Role of Carbohydrate Structures in the Binding of β1-Latency-Associated Peptide to Ligands†

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Received May 5, 1997; Revised Manuscript Received July 17, 1997⊗

ABSTRACT: Transforming growth factor β1 (TGF-β1) is a potent growth differentiation and morphogenesis factor. The amino-terminal 248 amino acid pro region of TGF-β1, the β1-latency-associated peptide (β1-LAP), is noncovalently associated with TGF-β1 in an inactive complex. Previous studies suggested that deglycosylated β1-LAP can not form this latent complex with TGF-β1. To study the role of the carbohydrate structures of β1-LAP in its biological functions, we expressed simian β1-LAP in *Escherichia coli* with a 10 histidine residue tag on the N-terminus. This polypeptide was solubilized from inclusion bodies with 6 M guanidine hydrochloride and purified by metal chelate affinity chromatography. Purified β1-LAP was refolded to its dimeric form using a chaotrope-mediated folding procedure. The dimeric β1-LAP forms 90 kDa complexes with TGF-β1, TGF-β2, and TGF-β3, and reverses the inhibitory activity of TGF-β1 on Mv1Lu cells. Solid phase binding assays demonstrate that refolded β1-LAP binds to heparin and thrombospondin 1. FET cell adhesion promoted by refolded β1-LAP was blocked by an RGD peptide. Purified β1-LAP produced in Chinese hamster ovary cells, deglycosylated with *N*-glycosidase F, forms a 80−90 kDa complex with mature TGF-β1. The carbohydrate structures of β1-LAP are not required for binding to ligands or for its biological activity.

Transforming growth factor β1 (TGF-β1)[1] belongs to a family of at least 25 cytokines (*1*). Included in this group are TGF-β1 and the related TGF-β2 and TGF-β3 isoforms (*2*−*4*), which mediate key events in cell growth and differentiation (*5, 6*).

TGF-β1 is synthesized as a precursor with a 29 amino acid leader sequence followed by a 248 residue pro region with the 112 residue mature TGF-β1 in the carboxy terminus (*7, 8*). Cleavage at a dibasic motif releases the C-terminal homodimeric 25 kDa TGF-β1. Recombinant TGF-β1 (*9*−*11*) and TGF-β1 derived from bone cultures (*12*) are noncovalent complexes of the C-terminal TGF-β1 dimer and a dimer of the N-terminal pro region. The N-terminal pro domain, designated β1-latency-associated peptide (β1-LAP), is essential for the folding and transport of TGF-β1 (*13*). Recombinant β1-LAP expressed in COS-1 cells (*10*) or in Chinese hamster ovary (CHO) cells (*11*) is a TGF-β1 binding protein. β-1-LAP expressed in a baculovirus system blocks the antiproliferative effects of TGF-β1 in a transgenic mouse model system (*14*). TGF-β1 latent complex is also secreted linked to the TGF-β binding protein (LTBP) through a disulfide bond to β1-LAP (*15*−*19*). These complexes are activated by low or high pH, heat, urea or SDS treatment (*20*−*22*), and proteolysis by plasmin, cathepsin D, or thrombin (*23, 24*). Plasmin may mediate activation *in vivo*, based on the activation of latent TGF-β1 in cocultures of endothelial and smooth muscle cells (*25*).

Three N-glycosylation sites at residues 82, 136, and 176 in β1-LAP contain mannose 6-phosphate (*26, 27*). Recombinant LTGF-β1 and platelet-derived LTGF-β1 bind to the mannose 6-phosphate/IGF-II receptor with high affinity (*26, 28*), directing LTGF-β1 to the cell surface, resulting either in activation of the complex or in its internalization (*29*). Sha et al. (*30*) demonstrated that inhibitors of N-glycosylation block secretion of TGF-β1 in Chinese hamster ovary (CHO) cells, while inhibitors of later stages of glycosylation had no effect. An Asn to Gln substitution at the second N-glycosylation site inhibited secretion of mature TGF-β1 in human embryonic kidney cells (*31*). An Asn to Ser substitution at the first N-glycosylation site reduced secretion of TGF-β1, and secretion was undetectable after removal of all three N-glycosylation sites (*32*). Other studies indicated that removal of β1-LAP carbohydrate structures dissociates TGF-β1 from the latent complex (*33*), implicating the carbohydrate structures in maintenance of the latent complex. Some features of carbohydrate structures of β1-LAP are not critical for the latent complex as β1-LAP with varying degrees of sialylation forms a complex with TGF-β1 (*34*).

The latent TGF-β-1 complex binds to three physiologically important ligands: heparin, thrombospondin 1, and integrins. Heparin inhibits the binding of the latent TGF-β1 complex to α_2-macroglobulin (*35*), consistent with heparin binding activity of the complex (*36*). Heparin also blocks the degradation of the latent TGF-β1 complex by plasmin and

† This work was supported by a grant to L.E.G. from the National Institutes of Health, National Cancer Institute (CA60848).

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⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1997.

[1] Abbreviations: TGF-β, transforming growth factor β; β1-LAP, β1-latency-associated peptide; LTGF-β, latent TGF-β; LTBP, latent TGF-β binding protein; ECM, extracellular matrix; TSP1, thrombospondin 1; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; Mv1Lu, mink lung epithelial; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IPTG, isopropyl 1-thio-β-D-galactopyranoside; RT, room temperature; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; Tris, tris (hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; Gnd·HCl, guanidine hydrochloride.

S0006-2960(97)01047-7 CCC: $14.00

trypsin (*37*), and the platelet and recombinant latent TGF-β1 complex bind to heparin−Sepharose (*38*). Thrombospondin 1 (TSP1), which is released with TGF-β1 after platelet degranulation (*39, 40*), activates the small and large forms of latent TGF-β1 (*41*). An RFK sequence in TSP1 mediates this effect (*42*). Although β1-LAP rather than mature TGF-β1 has been proposed as the target in TSP1 activation of the β1-LAP−TGF-β1 complex (*43*), no direct evidence supports this hypothesis. β1-LAP contains one Arg-Gly-Asp (RGD) integrin binding motif (*44*) which mediates β1-LAP association with integrins. Though the physiological significance of β1-LAP binding to integrins is unclear, it may anchor the latent TGF-β1 complex on the surface of target cells.

To analyze the role of β1-LAP carbohydrate structures in binding to physiologically significant ligands and in its biological activities, we produced the protein in *E. coli* since it contains no system for glycosylation like that of animal cells.

EXPERIMENTAL PROCEDURES

Materials. Tissue culture media, serum, and prestained and unstained protein molecular weight markers were from GIBCO BRL. Restriction enzymes, enzymes for DNA sequencing, and reagents for cloning were from U. S. Biochemical Corp., GIBCO BRL, New England Biolabs, or Promega Biotech. Oligonucleotides used for polymerase chain reaction (PCR) were from Genosys Biotech. Poly(vinylidene difluoride) (PVDF-plus) membranes were from Micron Separations Inc. Nitro-blue tetrazolium/5-bromo-4-chloroindolyl phosphate (BCIP/NBT) was from Bio-Rad Laboratories, Inc. Alkaline phosphatase conjugated goat anti-rabbit IgG was from Cappel. $Na^{125}I$ was from Amersham. TGF-β1 and CHO (Chinese hamster ovary) β1-LAP were prepared in our laboratory (*11, 45*). TGF-β2 and TGF-β3 were obtained from R & D Systems. Disuccinimidyl suberate (DSS) was from Pierce. A Thermo Max Microplate reader was from Molecular Devices Corp. Guanidine hydrochloride, carbenicillin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), isopropyl 1-thio-β-D-galactopyranoside (IPTG), and heparin were from Sigma Chemical Co. Thrombospondin 1 and the synthetic peptides GRGDSP and GRGESP were from GIBCO BRL. Ninety-six-well plates were from Corning. *p*-Nitrophenyl phosphate (pNPP) was from Amresco. Factor Xa and *N*-glycosidase F were from Boehringer Mannheim. Ni-NTA silica was from Qiagen Inc.

Construction of the Expression Vector pET-16b/β1-LAP. The β1-LAP sequence (residues 30−278) was amplified by PCR from a simian cDNA using the primers 5′-ACGTCTCGAGGGACTATCCACCTCCAAGACTATC-3′ (5′-primer) and 5′-TGCAGGATCCTCAGCGGTGCCGGGAGCTTTG-3′ (3′-primer) and inserted between the *Xho*I and *Bam*HI restriction sites of pET-16b (Novagen). The encoded protein has a 24 amino acid residue N-terminal extension with a 10 residue His-tag. The N-terminal extension can be removed by factor Xa cleavage.

Production of β1-LAP in E. coli. Transformed *E. coli* strains [HMS174(DE3), HMS174(DE3)plysS, BL21(DE3), and BL21(DE3)plysS (Novagen)] were grown overnight at 37 °C in 2 mL of LB medium containing 100 μg/mL carbenicillin. Cultures were diluted into 500 mL of LB medium and grown with shaking at 37 °C. At an OD_{600} of 0.6−0.8, IPTG was added to 1 mM, and growth was continued for 4 h. Cells were collected by centrifugation (5000*g*, 10 min) and suspended in 10 mL of 50 mM Tris-HCl (pH 8.0 at 25 °C) and 1 mM EDTA. Cells were harvested and stored at −70 °C.

Isolation and Solubilization of Inclusion Bodies. Reported pH values were determined at 25 °C. Frozen cells were thawed on ice, resuspended in 10 mL of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and subjected to sonication (3 × 20 s, 40 W, microtip, 550 Sonic Dismembrator). After centrifugation at 5000*g* for 10 min, the pellet was washed 3 times in 10 mL of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.5% (v/v) Triton X-100 and once with 10 mL of 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 2 M urea at 25 °C. The pellet was washed twice in deionized water and suspended in 5 mL of buffer A [6 M guanidine hydrochloride, 400 mM NaCl, 50 mM potassium phosphate (pH 8.0), 10 mM 2-mercaptoethanol, 1% Triton X-100, and 10% glycerol]. The material was solubilized by mixing gently at room temperature overnight and centrifuged at 4 °C at 12000*g* for 30 min, and the supernatant was used for purification.

Purification of His-β1-LAP by Metal Chelate Chromatography. Purification on Ni-NTA was performed at room temperature. His-β1-LAP from the solubilization step was added to 2 mL of Ni-NTA resin equilibrated with buffer A and mixed gently overnight. The resin was packed into a column, washed with 10 volumes of buffer A with 10 mM imidazole (pH 8.0), and eluted successively with 3 volumes of buffer A plus 25 mM imidazole, 50 mM imidazole, 60 mM imidazole, 75 mM imidazole (pH 8.0), and 250 mM imidazole (pH 7.0). Based on SDS−PAGE analysis, fractions with His-β1-LAP were pooled.

Refolding of His-β1-LAP. Dialyses were performed at 4 °C against 100 volumes of buffer. Purified monomeric β1-LAP (0.1 mg/mL) was dialyzed against 4.5 M Gnd·HCl, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.1% PEG 3350 for 30 min, then 4.0 M Gnd·HCl, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.1% PEG for 30 min, and then back to 4.5 M Gnd·HCl for 30 min. This sequence of dialyses between 4.5 M Gnd·HCl and 4.0 M Gnd·HCl was repeated 3 times. The sample was dialyzed stepwise (30 min at each concentration) against 3.75, 3.5, 3.25, 3, and 2 M Gnd·HCl, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% PEG, 0.3 M Na_2SO_3, and 0.03 M $Na_2S_4O_6$. The dialysis against 2 M Gnd·HCl was for 12−16 h. The buffer was changed to 1 M Gnd·HCl, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, and 0.1% PEG, and dialysis was continued for 6 h. The sample was then dialyzed against renaturation buffer [2 mM GSH, 0.5 mM GSSG, 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.2 M L-arginine hydrochloride, 2 mM EDTA, and 0.1% PEG] for 4 days. The buffer was changed to 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and dialysis was continued for 12−16 h. Purified monomeric β1-LAP was kept at low concentrations to prevent precipitation, since it aggregates above 125 μg/mL with a resulting low yield of dimeric β1-LAP. Since the optimal pH range for the formation of disulfide bonds is 7.0−10.0, the pH of dialysis buffers was adjusted to 8.0; at pH 5.0, no dimeric β1-LAP was detected. More precipitation occurred at RT than at 4 °C. Protection by S-sulfonation of Cys223 and Cys225, which are responsible for forming two interchain disulfide bonds, resulted in

higher yields presumably by preventing the formation of non-native intrachain disulfide bonds (*46*). Reduced glutathione (GSH) and oxidized glutathione (GSSG) were included in the renaturation buffer to promote disulfide bond formation and rearrangement. Optimum oxido shuffling conditions (2 mM GSH and 0.5 mM GSSG) were identified by varying the concentration of GSH and GSSG. Net reducing conditions were chosen because more monomers will have free cysteines available, and random inter- or intra-chain disulfide bonds are easier to reduce than native disulfide bonds. The renaturation buffer was supplemented with PEG, which increases molecular density and solvent viscosity and prevented aggregation of the refolding intermediates (*47*). L-Arginine hydrochloride destabilizes incorrectly folded or disulfide-bonded structures (*48, 49*).

Cell Culture. Mink lung epithelial (Mv1Lu) cells (ATCC CCL64) were grown in Dulbecco's Modified Eagle's medium (DMEM), with fetal bovine serum (10%, v/v), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were passaged every 3 days by treatment with 0.05% (w/v) trypsin in normal saline containing 0.02% (w/v) EDTA and replating at a lower density. Human colon carcinoma FET cells were maintained in chemically defined McCoy's 5A serum-free medium, with 20 μg/mL insulin, 4 μg/mL transferrin, and 10 ng/mL epidermal growth factor. Cells were replated at lower density after treatment with 0.125% (w/v) trypsin in Joklik's medium.

Growth Inhibition Assay for β1-LAP. The biological activity of refolded β1-LAP was determined using an Mv1Lu mink lung epithelial cell growth inhibition assay (*11*). Plates (96-well) were seeded with 3000 cells per well from confluent cultures. Serial dilutions of refolded β1-LAP or control proteins were incubated with 80 pM TGF-β1 for 1 h at RT. These proteins were added to 96-well plates containing cells and incubated at 37 °C for 72 h. Cells were stained with MTT, and stain was measured by the absorbance at 595 nm with a THERMO-max microplate reader. Purified CHO TGF-β1 was used to standardize the assay; 3−5 pM TGF-β1 inhibited Mv1Lu cell growth by 50%.

Solid-Phase Heparin Binding Assay. Plates (96-well) were coated with 100 μL of heparin at 20 μg/mL in TBS at RT overnight. After blocking unoccupied sites with 3% BSA in TBS for 4−6 h and washing 3 times with PBS, 1 μg of CHO β1-LAP, refolded β1-LAP, monomeric β1-LAP, BSA, or control proteins [total proteins from HMS174(DE3)plysS cells transformed with pET-16b without insert] was added to each well and incubated for 4 h at 37 °C. Unbound proteins were removed by washing 3 times with TBS−0.1% Tween. One hundred microliters of anti-TGF-$\beta1_{81-94}$ antibody (designated 1125 antibody) was added and incubated 12−16 h at 4 °C. The wells were washed 5 times with 150 μL of PBS, and 100 μL of alkaline phosphatase−goat anti-rabbit secondary antibody (diluted 1:500 as recommended by the supplier) was added per well, incubated 2 h at RT, and washed 3 times with TBS−0.1% Tween. One hundred microliters of 1 mg/mL *p*-nitrophenyl phosphate was added per well, and incubated at 37 °C for 10 min. Reactions were stopped with 25 μL of 2 N NaOH, and the absorbance at 405 nm was measured.

Solid-Phase Thrombospondin Binding Assay. Plates (96-well) were incubated with 100 μL of thrombospondin 1 (10 μg/mL in TBS) per well at RT overnight. The plates were blocked with 3% BSA in TBS and washed 3 times with PBS. Test samples, 200 ng of CHO β1-LAP, refolded β1-LAP, monomeric β1-LAP, BSA, or control proteins were added to the wells, either without treatment or after incubation with Ab1125 for 30 min at 37 °C. Plates were incubated for 4 h at 37 °C, and then washed with TBS−0.1% Tween. Polyclonal anti-TGF-β1 LAP antibody (1:500 dilution) was added and incubated 12−16 h at 4 °C, followed by goat anti-rabbit secondary antibody. After washing with TBS−0.1% Tween, 100 μL of 1 mg/mL p-nitrophenyl phosphate was added per well, and incubated at 37 °C 10 min. Reactions were stopped with 25 μL of 2 N NaOH, and OD_{405} was measured.

Human Colon Carcinoma FET Cell Adhesion Assay. Plates (96-well) were coated with 100 μL of control proteins, β1-LAP, or BSA at 10 μg/mL in TBS at RT overnight. The wells were washed 3 times with PBS, blocked by incubation for 3−5 h with 150 μL of 3% BSA at RT, and washed again with PBS. FET cells were harvested from confluent cultures, collected by centrifugation, resuspended in 10 mL of serum-free (SF) medium, and diluted to 6×10^5 cells/mL in SF medium. After a 30 min recovery period, 100 μL of SF medium and 100 μL of cell suspension were added per well, and incubated for 90 min at 37 °C in 5% CO_2 and 100% humidity. Unattached cells were removed, and the wells were washed gently 3 times with SF medium. Fifty microliters of SF medium containing 2 mg/mL MTT was added per well, the plates were incubated at 37 °C for 2 h, the medium was discarded, 150 μL of DMSO was added, and OD_{595} was measured. In the attachment inhibition assays, 50 μM RGD or RGE peptide was included in the cell suspensions and incubated for 30 min at 37 °C during the recovery period prior to the adhesion assay.

Quantitation of Recombinant Monomeric β1-LAP and Refolded Dimeric β1-LAP. Levels of 32 kDa monomeric and 64 kDa dimeric β1-LAP were determined by densitometry of immunoblots using an Image Acquisition and Analysis System (AMBIS). The β1-LAP dimer and monomer were determined by densitometry and absolute amounts were determined by comparison to known amounts of CHO β1-LAP.

Electrophoresis and Western Blotting. SDS−PAGE was performed on 12% polyacrylamide slab gels using a Tris−glycine buffer (*50*). Samples in guanidine hydrochloride were diluted to 100 μL, mixed with 100 μL of 10% TCA, and after 20 min on ice subjected to centrifugation at 15 000 rpm for 15 min in a microcentrifuge. Pellets were washed twice with 100 μL of cold ethanol, dried, and dissolved in SDS sample buffer. Gels were stained with Coomassie brilliant blue R-250 or electrophoretically transferred to poly(vinylidene difluoride) (PVDF-plus) membranes for antibody staining (*45*). Rabbit antiserum against residues 81−94 of the pro portion of TGF-β1 (Ab_{1125}) (described in reference 45) was affinity-purified (*51*). Affinity-purified, goat anti-rabbit IgG−alkaline phosphatase conjugate was used as a secondary antibody.

Cross-Linking of Refolded β1-LAP with Radioiodinated TGF-β1, TGF-β2, and TGF-β3. TGF-βs were iodinated ($Na^{125}I$) using a Chloramine-T method (*52, 53*). Cross-linking was performed as described previously (*10, 54*).

Digestion of the Refolded β1-LAP with Factor Xa. Refolded β1-LAP (10 μg) was incubated in 200 μL of 100 mM NaCl, 50 mM Tris-HCl (pH 8.0) and 1 mM $CaCl_2$ with factor Xa (0.3−1 μg) for 18 h at RT.

Deglycosylation of CHO β1-LAP by N-Glycosidase F. Purified CHO β1-LAP (50 μg) in 120 μL of 10 mM phosphate buffer (pH 7.4), 25 mM EDTA was incubated with 0.75% NP-40 and 6 units of *N*-glycosidase F at 37 °C for 19 h (native β1-LAP) or heated at 95 °C for 5 min in 0.1% SDS (denatured β1-LAP), and then incubated with 0.75% NP-40 and 6 units of *N*-glycosidase F at 37 °C for 19 h.

RESULTS

Construction of the β1-LAP Expression Plasmid. A PCR product encoding simian β1-LAP was introduced into pET-16b, which has regulated transcription from a T7 promoter, efficient translation initiation signals, and the *lac* repressor gene and the *lac* operator to ensure low levels of background expression. The fusion protein has 10 consecutive histidine residues (His−tag) for affinity purification. A factor Xa site allows for removal of the His−tag. Sequence analysis verified that the insert was identical to that published by Sharpes et al. (*8*), except that Cys33 (TGC) was changed to Ser (TCC) to prevent improper disulfide cross-linking.

Expression of Recombinant β1-LAP. Four *E. coli* strains, HMS174(DE3), HMS174(DE3)plysS, BL21(DE3), and BL21-(DE3)plysS (*55*−*57*), were transformed with pET-16b/β1-LAP and tested to optimize the expression of the protein. Bacterial lysates were examined by SDS−PAGE after Coomassie blue staining or Western blot analysis using anti-TGF-$\beta 1_{81-94}$ antipeptide antibody. The predicted molecular mass of monomeric His-tagged β1-LAP is 32 kDa (*8*, *45*); the mass of the N-terminal extension is 3.3 kDa. A β1-LAP immunoreactive polypeptide of 32 000 M_r was observed in three of the strains at up to 15% of the total protein. HMS174(DE3)plysS was chosen for purification due to the higher yield of the expressed protein. Uninduced bacteria harboring the expression plasmid or pET16b lacked detectable levels of β1-LAP. β1-LAP accumulated in HMS174-(DE3)plysS cells over 4 h after induction, with longer induction not resulting in higher yield.

Purification of β1-LAP. Insoluble β1-LAP was in inclusion bodies in *E. coli*. Several strategies were tested to obtain soluble β1-LAP, including growth at lower temperatures (30 and 22 °C), reducing the IPTG concentration, changing host strains, and growing in different media [e.g., LB containing betaine/sorbitol (*58*)]; however, no soluble β1-LAP was detected. However, β1-LAP solubilized with high concentrations of Gnd·HCl could be purified by affinity chromatography on Ni-NTA (Figure 1). About 2.0 mg of His-β1-LAP was obtained from a 50 mL bacterial culture for renaturation to its biologically active, dimeric form (*10*, *59*).

Renaturation of Recombinant β1-LAP. The renaturation of recombinant β1-LAP was performed by slow removal of Gnd·HCl by dialysis and slow oxidation of cysteines to disulfides by a glutathione-redox system. Under optimal conditions, refolding and dimerization of β1-LAP were complete after 4 days with 40% of the monomeric β1-LAP converted to its dimeric form (Figure 2). SDS−PAGE revealed a 32 kDa species in the presence of mercaptoethanol and a dimeric 64 kDa species in its absence. The 32 kDa band migrated as a doublet; the faster migrating species may be a degraded form of the monomeric β1-LAP. The 64 kDa species did not migrate as a doublet, suggesting that only the undegraded monomer dimerizes. In experiments presented in this report, the concentration of β1-LAP given is

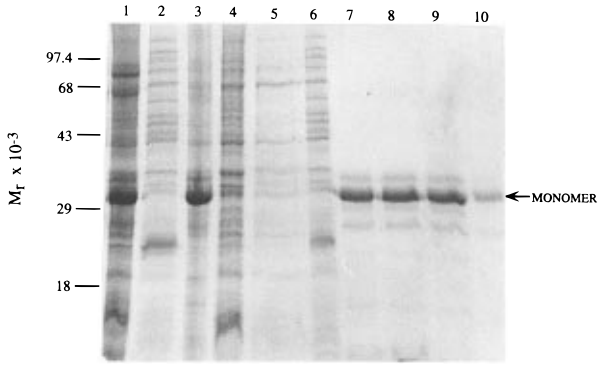

FIGURE 1: Purification of recombinant β1-LAP. HMS174(DE3)-plysS with pET-16b/β1-LAP from 500 mL of culture was grown and extracted as described under Experimental Procedures. Fractions from the solubilization and purification steps were analyzed by 12% SDS−PAGE and stained with Coomassie blue: lane 1, total cell extract after induction; lane 2, soluble cell fraction; lane 3, insoluble cell fraction solubilized in 6 M Gnd·HCl; lane 4, column flow-through fraction; lanes 5−10, fractions eluted with 10, 25, 50, 60, 75, and 250 mM imidazole, respectively. The arrow on the right indicates the migration of recombinant monomeric β1-LAP. The position and mass (in kDa) of molecular mass markers are indicated at the left.

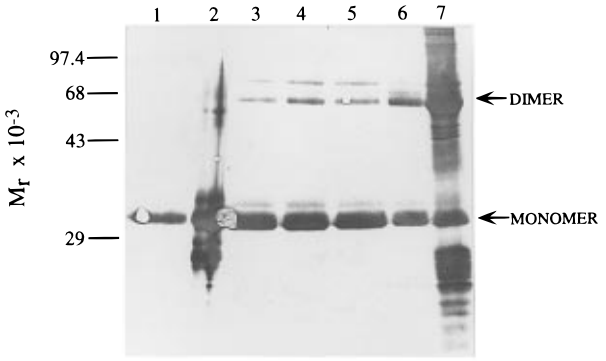

FIGURE 2: Dimerization of β1-LAP-His-tag. Samples were applied to a 12% nonreducing SDS−PAGE and analyzed by immunoblotting using anti-TGF-$\beta 1_{81-94}$ antibody: lane 1, supernatant after Gnd·HCl was reduced to 1 M; lane 2, pellet after Gnd·HCl reduced to 1 M; lanes 3−6, supernatants after 24, 48, 72, and 96 h of renaturation, respectively; lane 7, pellet after 96 h in renaturation buffer. Pellets (lanes 2 and 7) were from 1 mL aliquots. Supernatants (25 μL) were loaded in the other lanes. The arrows at the right indicate the position of recombinant monomeric and dimeric β1-LAP. The position and mass (in kDa) of molecular mass markers are indicated at the left.

that of the dimeric species, which was determined by densitometry of samples run on SDS−PAGE as described under Experimental Procedures.

Biological Activity of Refolded β1-LAP. The biological activity of refolded β1-LAP was measured using a mink lung epithelial cell (Mv1Lu) growth inhibition assay (11). The sensitivity of Mv1Lu cells to TGF-β1 is shown by the dose−response inhibition curve in Figure 3A. Fifty percent inhibition was observed at 3−5 pM TGF-β1 and near full inhibition at 80 pM TGF-β1. Control proteins from HMS174-(DE3)plysS cells transformed with pET-16b, refolded β1-LAP, and monomeric β1-LAP had little effect on cell growth unless added in large amounts (Figure 3B). When 800 pM TGF-β1 was combined with 35.2 μM control proteins or monomeric β1-LAP, incubated for 1 h, and serially diluted for a bioassay (Figure 3C), both controls showed dose−response inhibition curves similar to TGF-β1 alone. The control proteins and unfolded His-tagged β1-LAP did not

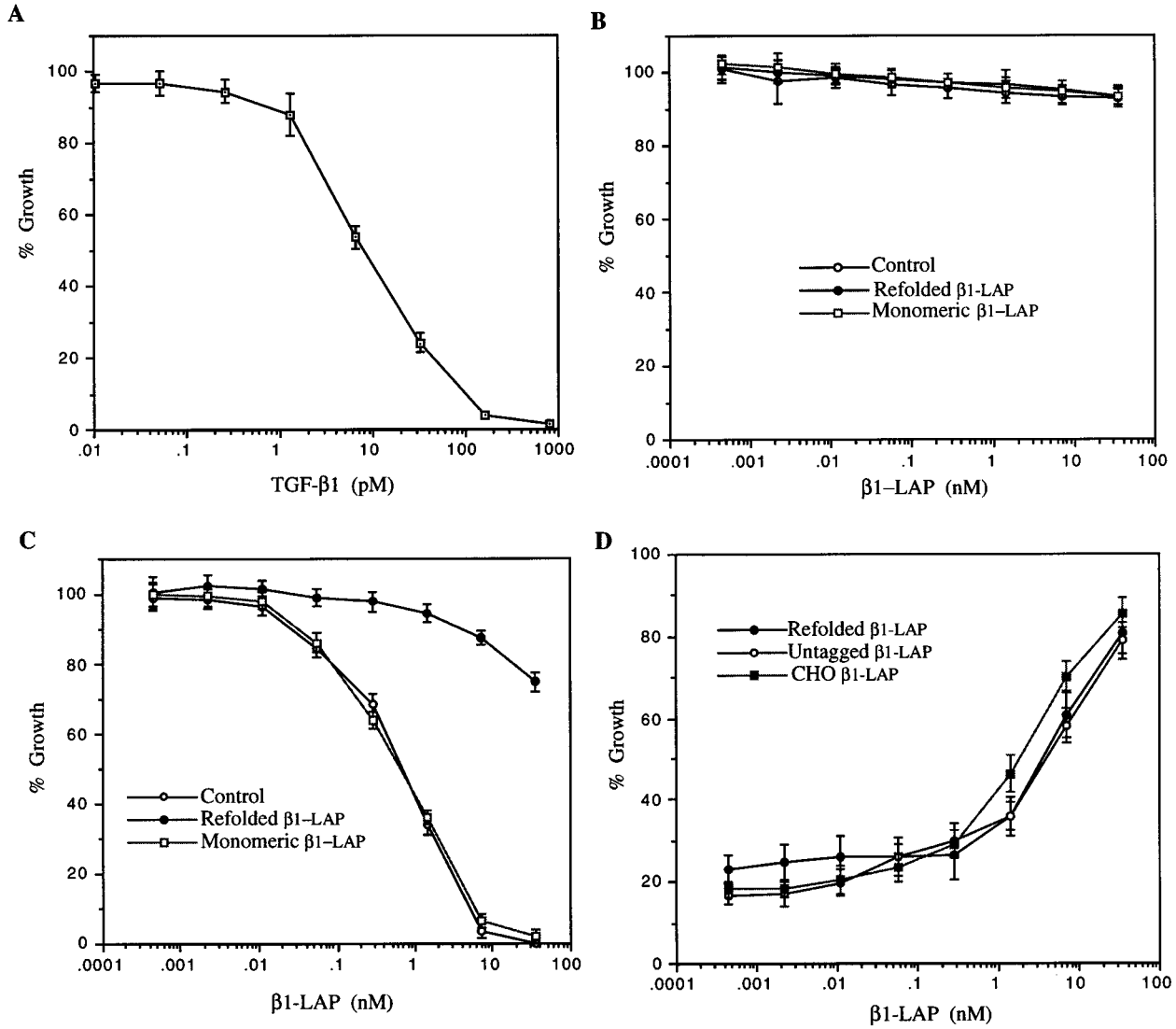

FIGURE 3: Effect of refolded β1-LAP, monomeric β1-LAP, CHO β1-LAP, and control proteins on growth of mink lung epithelial cells. (A) Effect of TGF-β1 on growth of mink lung epithelial cells. (B) Effect of control proteins, refolded β1-LAP, or monomeric β1-LAP on growth of mink lung epithelial cells. (C) The same as (B) except that TGF-β1 was added to control proteins or β1-LAPs at 800 pM and incubated for 1 h prior to dilution to the concentrations of β1-LAP indicated in the figure. (D) Serial dilutions of refolded β1-LAP, untagged β1-LAP after factor Xa cleavage, and β1-LAP purified from CHO cells were incubated with 80 pM TGF-β1 for 1 h prior to plating for assay. Results are the average of three experiments performed in triplicate ± SD.

reverse the effect of TGF-β1 on cell growth. Refolded β1-LAP, however, blocked the inhibitory activity of TGF-β1. Figure 3D shows the dose-response curves of Mv1Lu cells to refolded β1-LAP, untagged β1-LAP (described later under Results), and β1-LAP produced in CHO cells as a positive control. TGF-β1 was combined with serial dilutions of refolded β1-LAP and CHO β1-LAP, incubated for 1 h, and then tested in the mink lung epithelial cell bioassay. Both forms of β1-LAP increased Mv1Lu cell growth in a dose-dependent manner. The EC_{50} value for refolded β1-LAP was 4.3 ± 0.7 nM, and that for CHO β1-LAP was 1.8 ± 0.2 nM. Since the concentration of β1-LAP is based on the amount of the dimer in the preparation, the difference in EC_{50} values between the refolded and CHO β1-LAP may be significant. However, we do not know if all of the dimeric species is native, though monomeric β1-LAP clearly is not.

Characterization of Deglycosylated CHO β1-LAP. To examine the effect of deglycosylation on its activity, we treated CHO β1-LAP with *N*-glycosidase F, which cleaves both high-mannose and complex glycoproteins (60). CHO β1-LAP deglycosylated under native conditions gave a species of 60 kDa under nonreducing conditions, corresponding to the size of deglycosylated dimeric CHO β1-LAP and a smaller species that may be a degraded form of CHO β1-LAP (data not shown). Under reducing conditions, deglycosylated CHO β1-LAP migrated as a 30 kDa doublet, the molecular mass predicted for monomeric β1-LAP. Lower concentrations of *N*-glycosidase F gave incomplete deglycosylation. Deglycosylated CHO β1-LAP formed an 80−90 kDa DSS cross-linked complex with TGF-β1 (Figure 4, lane 5), indicating that deglycosylated β1-LAP binds to TGF-β1. The Mv1Lu growth inhibition assays could not be employed because components of the reaction used for *N*-glycosidase F digestion inhibited the growth of CCL64 cells (data not shown).

Binding of β1-LAP to TGF-β1. We investigated the formation of the latent TGF-β1 complex with refolded β1-LAP by chemical cross-linking. Radioiodinated TGF-β1 was incubated with refolded β1-LAP for 2 h at RT, and then treated with the cross-linker DSS for 10 min. Refolded β1-LAP and TGF-β1 formed a 90 kDa species which was detected by SDS−PAGE and autoradiography (Figure 5A,

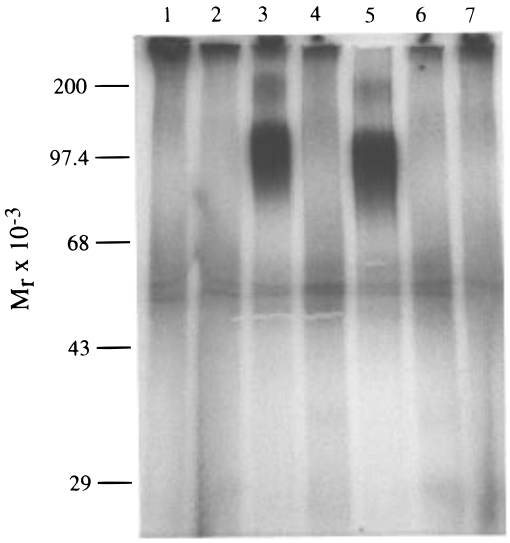

FIGURE 4: Cross-linking of deglycosylated CHO β1-LAP to ^{125}I-TGF-β1. The controls, CHO β1-LAP, and deglycosylated CHO β1-LAP were incubated with ^{125}I-TGF-β1 for 2 h and cross-linked with DSS as described under Experimental Procedures. Samples were resolved on 9% SDS PAGE and detected by autoradiography: lane 1, no addition; lane 2, conditioned medium from parental CHO cells; lane 3, CHO β1-LAP; lane 4, CHO β1-LAP with 20 nM cold TGF-β1; lane 5, deglycosylated CHO β1-LAP; lane 6, deglycosylated CHO β1-LAP with 20 nM cold TGF-β1; lane 7, *N*-glycosidase F. The position and mass (in kDa) of molecular mass markers are indicated at the left.

lanes 4 and 5). Control proteins did not form this complex with TGF-β1, nor did monomeric β1-LAP. CHO β1-LAP formed a 110 kDa complex with TGF-β1 (Figure 5A, lanes 8 and 9). The complex formed between refolded β1-LAP or CHO β1-LAP with TGF-β1 could be competitively displaced with 20 nM unlabeled TGF-β1 (Figure 5A, lanes 6 and 10). Refolded β1-LAP forms complexes with iodinated TGF-β2 and TGF-β3 (Figure 5B,C), similar to those formed with β1-LAP produced in COS-1 cells (54). Bioassays demonstrated that the TGF-β1−refolded β1-LAP complex was activated by heat or acid treatment, similar to the latent complex from platelets or expressed in CHO cells (10, 21) (data not shown).

Interaction with Extracellular Matrix. Heparin inhibits the proliferation and differentiation of vascular smooth muscle cells *in vivo* (61) and *in vitro* (62). Platelet and recombinant latent TGF-β1 complexes bind to heparin attached to Sepharose through specific interaction with this polyanion (38). To determine if heparin interacts with β1-LAP, we analyzed refolded β1-LAP using a solid-phase heparin binding assay. Refolded β1-LAP, CHO β1-LAP, monomeric β1-LAP, BSA or control proteins were added to 96-well plates coated with heparin. After removing unbound protein, Ab1125 primary antibody and alkaline phosphatase secondary antibody were used to detect bound β1-LAP as shown in Figure 6. The binding of CHO β1-LAP to heparin was set as 100%. Refolded β1-LAP showed 112% binding relative to CHO β1-LAP; binding of monomeric β1-LAP was not detected. These results indicate that recombinant, dimeric β1-LAP contains a heparin binding site(s) that is (are) not detected in the monomer.

Thrombospondin 1 is a 450 kDa homotrimeric, multifunctional glycoprotein with three disulfide-linked subunits which binds to β1-LAP. We investigated the binding of TSP1 to CHO β1-LAP and refolded β1-LAP using a solid-phase

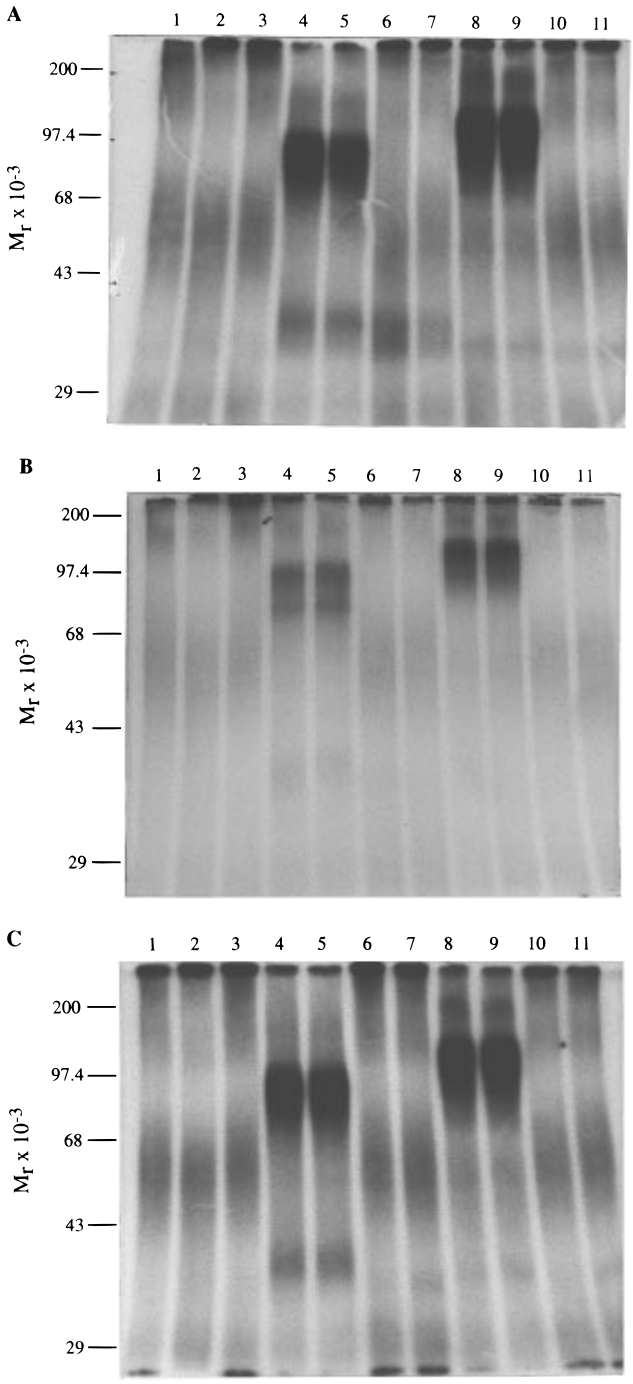

FIGURE 5: Cross-linking of recombinant β1-LAP to ^{125}I-TGF-β1 (A), ^{125}I-TGF-β2 (B), and ^{125}I-TGF-β3 (C). Samples were incubated with ^{125}I-TGF-βs for 2 h and cross-linked with DSS as described under Experimental Procedures. Samples were fractionated on 9% SDS−PAGE and detected by autoradiography: lane 1, no addition; lane 2, control proteins; lane 3, monomeric β1-LAP; lane 4, refolded β1-LAP; lanes 5−7, refolded β1-LAP with 2, 20, or 100 nM unlabeled TGF-βs, respectively; lane 8, CHO β1-LAP; lanes 9−11, CHO β1-LAP with 2, 20, or 100 nM cold TGF-βs, respectively. The position and mass (in kDa) of molecular mass markers are indicated at the left.

TSP1 binding assay. CHO β1-LAP, refolded β1-LAP, monomeric β1-LAP, BSA, or control proteins were added to microtiter dishes coated with TSP1. Assays were developed similarly to heparin binding assays using a polyclonal antibody against the pro region of TGF-β1. Refolded β1-LAP interacts with TSP1 (Figure 7) with 88% of the binding activity relative to CHO β1-LAP. This suggests that most of the TSP1 binding sites are present in refolded β1-LAP,

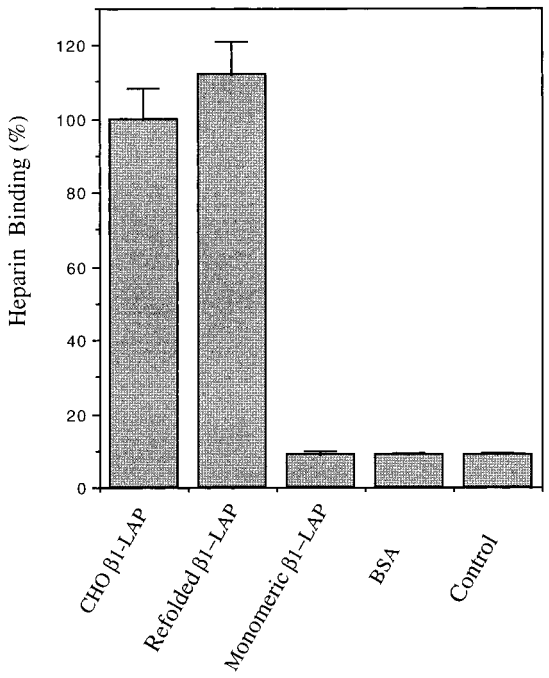

FIGURE 6: Heparin binding to β1-LAP. Binding of β1-LAP to heparin immobilized on microtiter dishes was detected using anti-TGF-$\beta 1_{81-94}$ antibody and alkaline phosphatase-conjugated goat anti-rabbit secondary antibody as described under Experimental Procedures. Binding was relative to that of CHO β1-LAP. Results are the average of three experiments performed in triplicate ± SD.

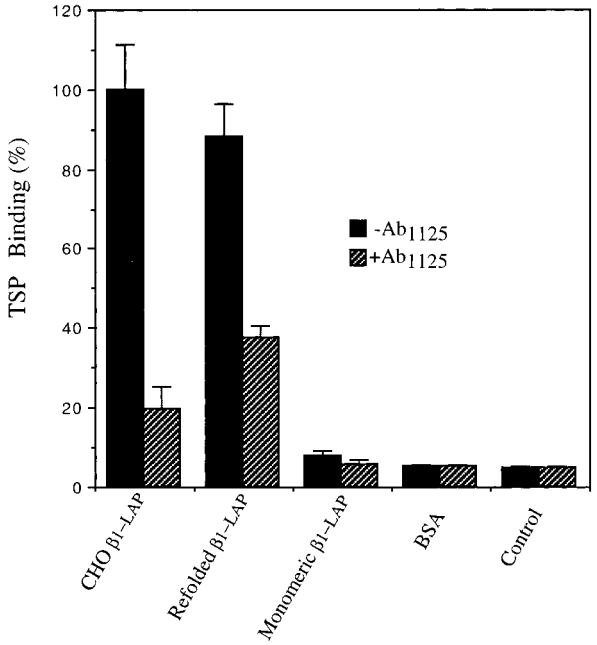

FIGURE 7: Thrombospondin 1 binding to β1-LAP. Binding of β1-LAP to TSP1 immobilized on microtiter dishes was detected by polyclonal anti-TGF-β1-LAP antibody and alkaline phosphatase-conjugated goat anti-rabbit antibody as described under Experimental Procedures. Binding was relative to that of CHO β1-LAP. Results are the average of three experiments performed in triplicate ± SD.

but are not detected in monomeric β1-LAP. Interaction between TSP1 and latent TGF-β1 is blocked by Ab1125, implying that the binding site(s) is (are) near the amino terminus of β1-LAP (43). CHO β1-LAP, refolded β1-LAP, monomeric β1-LAP, and negative control proteins were incubated with Ab1125 and assayed for TSP1 binding. As illustrated in Figure 7 (hatched bars), 80% of the binding activity of CHO β1-LAP and 57% of the binding activity of refolded β1-LAP were blocked, indicating that the TSP binding site(s) is (are) near residues 81−94.

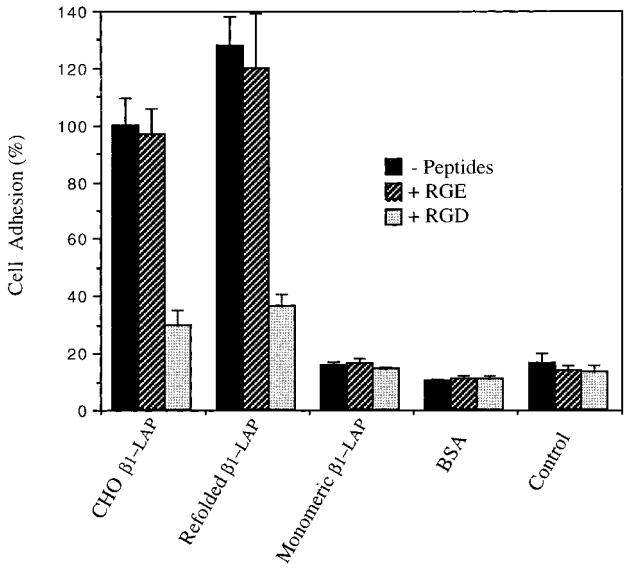

FIGURE 8: Effect of β1-LAP on human colon carcinoma FET cell adhesion. FET cells were seeded into microtiter dishes coated with control proteins, β1-LAP proteins, or BSA as described under Experimental Procedures. Cell adhesion was relative to that of the wells coated with CHO β1-LAP. Results are the average of three experiments performed in triplicate ± SD.

Cell Adhesion. Cell surface integrins recognize the tripeptide Arg-Gly-Asp (RGD), a sequence found in many adhesion proteins of extracellular matrices and in some serum proteins (44). The conformation around the RGD sequence appears to determine whether it is recognized and to which integrin it binds (63). Since β1-LAP contains an RGD motif, we tested it in a human colon carcinoma FET cell adhesion assay. FET cells were added to 96-well plates coated with CHO β1-LAP, refolded β1-LAP, monomeric β1-LAP, BSA, or control proteins. After removing unattached cells, the number of attached cells was determined. As shown in Figure 8 (solid bars), CHO β1-LAP increased cell adhesion by 9-fold, and the refolded β1-LAP increased cell adhesion by 11-fold while the monomeric β1-LAP did not promote cell adhesion. To confirm that the RGD motif is responsible for the cell adhesion promoting activity of refolded β1-LAP, GRGDSP and GRGESP peptides were used as competitors in the attachment inhibition assays. RGD peptides inhibited cell adhesion in wells coated with CHO β1-LAP and refolded β1-LAP. RGE peptides had little effect on cell adhesion (Figure 8, hatched and dotted bars). This result suggests that increased cell adhesion in wells coated with CHO β1-LAP and refolded β1-LAP is due to the interaction with integrin through the RGD sequence. The dose−response curve in the FET cell adhesion assay (Figure 9) indicated that when the concentration of refolded β1-LAP was below 8 μg/mL, FET cell adhesion increased in response to β1-LAP concentration; when the concentration of refolded β1-LAP was over 8 μg/mL, no additional increase in cell adhesion was observed. This implies that the RGD motif within the refolded β1-LAP and CHO β1-LAP mediates cell adhesion through interaction with integrin, while this sequence in monomeric β1-LAP cannot mediate cell adhesion, implicating the importance of conformation in binding. Although the refolded β1-LAP clearly mediates cell adhe-

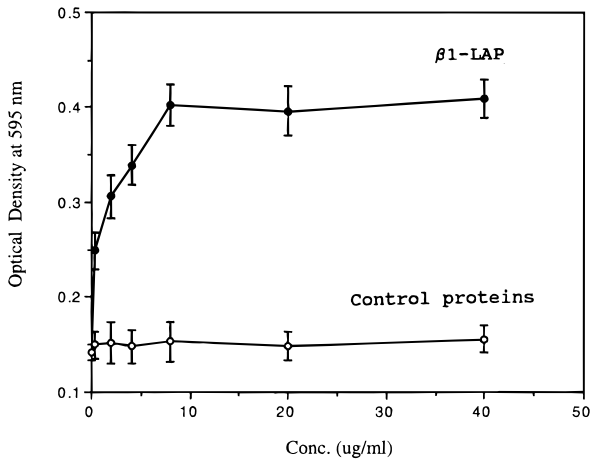

FIGURE 9: Dose-response curve for the effect of refolded β1-LAP on FET cell adhesion. Microtiter plates were coated with control proteins (○) or refolded β1-LAP (●) at the indicated concentrations, and FET cell adhesion was measured as described under Experimental Procedures. Relative attached cells were expressed as optical density units. Results are the average of three experiments performed in triplicate ± SD.

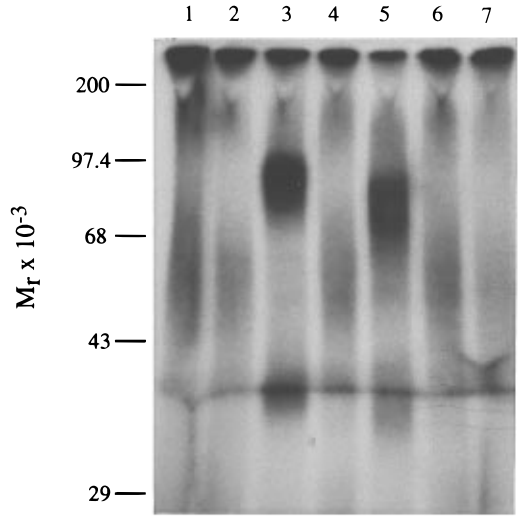

FIGURE 10: Cross-linking of untagged refolded β1-LAP to ^{125}I-TGF-β1. The controls, refolded β1-LAP, and untagged β1-LAP were incubated with ^{125}I-TGF-β1 for 2 h and cross-linked with DSS as described under Experimental Procedures. Samples were resolved on 9% SDS−PAGE and detected by autoradiography: lane 1, no addition; lane 2, control proteins; lane 3, refolded β1-LAP; lane 4, refolded β1-LAP with 20 nM cold TGF-β1; lane 5, untagged β1-LAP; lane 6, untagged β1-LAP with 20 nM cold TGF-β1; lane 7, factor Xa. The position and mass (in kDa) of molecular mass markers are indicated at the left.

sion, we cannot rule out the possibility that the monomeric protein is rapidly degraded by proteases secreted by FET cells and does not promote adhesion for this reason.

Removal of the N-Terminal His-tag of Refolded β1-LAP by Factor Xa. The 24 residue N-terminal tag added to aid in purifying β1-LAP could alter its biological activities. To address this issue, we removed the N-terminal His-tag by factor Xa treatment (Figure 10), leaving four residues (His-Met-Leu-Glu) beyond what is found in authentic β1-LAP. After factor Xa digestion, the monomeric β1-LAP doublet still migrated as a doublet (data not shown). That both bands of the doublet were affected suggests that the N-terminal His tag is present on both species. The factor Xa cleaved protein formed a DSS cross-linked complex of approximately 84 kDa with radioiodinated TGF-β1 (Figure 10, lane 5). The factor Xa cleaved material reversed the inhibitory activity of TGF-β1 on the growth of CCL64 cells (Figure 3D) with an EC_{50} value of 4.5 ± 0.8 nM, similar to the value obtained with tagged β1-LAP.

DISCUSSION

Recombinant β1-LAP-His-tag was produced in *E. coli* and purified from inclusion bodies as a monomeric protein to examine the role of complex oligosaccharides in the interaction of this protein with its ligands. Renatured, unglycosylated β1-LAP forms a latent complex with TGF-β1, as evidenced by its reversal of the inhibitory activity of TGF-β1 in a cell growth inhibition assay with a potency similar to that of glycosylated β1-LAP and by its forming 80−90 kDa cross-linked complexes with iodinated TGF-β1, TGF-β2, and TGF-β3.

Biological roles for protein oligosaccharides include contributing to conformational stability of proteins, providing ligands for protein targeting, contributing to cell−matrix or cell−cell interactions (64), stabilization of proteins against proteolytic degradation, and increasing protein solubility (65−67). Previous reports suggested β1-LAP carbohydrate structures were essential for formation of the latent TGF-β1 complex, since treatment of the complex with endoglycosidase F, sialidase (33), and *N*-glycanase (22) activated latent TGF-β1. The present studies do not support this conclusion. Cleavage of β1-LAP by contaminating proteases in glycosidases used in these studies is a more likely explanation for the earlier results. Carbohydrate structures of β1-LAP are more likely involved in the processing and secretion of the precursor. This idea is supported by the impaired secretion of TGF-β1 after inhibition of N-glycosylation by tunicamycin (30), the reduced secretion of TGF-β1 in embryonic kidney cells associated with elimination of the second N-glycosylation site of the TGF-β1 precursor (31), and the reduced secretion of β1-LAP in COS-1 cells associated with elimination of either the first or the second N-glycosylation site (32). Mannose 6-phosphate of TGF-β1 may act as a sorting signal by interaction with mannose 6-phosphate/IGF-II receptors directing LTGF-β1 to cellular organelles where LTGF-β1 is activated, TGF-β1 is secreted, and β1-LAP is internalized by endocytosis (28, 29).

TGF-β1 binds to several extracellular matrix (ECM) components including type IV collagen (68) and decorin (69) and is inactivated. LTGF-β1 binds to fibroblast ECM through the LTBP (70), and matrix-associated LTGF-β1 can be activated (24, 71). Platelet and recombinant LTGF-β1 bind heparin, and a polyclonal antibody against mature TGF-β1 does not bind to the latent TGF-β1 complex from platelets or recombinant TGF-β1 complex, suggesting that the TGF-β1 is masked by the pro region or that the epitopes on TGF-β1 are not recognized due to conformational differences between free TGF-β1 and the latent complex (38). These earlier results are consistent with our finding that heparin binds to β1-LAP in the absence of TGF-β1. Our findings also demonstrate that native structure is required for heparin binding since only the renatured protein bound this ligand; in addition, the carbohydrate structures are not essential. Sequences of the basic residues-B-B-B-X-X-B-, -B-B-X-B- (72), and -B-X-X-B-X-X-B-X-X-B- (36) have been implicated in heparin binding; three sequences in β1-LAP, KRKR (41−45), RLKLK (159−163), and RHRR (275−278), are potential heparin binding sites.

Thrombospondin 1, an abundant protein of platelet α-granules, is produced by a variety of cells, and incorporated into the extracellular matrix (73, 74). TSP1, which is noncovalently associated with TGF-β1 (75), may serve as a carrier to prolong the biological effects of TGF-β1 by protecting it from extracellular inactivators such as α_2-macroglobulin and decorin. TSP binds to the large and small forms of LTGF-β1, and activation of LTGF-β1 occurs upon interaction with TSP1 (41). Our results provide direct evidence for a previous hypothesis which suggested that TSP binds directly to β1-LAP. Only dimeric β1-LAP binds to TSP1 and N-glycosylation is not required for binding.

Integrins are a family of structurally related transmembrane glycoprotein receptors consisting of noncovalently associated α and β subunits (44), which mediate cell−matrix and cell−cell adhesion. Many recognize RGD, a sequence present in many extracellular ligands, which is crucial for cell adhesion (76). LTBP and β1-LAP contain a potential RGD motif, suggesting that they may interact with integrins. The role of these structures is supported by TGF-β1 LAP promoting interaction of FET cells in a manner which is blocked by RGD peptides. This may reflect a mechanism to target LTGF-β1 to the cell surface for TGF-β1 release.

Renatured β1-LAP reverses the inhibitory activity of TGF-β1 on mink lung epithelial cells, forms complexes with TGF-β1, TGF-β2, and TGF-β3, binds to heparin and thrombospondin, and promotes FET cell adhesion by an RGD-dependent integrin interaction. These properties depend on β1-LAP being a native dimer, but do not require N-glycosylation.

ACKNOWLEDGMENT

We thank Jiurong Liang and Manoranjani P. M. Tillekeratne for providing confluent FET cells. We are grateful to Erwin M. Reimann and Susan S. Dignam for their critical comments on the manuscript.

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BI9710479