL assay. Data are means ± S.E. for triplicate determinations (n = 2).

Site-directed Mutagenesis and DNA Purification—Using FLAG-tagged and LAP-FLAG vectors as templates, primers were designed to introduce a FLAG epitope/spacer, which separated it from the LAP cleavage site. The purified PCR product was then inserted according to manufacturer’s instructions into the mammalian expression vector pEF6/V5-His-TOPO (Invitrogen). Resulting plasmids were verified by DNA sequencing as a template, primers were designed to introduce a FLAG epitope/spacer, which separated it from the LAP cleavage site. The purified PCR product was then inserted according to manufacturer’s instructions into the mammalian expression vector pEF6/V5-His-TOPO (Invitrogen). Resulting plasmids were verified by DNA sequencing at a DNA sequencing core facility at the University of Alabama at Birmingham. Placement of the FLAG epitope at this site has been shown not to affect expression and function of active TGF-β (14). Using similar protocols, we expressed the construct in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot. In addition, the plasminogen activator inhibitor-1 promoter luciferase (PAIL) assay was expressed in COS-1 cells and assayed for proper processing and secretion by immunoblot.

Production of LAP-FLAG—Using the human TGF-β latent complex as a template, primers were designed to introduce a FLAG epitope/terminus codon 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 Western blot, and the ability to confer latency to active TGF-β was verified by the PAIL assay.

Transfection, Cell Lysate, and Conditioned Medium Collection—Upon reaching 70% confluence in 6-well plates, COS-1 cells were washed in serum-free medium and transfected with 1 μg of DNA and 3 μl of FuGENE 6 (Roche Applied Sciences) per the manufacturer’s instructions in a final volume of 1 ml of serum-free Dulbecco’s modified Eagle’s medium with ITS supplement (Sigma). After 48 h, the conditioned medium was collected, centrifuged free of cell debris, and stored for analyses. Cells were washed in phosphate-buffered saline, harvested in 100 μl of SDS sample buffer, sonicated, and centrifuged. The resultant cell lysate supernatant was stored at −80 °C until analysis. TGF-β1 (−/−) cells, 70% confluent on 6-well plates, were transfected overnight with 1 μg of DNA using LipofectAMINE/Plus and Opti-MEM (Invitrogen). Wells were then washed with serum-free medium followed by the addition of 1 ml/well serum-free medium containing ITS. After 48 h, medium was collected, centrifuged free of cell debris, and stored. Bovine aortic endothelial cells were seeded in 100-mm dishes, grown to 70% confluence, and washed with serum-free medium. A final volume of 5 ml of serum-free medium was placed on the cells for 48 h, collected, centrifuged free of cell debris, and stored for analysis.

Immunoblotting—Conditioned medium was added to 6× Laemmli sample buffer, and 50 μl final volume was separated by SDS-PAGE (4–20% gradient) under non-reducing conditions. Cell lysates were analyzed by Bradford assay to determine total protein for loading of equal amounts of protein. 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, 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 TBST, 2.5% nonfat dry milk. For anti-FLAG blots, the membrane was blocked overnight, washed, and incubated with 10 μg of anti-FLAG-M2-peroxidase conjugate (Sigma) in 10 ml of TBST for 45 min. Immunoblots were developed by enhanced chemiluminescence (PerkinElmer Life Sciences). Blots were stripped between developments with Re-blot Plus (Chemicon). Densitometry was performed using the one-dimensional gel analysis function of NIH ImageJ.

**PAIL Assay for TGF-β Activity**—TGF-β activity in conditioned medium was determined by an established bioassay using mink lung epithelial cells expressing the TGF-β response element of the PAI-1 promoter fused to luciferase as a reporter (12). Human TGF-β1 (R&D Systems) was used to establish the standard curve. Total TGF-β levels were assayed in conditioned medium that had been heat-activated at 100 °C for 3 min and then diluted 1/5 in serum-free medium. Levels of active TGF-β were determined in medium diluted 1/5 that received no heat treatment. Where indicated, peptides were preincubated with medium for 30 min at room temperature. Samples were assayed in triplicate.

**Immunoprecipitation**—To detect soluble protein-protein interactions, 500 μl of COS-1 conditioned medium expressing LAP-FLAG constructs was incubated for 1 h at room temperature with 20 μl (final volume) of prewashed Anti-FLAG M2 affinity gel according to the manufacturer’s instructions (Sigma). Resin was centrifuged and washed in Tris-buffered saline three times. The washed resin was incubated with 20 μl of 0.1 M glycine HCl, pH 3.5, to release bound LAP-FLAG with associated TGF-β. The releasate was combined with 2.2 μl of 10× Tris-buffered saline and 6× Laemmli sample buffer and analyzed by immunoblotting. In some experiments, peptides were added and incubated for 30 min at room temperature prior to addition of the affinity gel. Equal pull-down of LAP-FLAG was verified by FLAG immunoblot/densitometry. Negative controls included resin alone and resin incubated with empty vector conditioned medium (containing endogenous TGF-β). Phosphate-buffered saline containing 40 ng of recombinant TGF-β was used as a positive control.

**Modified Enzyme-linked Immunosorbent Assay**—To detect immobilized protein-protein interactions, we developed a novel modified enzyme-linked immunosorbent assay for these studies. Briefly, either biotin-tagged peptides (10 μg/ml) or biotinylated TGF-β (0.2 μg/ml) was incubated on preblocked neutravidin-coated plates overnight at 4 °C (Pierce). After three washes with TBST, conditioned medium containing LAP-FLAG constructs was added and incubated for 3 h at 4 °C. Following three washes with TBST, wells were incubated in 1 μg/ml anti-FLAG M2 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 by a spectrophotometric plate reader (Bio-Tek). After background subtraction, the mean absorbance at 450 nm of triplicate wells was reported. In some experiments, conditioned medium was incubated with peptides

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**Fig. 2. Binding of LAP to mature TGF-β.** A, FLAG-tagged LAP constructs. B, Western blots of anti-FLAG immunoprecipitates (IP) from conditioned medium of COS-1 cells expressing indicated LAP-FLAG constructs and endogenous TGF-β immunoblotted with either anti-FLAG or anti-TGF-β antibodies (n = 2). C, LAP-FLAG construct in conditioned medium of TGF-β1 (−/−) cells binding to immobilized, biotinylated TGF-β1. Data (absorbance at 450 nm) are expressed as the means of triplicate determinations ± S.D. Serum-free medium containing FLAG peptide was added to measure non-specific binding of the FLAG epitope to TGF-β (n = 3). **WB**, Western blot.
for 45 min at 4 °C prior to addition to the wells. Biotin-tagged peptides were analyzed for equal binding to the plate by detection with streptavidin-horseradish peroxidase. Samples were assayed in triplicate, and controls included wells with each reagent alone, wells with non-biotin-tagged peptides or non-biotinylated TGF-β/H9252 and finally, to control for any effects of the FLAG epitope, wells with 5/H9262/M FLAG peptide (Sigma) incubated with biotin-immobilized peptides or TGF-β/H9252 were also included.

**Statistics**—Significance was determined using paired, 2-tailed Student’s t test. All error bars shown are mean ± standard deviation.

**RESULTS**

Mutation of the LSKL Sequence of LAP Results in Impaired Secretion of Mature TGF-β—To ensure proper folding and post-translational processing, the recombinant SLC was expressed using mammalian expression systems. To distinguish the engineered latent complex from the endogenous latent complex secreted by COS-1 cells, the SLC was expressed with a FLAG tag inserted at the N terminus of the mature domain according to previously published approaches (14). Site-specific mutations of the LSKL (amino acids 54–57) site of LAP were introduced into the vector containing the expression sequence for FLAG-TGF-β latent complex (FLAG-TGF-β). These mutations (Fig. 1A) include a complete deletion of the LSKL sequence (ΔLSKL) and an alanine substitution for lysine at amino acid 56 (LSA56L). The latter substitution was used because peptides of the LSAL sequence do not inhibit TSP1-mediated TGF-β activation as do LSKL peptides (11). These tagged constructs and the unmodified wild type latent TGF-β were expressed in COS-1 cells. Conditioned medium and cell lysates were analyzed for expression and secretion of the SLC by immunoblotting (Fig. 1B). The unmodified TGF-β and the FLAG-TGF-β were both expressed and secreted. The resulting FLAG-TGF-β latent complex (Fig. 1A) was secreted as a latent molecule but retained its ability to be activated by heat, acid, and TSP1 (not shown). Introducing the LSA56L mutation into the LAP resulted in a 70% decrease (by densitometry) in secretion of the mature TGF-β domain as detected in immunoblots for both TGF-β and the FLAG epitope. The decrease was also confirmed by the lower levels of total TGF-β detected in the conditioned medium of cells expressing LSA56L FLAG-TGF-β, as compared with cells expressing unmodified FLAG-TGF-β. LAP secretion also appeared slightly reduced in LAP-modified SLC-expressing cells, although levels were still higher than in the empty vector control. The lack of secretion of tagged TGF-β in cells expressing the SLC with LSKL modifications was not the result of defective protein translation because cell lysates probed with anti-FLAG antibody showed FLAG-TGF-β precursors expressed in all FLAG-TGF-β latent complex construct-expressing cells. Protein processing and secretion appears intact because both recombinant LAP and endogenous TGF-β were secreted. Thus, mutation of the latent complex at the LSKL sequence of LAP results in impaired secretion of mature TGF-β.

Mutation of the LSKL Sequence Impairs Association of LAP and TGF-β—To determine whether the impaired secretion of
mature TGF-β in the SLC mutated in the LSKL sequence is because of altered binding between LAP and the mature domain, the ability of LAP to bind TGF-β was assessed. FLAG-tagged LAP constructs with mutations in the LSKL sequence, either LSA56L or ΔLSKL, were engineered and expressed in COS-1 cells (Fig. 2A). The FLAG tag distinguishes engineered LAP from endogenous LAP. Conditioned medium from transfected COS-1 cells expressing wild type LAP-FLAG, LSA56L LAP-FLAG, or ΔLSKL LAP-FLAG was collected. Initially, the medium was assessed by immunoblotting and densitometry to confirm that the LAP-FLAG constructs were expressed equivalently (data not shown). Then, LAP-FLAG and mutants were isolated from the conditioned medium by immunoprecipitation with anti-FLAG antibody coupled to resin. LAP-FLAG and mutants with associated endogenous TGF-β was detected by immunoblot with anti-TGF-β antibody. The blot was stripped and reprobed with anti-FLAG antibody to determine equal immunoprecipitation of LAP-FLAG protein. Conditioned medium from cells expressing the empty vector (Cos EV CM) is a negative control. Recombinant TGF-β is a positive control for the Western blot (WB) (n = 3).
TGF-β association (72% of wild type by densitometry normalized to LAP-FLAG). These results were not because of defective production of endogenous TGF-β, as PAIL assays of the conditioned medium showed equal levels of total (heat-activated) TGF-β secreted with each LAP construct (data not shown). To confirm these findings, an alternate approach was used to assess LAP-TGF-β binding. TGF-β1 (−/−) fibroblasts were transfected with the LAP-FLAG and LSKL-modified constructs. Equivalent secretion of LAP was confirmed by immunoblotting and densitometry. Analysis by PAIL assay confirmed lack of TGF-β activity in the conditioned medium (not shown). Biotinylated TGF-β1 was immobilized on a neutravidin-coated plate. Binding of LAP-FLAG to immobilized TGF-β1 was then assessed through detection of the FLAG epitope with an anti-FLAG antibody conjugated to peroxidase. Unmodified LAP-FLAG had the highest level of binding to immobilized TGF-β (Fig. 2C). Binding to LSA56L LAP-FLAG was only partially reduced, which was consistent with the immunoprecipitation experiments. However, deletion of the LSKL sequence from LAP completely abrogated binding to TGF-β. The FLAG peptide control or conditioned medium from cells transfected with an empty vector did not bind TGF-β, indicating that LAP binding to TGF-β is specific. Thus, these studies show that the LSKL sequence in LAP is critical for binding to the mature domain of TGF-β.

Mutation of the LSKL Sequence Impairs the Ability of LAP to Confer Latency—Because LSKL is necessary for LAP binding to mature TGF-β and LAP association with the mature domain is critical for latency, we asked whether the LSKL sequence is required for LAP induction of latency. To examine the effects of LSKL mutations on latency, COS-1 conditioned medium expressing LAP-FLAG constructs was assayed for the ability to inactivate 20 pM active TGF-β in the PAIL assay. As predicted, the LAP-FLAG construct in COS-1 conditioned medium reduced levels of active TGF-β (Fig. 3A). LSA56L LAP-FLAG also retained its ability to confer latency, though slightly impaired, which is consistent with its impaired binding to mature TGF-β. In contrast, ΔLSKL LAP-FLAG failed to confer latency as predicted from its inability to bind mature TGF-β. Conditioned medium from cells transfected with the empty vector had no effect on TGF-β activity. Finally, increasing amounts of active TGF-β1 were added to conditioned medium collected from TGF-β1 (−/−) cells transfected with the LAP constructs, and TGF-β activity was assessed by the PAIL assay (Fig. 3B). The results show that LAP-FLAG conferred latency and that LSA56L LAP-FLAG was slightly less effective in conferring latency. In contrast, ΔLSKL LAP-FLAG was unable to confer latency. These studies show that the LSKL sequence of LAP is important not only for binding to the mature domain but also for conferring latency and formation of the latent complex.

The LSKL Sequence of LAP Recognizes the RKPK Sequence of Mature TGF-β—We next sought to identify the sequence in mature TGF-β recognized by the LAP LSKL sequence. Prior studies showed that the LSKL sequence binds to the KRFK sequence of TSP1 and that this interaction is necessary for TSP1-dependent TGF-β activation (11). Reasoning that the LSKL sequence might bind a sequence that has a similar arrangement of basic and hydrophobic residues as KRFK, the amino acid sequence of mature TGF-β was scanned, and the sequence RKPK (amino acids 94–98), which lies in the type II receptor binding region (15), was identified as a potential cognate of KRFK. To ascertain whether LAP binds the RKPK sequence, biotin-tagged RKPK and RKAK peptides were immobilized on neutravidin plates, and binding of LAP-FLAG protein in conditioned medium of TGF-β1 (−/−) cells was assayed by immunodetection of the FLAG epitope. LAP-FLAG bound to RKPK but not the control RKAK peptide (Fig. 4A). Binding of LSA56L LAP-FLAG to RKPK was reduced, and ΔLSKL LAP-FLAG showed no binding to the RKPK peptide. Serum-free medium containing the FLAG peptide did not bind RKPK, demonstrating that LAP-FLAG binding is not due to the FLAG epitope. RKPK peptides, but not the control peptides (RKAK and RPPK), also inhibited the association of immobilized biotinylated TGF-β1 and LAP-FLAG from TGF-β1 (−/−) conditioned medium (Fig. 4B). Association of LAP-FLAG with endogenous TGF-β in COS-1 conditioned medium was competitively blocked by increasing concentrations of RKPK peptide but not by RKAK or RPPK control peptides (Fig. 4C). Experiments were controlled for equal LAP-FLAG pull-down (anti-FLAG immunoblot). There was no association between anti-FLAG resin and endogenous TGF-β (empty vector conditioned medium). These data show that peptides containing the
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FIG. 6. Secretion of TGF-β latent complex. Conditioned medium from COS-1 cells expressing TGF-β SLC constructs was probed with anti-TGF-β, anti-FLAG, or anti-LAP antibodies. TGF-β is found in the conditioned medium of wild type unlated and FLAG-tagged TGF-β but not in SLC constructs expressingΔLSKL LAP or ΔRKPK mature TGF-β. The FLAG-tagged mature domain is detected in the cell lysates. LAP is secreted by all constructs. Cells expressing SLC constructs of the ΔLSKL LAP or ΔRKPK mature domain have reduced levels of total TGF-β in their conditioned medium as measured by the PAIL assay (lower panel). Results are the means of triplicate determinations ± S.D. (n = 2).

RKPK sequence bind LAP and inhibit association of LAP with mature TGF-β.

RKPK Peptides Activate the TGF-β Latent Complex—Because RKPK prevents LAP from binding to mature TGF-β, the RKPK peptide is predicted to activate the latent complex, potentially through dissociation of the LAP from the mature domain. The ability of RKPK to activate endogenous latent TGF-β in conditioned medium from bovine aortic endothelial cells was measured in the PAIL assay (Fig. 5A). Control peptides (RKAK and RPPK) did not increase TGF-β activity above base-line levels. However, RKPK stimulated a dose-dependent increase in TGF-β activity. Peptides did not increase TGF-β activity when added to serum-free medium (not shown) or when added to active TGF-β (Fig. 5B), indicating that the ability of RKPK to increase TGF-β activity occurs through activation of the latent complex and not modulation of active TGF-β or cellular receptors. The ability of RKPK peptides to activate latent TGF-β is further supported by the observation that RKPK peptides, but not control peptides (RKAK), activate the purified recombinant TGF-β latent complex (Fig. 5C). Together, these data show that RKPK peptides activate both recombinant and cell-derived latent TGF-β, presumably by interfering with the ability of LAP to confer latency to the mature domain.

Deletion of the RKPK Sequence Results in Loss of Secretion of TGF-β—It is known that the mature domain must be associated with the LAP to be secreted (13). If the RKPK sequence is important for mature domain association with the LAP, then deletion of RKPK from the SLC should impair secretion of the mature domain as was observed when LSKL was deleted. FLAG-tagged SLC deleted in the RKPK sequence (FLAG-TGF-βΔRKPK) was made and expressed in COS-1 cells. Serum-free conditioned medium and cell lysates from these cells and from cells expressingΔLSKL FLAG-TGF-β, FLAG-TGF-β, the unmodified SLC, and an empty vector were collected for analysis. No FLAG-tagged TGF-β was detected in the conditioned medium of cells expressing latent complex deleted in either the LSKL (ΔLSKL FLAG-TGF-β) or the RKPK (FLAG-TGF-βΔRKPK) sequences (Fig. 6), although LAP was detected in the conditioned medium. The absence of FLAG-tagged TGF-β in the conditioned medium is not due to a lack of protein expression, because protein was detectable in lysates of all cells expressing FLAG-tagged latent complex constructs. The lack of a detectable mature domain in the conditioned medium when either LSKL or RKPK is deleted is consistent with the base-line levels of TGF-β activity measured in the conditioned medium using the PAIL assay. However, it is possible that deletion of the RKPK sequence could also impair mature TGF-β binding to its receptors, as RKPK lies within the receptor binding region (15). These studies provide further support for our conclusion that the RKPK sequence in the mature domain is important for binding to LAP and formation of the latent complex.

DISCUSSION

These data show that the LSKL sequence of LAP is critical for proper formation and function of the latent TGF-β complex. Mutations of the LSKL sequence reduced binding of LAP to the mature domain and significantly impaired the ability of LAP to confer latency to mature TGF-β. We identified the sequence RKPK in the mature domain as a potential recognition sequence for LSKL. Peptides containing RKPK bound LAP and activated the latent complex. Finally, SLC constructs with deletions in either the RKPK sequence of the mature domain or the LSKL sequence of LAP resulted in loss of secretion of mature TGF-β, presumably because of impaired association of LAP with the mature domain. This is the first report to identify specific complementary sequences in the SLC that are critical for LAP/TGF-β association and latency.

The LSKL sequence lies within the larger region (amino acids 50–85) previously identified to be important for latency and secretion of the mature domain (8). However, in those studies, the site in the mature domain important for LAP association was not identified. The LSKL sequence is conserved in the three mammalian TGF-β isoforms, suggesting its broad
importance for latency (11). The LSKL sequence is also conserved across species for which the TGF-β sequence is known; purified simian β1-LAP deleted in LSKL is also unable to confer latency to mature TGF-β (data not shown). Finally, known inactivators of TGF-β1 (α2-macroglobulin and decorin) contain LSKL motifs, which could potentially associate with the RKPK sequence in the mature domain and inhibit activity.

The LSA56L LAP mutation partially affected secretion of the mature domain but had only minimal effects on LAP binding to mature TGF-β and on the ability of LAP to confer latency. This was unexpected because peptides of this sequence do not disrupt TSP1-dependent activation by competing for TSP-LAP binding. It is possible that the LSAL sequence in the context of the SLC retains sufficient structure to mediate LAP interaction with the mature domain.

The RKPK sequence is also conserved in the mature domain of TGF-β2 and TGF-β3 contain similar conserved sequences, KTPK and RTPK, respectively. Although the ability of LSKL to bind these other motifs was not tested directly, previous studies showed that chimeric TGF-β1/β2 protein, with KTPKI substituted for RKPKV, associated with LAP and was secreted (15). This same study identified that the RKPK sequence lies within a region important for binding to signaling receptors. It is conceivable that association of LSKL with RKPK would sterically prevent receptor binding. This is consistent with the known structure of TGF-β1, which shows that the RKPK sequence lies at the end of a largely extended β-structure (16). However, additional structural information for LAP and the latent complex will be needed to determine the proximity of the LSKL sequence to the RKPK site.

These data also provide a molecular mechanism for TSP1-mediated TGF-β activation. Activation by TSP1 occurs through binding of the RKPK sequence of TSP1 to the LAP through its LSKL sequence (11). Thus, it is plausible that KRFK competes with the RKPK sequence in the mature domain for LSKL binding in the LAP, resulting in an altered interaction of the LAP and the mature domain.

Control of TGF-β activity by post-translational activation of the latent complex is documented to be disregulated in many disease states (5, 17). TGF-β activators are potentially useful in models of wound healing where TGF-β activity might be deficient (18). On the other hand, natural TGF-β inhibitors (including LAP and the soluble type II TGF-β receptor) and small molecules (including peptides derived from TSP1) can potentially attenuate pathology in diseases from fibrosis to neoplasia (18–20). The results of these studies provide a new molecular basis for understanding latency and activation, which should aid in the design of novel therapeutics to modulate inappropriate levels of TGF-β activity.

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