Growth Factor-Binding Sequence in Human α_2-Macroglobulin Targets the Receptor-Binding Site in Transforming Growth Factor-β†

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ABSTRACT: α_2-Macroglobulin (α_2M) binds transforming growth factor-β1 (TGF-β1) and TGF-β2, forcing these growth factors into a state of latency. The mechanism by which this occurs remains unclear. In this paper, we demonstrate that peptides, derived from the structure of human α_2M (amino acids 714−729), bind directly to TGF-β1 and block the binding of TGF-β1 to the type I and II TGF-β receptors. The α_2M-derived peptides are notable for hydrophobic tripeptide sequences (WIW or VVV) and acidic residues (Glu^{714} and Asp^{719} in the mature α_2M subunit), which may function analogously to the structural elements that mediate TGF-β-binding in the type II receptor. Mutating Glu^{714} and Asp^{719} in the α_2M-peptide-GST fusion protein, FP3, which contains the putative growth factor-binding site, significantly decreased the binding affinity of FP3 for TGF-β1. The α_2M-derived peptides, which bind TGF-β1, inhibited the interaction of TGF-β1 with its receptors in fetal bovine heart endothelial cells. The same peptides also inhibited the activity of TGF-β1 in endothelial cell proliferation assays. These results demonstrate that α_2M-derived peptides target the receptor-binding sequence in TGF-β.

α_2-Macroglobulin (α_2M)[1] is a 718 kDa homotetrameric glycoprotein, originally described as a broad-spectrum proteinase inhibitor (*1*−*3*). Proteinases react with α_2M by cleaving one of a number of target peptide bonds located near the center of α_2M subunit, in the bait region (*1*). Cleavage of the bait region induces a major conformational change in α_2M, trapping the proteinase in a nondissociable complex (*1*, *4*, *5*). Each α_2M subunit has a thiol ester bond that becomes exposed during a conformational change and reacts with nucleophiles, including the side chains of Lys residues in the attacking proteinase (*6*, *7*). This reaction is responsible for covalent bond formation between α_2M and the proteinase. α_2M thiol esters also react directly with small primary amines, causing a conformational change in α_2M, which is equivalent to that induced by proteinases (*8*, *9*). Conformationally modified α_2M is referred to as activated because it is recognized by the α_2M receptor/low-density lipoprotein receptor-related protein (LRP-1) (*10*, *11*).

The transforming growth factor-β (TGF-β) family of growth factors regulates diverse cellular processes, including proliferation, differentiation, and apoptosis (*12*). Biologically active molecules are homodimers, in which the monomers are linked by hydrophobic interactions and usually by interchain disulfide bonds (*13*). TGF-β activities are mediated by receptors with Ser/Thr kinase activity (*14*, *15*). These receptors are grouped into at least two subfamilies, referred to as Type I (TβRI) and Type II (TβRII) receptors, with molecular masses of 50 and 70−85 kDa, respectively (*16*−*20*). Additional cell surface proteins involved in TGF-β-binding include the type V receptor, the type III TGF-β receptor or betaglycan (250−350 kDa), and endoglin (190 kDa) (*21*−*25*).

TGF-β activity is controlled at multiple levels. Cells secrete TGF-β as a latent complex, which is activated by complicated reactions that may involve proteinases, thrombospondin, the integrin $\alpha_v\beta_6$, reactive oxygen species, or shifts in pH (*26*). Once activated, TGF-β binds to α_2M, forming complexes that are again latent (*27*). Because TGF-β-binding to α_2M is reversible, native α_2M may provide a reservoir of active TGF-β; however, when TGF-β binds to activated α_2M, the entire complex binds to LRP-1 and undergoes clearance and catabolism (*28*, *29*). Native and activated α_2M bind TGF-β2 with equal affinity whereas activated α_2M binds TGF-β1 with higher affinity than native α_2M (*30*, *31*). This difference appears to be conformational because when α_2M is denatured, the affinities of native and activated α_2M for TGF-β1 are the same (*32*). In cell culture, α_2M regulates cell phenotype by binding endogenously produced TGF-β and disrupting TGF-β autocrine signaling pathways (*33*−*35*). Furthermore, the α_2M gene knock-out mouse demonstrates abnormal responses to various forms

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[1] Abbreviations: α_2M, α_2-macroglobulin; α_2M-MA, α_2M-methylamine; TGF-β, transforming growth factor-β; TβR, TGF-β receptor; PDGF-BB, platelet-derived growth factor-BB; LRP-1, low-density lipoprotein receptor-related protein-1; GST, glutathione-*S*-transferase; FBHE cell, fetal bovine heart endothelial cell; DTT, dithiothreitol; IAM, iodoacetamide; IPTG, isopropylthio-β-D-galactoside; BSA, bovine serum albumin; DSS, disuccinimidyl suberate; PVDF, polyvinylidene fluride; DMEM, Dulbecco's modified Eagle's medium; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline, 0.1% Tween 20; EBSS, Earle's Balanced Salt Solution; EHB, Earle's Balanced Salt Solution containing 2 mg/mL BSA.

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of exogenous challenge that may be explained by aberrant TGF-β regulation (*36*−*38*). Thus, there is considerable evidence to support the hypothesis that regulation of TGF-β activity is a major function of α_2M in vivo.

α_2M binds a number of growth factors and cytokines that share little or no sequence identity with TGF-β. These interactions vary considerably in affinity, and it is reasonable to assume that the highest-affinity interactions are most likely to be physiologically significant in vivo (*30*, *31*). By analyzing a library of human α_2M-peptide-glutathione-*S*-transferase (GST) fusion proteins and a battery of synthetic peptides, we identified a 16 amino acid peptide (P3), corresponding to a sequence just C-terminal to the α_2M bait region, which binds TGF-β1, TGF-β2, and platelet-derived growth factor-BB (*32*, *39*). It is not known whether the P3 sequence functions in growth factor-binding in intact α_2M or whether P3 interacts with the TGF-β active site and thus may inhibit receptor-binding.

The crystal structure of the human TβRII ectodomain−TGF-β3 complex was recently reported (*40*). The binding site includes a hydrophobic interface flanked by Arg^{25} and Arg^{94} in TGF-β3, which form hydrogen-bonded ion pairs with Glu^{119} and Asp^{32} of TβRII. The structural features of the TGF-β-binding site in TβRII are interesting because P3 also includes a high density of hydrophobic amino acids flanked by negatively charged residues (*39*). Liu et al. (*41*) prepared synthetic peptides corresponding to the structure of TGF-β1 and identified peptides that inhibit TGF-β1-binding to α_2M (*41*). In the most active peptides, a tryptophan residue (Trp^{52}) played an essential role.

The major goal of the present investigation was to determine whether α_2M-derived peptides, which inhibit the binding of TGF-β to α_2M, also inhibit TGF-β-binding to its receptors. If so, this result would provide evidence that the peptides target the receptor recognition sequence in TGF-β. Another goal was to determine the structural characteristics of α_2M-derived peptides that are important for TGF-β-binding. Our results demonstrate that α_2M-derived peptides may be used to target and inhibit TGF-β receptor-binding and activity.

MATERIALS AND METHODS

Reagents and Proteins. TGF-β1, TGF-β2, acidic fibroblast growth factor (aFGF), and basic fibroblast growth factor (bFGF) were from R&D Systems (Minneapolis, MN). Methylamine HCl, dithiothreitol (DTT), iodoacetamide (IAM), isopropylthio-β-D-galactoside (IPTG), dimethyl sulfoxide (DMSO), and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL). $Na^{125}I$ was from Amersham Pharmacia Biotech (Piscataway, NJ). Polyvinylidene fluoride (PVDF) membranes were from Millipore Corporation (Bedford, MA). The QuikChange System for site-directed mutagenesis was from Stratagene (La Jolla, CA). Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, and Earle's balanced salts solution (EBSS) were from Life Technologies, Inc. (Rockville, MD). TGF-β1 was radioiodinated with chloramine T to a specific activity of 100−200 μCi/μg, as previously described (*42*).

Preparation of α_2M and α_2M-MA. Native α_2M was purified from human plasma according to the method of Imber and Pizzo (*43*). The concentration of α_2M was determined by the absorbance at 280 nm using an $A_{1\%,1.0cm}$ of 8.93 (*3*). α_2M-MA, a form of activated α_2M, was prepared by dialyzing native α_2M against 200 mM methylamine hydrochloride in 50 mM Tris-Cl, pH 8.2 for 16 h at 22 °C, followed by extensive dialysis against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS) at 4 °C (*9*). Reaction of native α_2M with methylamine was confirmed by nondenaturing PAGE. In this system, α_2M-MA and other forms of activated α_2M demonstrate a characteristic increase in electrophoretic mobility (*4*, *44*).

GST Fusion Proteins and Synthetic Peptides. GST-fusion proteins that include partial α_2M sequences have been previously described (*32*, *45*). The construct that encodes amino acids 591−774 in pGEX-2T (FP3) was subjected to site-directed mutagenesis using the QuikChange System. Asp^{714} and Glu^{719} were converted into Arg, to generate FP3-RR (the numbering system is based on the sequence of intact α_2M after signal peptide processing). BL21 cells harboring GST fusion protein expression constructs were induced for protein expression with 0.1 mM IPTG for 3 h at 37 °C. Fusion proteins were purified to homogeneity as previously described (*32*).

Multiple peptides that span the putative growth factor-binding site of human α_2M were synthesized by the University of Virginia Biomolecular Research Core Laboratory. N-termini were acetylated, and C-termini were amidated. Peptides were purified by reversed-phase high-performance liquid chromatography using a Phenomenex Jupiter C_{18} column. Accuracy and homogeneity of the peptides were confirmed by MALDI-TOF mass spectrometry. Synthetic peptides were dissolved in DMSO and were free of particulates by visual inspection. However, to more accurately assess the degree of solubilization, peptide preparations also were examined by phase-contrast microscopy at 400×. Specific preparations were subjected to centrifugation at 8000*g* for 1 min and then to amino acid analysis.

Nondenaturing PAGE Analysis of TGF-β-Binding to α_2M and α_2M-MA. ^{125}I-TGF-β1 (3 nM) was incubated with native α_2M (0.2 μM) and increasing concentrations of synthetic peptides (4−20 μM) or with α_2M-MA (0.05 μM) and increasing concentrations of GST-fusion proteins (0.02−0.5 μM) in PBS with 15 μM BSA for 60 min at 37 °C. The samples were then subjected to nondenaturing PAGE on 5% slabs (*44*). The gels were dried, and binding of ^{125}I-TGF-β1 to α_2M was assessed by PhosphorImager analysis (Molecular Dynamics). Assuming that intact α_2M directly competes with the fusion proteins or synthetic peptides for a common binding site in the structure of TGF-β1, then the concentration of each peptide or fusion protein, which inhibits ^{125}I-TGF-β1-binding to α_2M by 50% (the IC_{50}), is related to the apparent K_I is as follows:

$$K_I = IC_{50}/(1 + [\alpha_2M]/K_D)$$

In this expression, the K_D is the equilibrium dissociation constant for the binding of TGF-β1 to native α_2M (K_D of 0.33 μM) or α_2M-MA (K_D of 0.08 μM) (*30*). In control experiments, we demonstrated that free ^{125}I-TGF-β1 and ^{125}I-TGF-β1, which were preincubated with each synthetic peptide or fusion protein, do not comigrate with α_2M in the nondenaturing PAGE system.

^{125}I-TGF-β Binding to Immobilized α_2M. Native α_2M (1 μg in 100 μL) was incubated in 96-well microtiter plates for 4 h at 22 °C, as previously described (*46*). This procedure results in the immobilization of approximately 90 fmol of α_2M/well. The wells were washed three times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with PBS-T for 16 h at 4 °C. ^{125}I-TGF-β1 (0.1 nM) was incubated with the immobilized α_2M in the presence of 15 μM BSA and increasing concentrations of synthetic peptides (P3, P3.5, or P3.7) for 1 h at 22 °C. The wells were then washed three times with PBS-T. ^{125}I-TGF-β1 that was associated with the immobilized phase was recovered in 0.1 M NaOH, 2% SDS and quantitated in a γ-counter.

Ligand Blot Analysis. Ligand blotting was performed as previously described (*32*). Purified GST-fusion proteins were dialyzed against 2 mM DTT for 12 h at 22 °C and then treated with 10 mM IAM for 2 h at 22 °C. Equivalent amounts of each fusion protein (2 μg) were subjected to SDS−PAGE on 10% slabs and electrotransferred to PVDF membranes. The membranes were blocked with 5% milk in PBS-T, probed with ^{125}I-TGF-β1 (10 pM) for 2 h at 22 °C, washed with PBS-T, and subjected to PhosphorImager analysis.

Affinity-Labeling of TGF-β Receptors. FBHE cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% FBS, 20 ng/mL aFGF, and 80 ng/mL bFGF. Cells were harvested at subconfluence with trypsin-EDTA (0.05% trypsin, 0.5 mM EDTA) and passaged. In affinity-labeling experiments, the cells were plated at 2×10^5/well in 35 mm wells and cultured for 24 h. The cells then were washed three times with EBSS, 25 mM Hepes, pH 7.4, containing 2 mg/mL BSA (EHB) and incubated for 1 h with 0.1 nM ^{125}I-TGF-β1 at 4 °C, in the presence or absence of synthetic peptides (8 μM), native α_2M (0.5 μM), or unlabeled TGF-β1 (10 nM). After three washes with EHB, the cross-linking reagent, DSS, was added at a final concentration of 0.17 mM for 20 min. The cells were washed once for 5 min with 50 mM glycine, 100 mM NaCl, pH 5, and once with EHB. Cell lysates were denatured in the presence of DTT and subjected to SDS−PAGE on 8% slabs. ^{125}I-TGF-β1 was detected by PhosphorImager analysis.

Inhibition of Endothelial Cell Growth. FBHE cell proliferation assays were performed in dilute FBS (0.2%). The cells were plated in 24-well cell culture plates (2×10^4 cells per well) and incubated in DMEM with 10% FBS for 15 h. After washing, the cells were pulse-exposed to TGF-β1 (50 pM) in fresh DMEM containing 0.2% FBS for 1 h. The TGF-β1 was preincubated with synthetic peptides (15 μM) or vehicle for 15 min and then added to the cultures. After the pulse-exposure period, the cells were washed three times with serum-free DMEM and cultured for an additional 30 h in DMEM with 0.2% FBS. [^{3}H]Thymidine was added for 18 h. The cells then were washed with EBSS, 25 mM Hepes, pH 7.4, and fixed in 10% trichloroacetic acid. Cell-associated radioactivity was recovered in 1 M NaOH. HCl was used to neutralize the pH, and the extracts were combined with Ready-Safe scintillation fluid for counting in a Beckman scintillation counter.

Direct Binding of TGF-β1 to Synthetic Peptides. P3, P3.5, and P3.7 were dissolved in DMSO and diluted to a final a concentration of 4 μg/100 μL in PBS. The peptides were then immobilized in 96-well microtiter plates by incubation at 22 °C for 4 h. The wells were washed and blocked as described for the experiments with immobilized α_2M. ^{125}I-TGF-β1 (0.1 nM) was incubated with the immobilized peptides, in the presence or absence of the equivalent unlabeled peptide in solution, for 2 h at 37 °C. The wells were then washed. ^{125}I-TGF-β1, which was associated with the immobilized phase, was recovered in 0.1 M NaOH, 2% SDS and quantitated in a γ-counter.

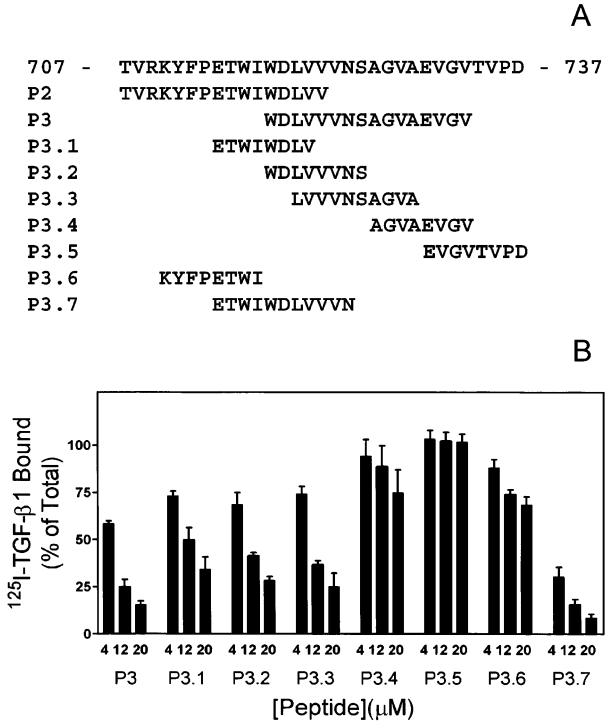

FIGURE 1: TGF-β1 competition binding experiments with synthetic peptides and α_2M. (A) The amino acid sequences of P2, P3, and P3.1-P3.7, which collectively correspond to amino acids 707−737 of the human α_2M subunit, are shown. (B) ^{125}I-TGF-β1 was incubated with native α_2M and the indicated concentrations of synthetic peptides in PBS with 15 μM BSA for 60 min at 37 °C. The samples were then subjected to nondenaturing PAGE. ^{125}I-TGF-β1-binding to α_2M was detected by PhosphorImager analysis and quantitated using ImageQuant software. Binding is expressed as a percentage of that observed in the absence of peptides.

RESULTS

^{125}I- TGF-β1 Binding to Synthetic Peptides. We previously localized a growth factor-binding sequence to amino acids 718−733 of α_2M (*39*). A 16 amino acid synthetic peptide (P3), corresponding to amino acids 718−733 (see Figure 1A), blocked the binding of both TGF-β1 and PDGF-BB to intact α_2M. Other peptides from the same region, including the partially overlapping 16mer, P2, were inactive. To identify amino acids in P3 that may be critical for TGF-β-binding, we prepared a series of overlapping 8mers (P3.1−P3.5). As shown in Figure 1B, peptides that overlapped with the N-terminus of P3 (P3.1, P3.2, P3.3) competed with intact α_2M for ^{125}I-TGF-β1-binding. The approximate IC_{50} values were less than 12 μM and only slightly higher than the IC_{50} determined with P3. By contrast, peptides that overlapped mainly with the C-terminus of P3 (P3.4 and P3.5) competed poorly with α_2M for TGF-β1-binding, suggesting that the

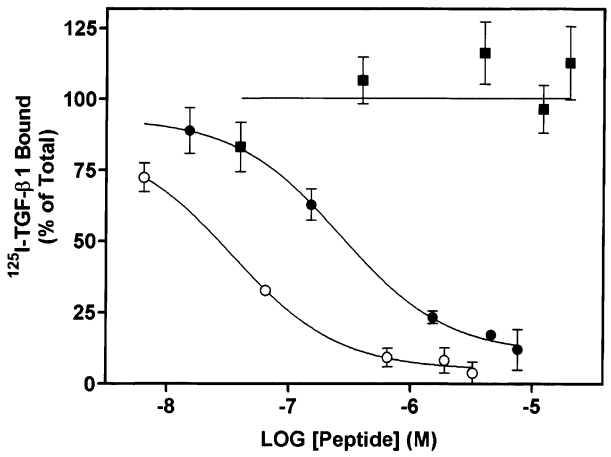

FIGURE 2: TGF-β1-binding to immobilized α_2M. ^{125}I-TGF-β1 (0.1 nM) was incubated in α_2M-coated wells in the presence of increasing concentrations of P3 (closed circle), P3.7 (open circle), or P3.5 (closed square) for 1 h at 22 °C. The wells were then washed, and radioactivity that was associated with the immobilized phase was recovered in 0.1 M NaOH, 2% SDS. Recovered radioactivity was quantitated in a γ-counter and is expressed as a percentage of that observed in the absence of peptides.

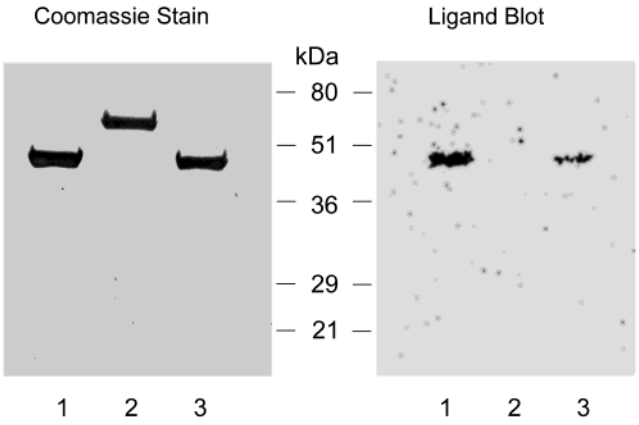

FIGURE 3: Ligand blot analysis of TGF-β1 binding to GST fusion proteins. The purified fusion proteins, FP3, FP3-RR, and FP4 (2 μg), were subjected to SDS−PAGE and electrotransferred to PVDF membranes. The membranes were stained with Coomassie Blue or subjected to ligand blot analysis using 10 pM ^{125}I-TGF-β1. After incubation with the membranes for 2 h at 22 °C, ^{125}I-TGF-β1-binding was detected by PhosphorImager analysis. Lanes 1−3 show FP3, FP4, and FP3-RR, respectively.

N-terminus of P3 is principally responsible for this interaction.

All of the peptides that inhibited TGF-β1-binding to α_2M included one of two hydrophobic tripeptide-sequences (WIW or VVV), and most also had acidic amino acids. P3.1 not only overlapped with P3 but also was contained entirely within P2. This is intriguing because P2 demonstrated no activity in TGF-β-binding experiments (*39*). One explanation for this result is that the basic amino acids in P2 (Arg^{709} and Lys^{710}) interact internally with the acidic residues, neutralizing its TGF-β-binding activity. To test this hypothesis, we synthesized an 11 amino acid peptide (P3.7), which includes the two hydrophobic tripeptide sequences, two acidic residues, and no basic residues. P3.7 was more effective at inhibiting ^{125}I-TGF-β1-binding to α_2M than any other peptide, including P3. By contrast, P3.6, which partially overlaps with P3.7 but lacks the hydrophobic sequences and includes Lys^{710}, demonstrated little or no activity.

Determination of K_d Values for the Binding of TGF-β1 to P3 and P3.7. After dilution out of DMSO, some of the hydrophobic peptides, including P3 and P3.7, demonstrated slow self-association to form aggregates. This was not the case with the less hydrophobic peptides, such as P3.5. When peptide aggregates were cleared by centrifugation, immediately prior to performing experiments, the residual soluble component demonstrated slightly improved ability to compete for TGF-β-binding. This is evident in the studies shown in Figure 2.

In these experiments, we compared the ability of peptides in solution to bind ^{125}I-TGF-β1 and thereby inhibit ^{125}I-TGF-β1-binding to immobilized native α_2M. Because the ^{125}I-TGF-β1 concentration (0.1 nM) was significantly lower than the K_D for TGF-β1-binding to immobilized α_2M, TGF-β1-binding to immobilized α_2M was a linear function of the free TGF-β1 concentration, and the IC_{50} was a direct estimate of the apparent K_I for peptide-binding to TGF-β1 (*32*). P3.5 did not inhibit ^{125}I-TGF-β1-binding to immobilized α_2M, as was anticipated based on the results shown in Figure 1. By contrast, significant competition was observed with P3, and even greater competition was observed with P3.7. The IC_{50} values were 260 and 34 nM for P3 and P3.7, respectively. The IC_{50} determined with P3 is in good agreement with our previously reported value (*39*). The apparent K_I values, which are based on the IC_{50}s, do not account for the fact that TGF-β is a homodimer and thus may require two mol/mol of bound peptide to completely block binding to immobilized α_2M. Also, we cannot determine whether residual self-aggregation of P3 or P3.7 compromised the effectiveness of these peptides in TGF-β1-binding, even after clearing the solutions by centrifugation.

Binding of TGF-β to GST Fusion Proteins. The presence of acidic amino acids in the context of hydrophobic residues, in both P3 and P3.7, raised the hypothesis that these peptides may mimic the TGF-β-binding sequence in TβRII (*40*). To test the role of the acidic residues, we mutated Glu^{714} and Asp^{719} to Arg in FP3, the GST-fusion protein that contains the putative growth factor-binding sequence of α_2M (*32*). In ligand-blotting experiments, the mutated form of FP3 (FP3-RR) still bound ^{125}I-TGF-β1; however, binding was substantially decreased as compared with unaltered FP3 (Figure 3). As a negative control, we also studied FP4, which contains amino acids 775−1059 of the mature α_2M subunit. FP4 failed to bind ^{125}I-TGF-β1, confirming our previous results (*32*). Equivalent results were obtained when ligand blotting experiments were performed with ^{125}I-TGF-β2 instead of ^{125}I-TGF-β1 (results not shown).

To more quantitatively assess the effects of charge reversal in FP3−RR, we compared the ability of purified FP3 and FP3−RR to compete with α_2M-MA for binding to ^{125}I-TGF-β1 in solution (Figure 4). Concentration-dependent inhibition of ^{125}I-TGF-β1-binding to α_2M-MA was observed with both fusion proteins. The IC_{50} for inhibition of TGF-β1-binding to α_2M-MA by FP3 was 125 nM, corresponding to an apparent K_I of 80 nM, in good agreement with our previous determination (*32*). In the concentration range studied, FP3-RR failed to inhibit TGF-β1-binding to α_2M-MA by greater than 50%; however, by extrapolation, the IC_{50} was approximately 650 nM, corresponding to an apparent K_I of 0.4 μM. From these results, we conclude that the acidic residues

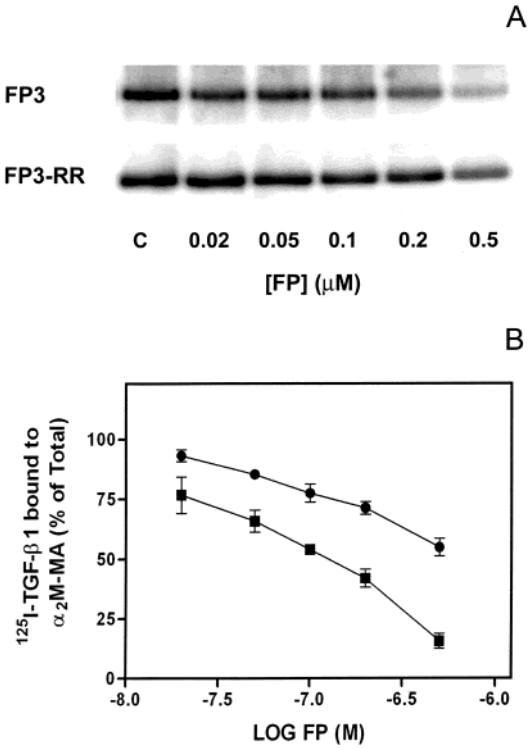

FIGURE 4: Binding of TGF-β1 to FP3 and FP3-RR. (A) ^{125}I-TGF-β1 (3 nM) was incubated with 0.05 μM α_2M-MA and the indicated concentrations of FP3 or FP3-RR. The samples were then subjected to nondenaturing PAGE. TGF-β-binding to α_2M-MA was detected by measuring the radioactivity comigrating with the α_2M-MA band. The PhosphorImager analysis is shown. The lane labeled C indicates ^{125}I-TGF-β1-binding to α_2M-MA in the absence of fusion proteins. Coomassie-stained gels demonstrated equivalent loading of α_2M-MA in each lane (not shown). (B) The results of four separate experiments with FP3 (closed square) and FR3-RR (closed circle) were averaged to generate the results that are shown. Binding is expressed as a percentage of that observed in the absence of fusion proteins.

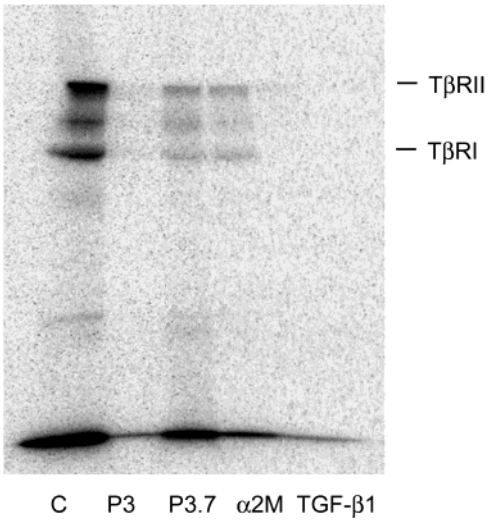

FIGURE 5: Affinity-labeling of TGF-β receptors. Confluent monolayers of FBHE cells were affinity-labeled with 100 pM ^{125}I-TGF-β1 for 1 h at 4 °C, in the presence or absence of synthetic peptides (8 μM), native α_2M (0.5 μM), or unlabeled TGF-β1 (10 nM). Cell lysates were denatured in the presence of DTT and subjected to SDS−PAGE on 8% slabs. ^{125}I-TGF-β1 was detected by PhosphorImager analysis. The lane labeled C indicates ^{125}I-TGF-β1-labeling of the receptors in the absence of competing agents.

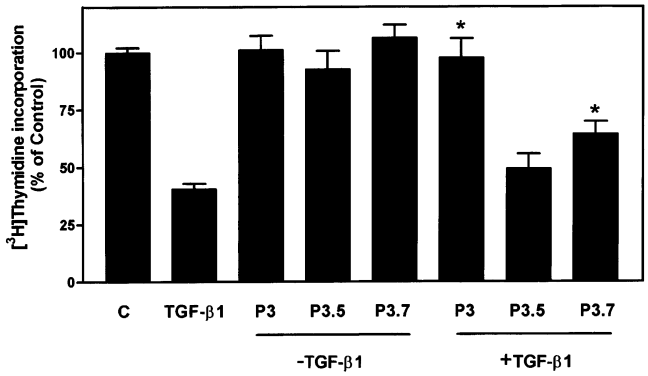

FIGURE 6: FBHE cell proliferation assays. FBHE cells were pulse-exposed to TGF-β1 (50 pM), synthetic peptides (15 μM), or synthetic peptides + TGF-β1 for 1 h. Synthetic peptides were preincubated with TGF-β1 for 15 min at 37 °C before addition to FBHE cultures. After 30 h, 1 μCi/mL [^{3}H]thymidine was added for an additional 18 h. [^{3}H]Thymidine incorporation was determined as a percentage of that observed in control cultures (C), which were not treated with TGF-β1 or synthetic peptides. Statistical analysis was performed to compare the effects of synthetic peptides on the TGF-β response. Significant reversal of the TGF-β response ($p < 0.05$) is indicated by *.

contribute to TGF-β1-binding but are not absolutely necessary.

α_2M-Derived Peptides Block the Binding of TGF-β to Its Receptors in FBHE Cells. The biological activity of α_2M-derived peptides, as antagonists of TGF-β activity, has not been previously tested. To determine whether the peptides block TGF-β-binding to its cell-surface receptors, we performed affinity-labeling experiments in FBHE cells. In the absence of competing agents, ^{125}I-TGF-β1 (0.1 nM) was cross-linked to two major receptors (Figure 5), which based on molecular mass were TβRI and TβRII (*16*−*20*). A similar affinity-labeling pattern was observed with ^{125}I-TGF-β2 (results not shown). Radioactivity that migrated near the dye front probably represented ^{125}I-TGF-β1 that bound to the FBHE cell surfaces but was not cross-linked by DSS. In the presence of a 100-fold molar excess of unlabeled TGF-β1, receptor labeling by ^{125}I-TGF-β1 was entirely blocked, providing evidence that the interactions are saturable.

Native α_2M (0.5 μM), P3 (8 μM), and P3.7 (8 μM) all substantially inhibited receptor affinity-labeling by ^{125}I-TGF-β1 and also decreased the amount of radioactivity recovered near the dye front (Figure 5). P3.7 was less effective than P3, which was interesting because our data indicate that P3.7 binds to TGF-β1 with higher affinity than P3. Because intact α_2M may bind TGF-β within its central cavity, the mechanism by which α_2M inhibits TGF-β-binding to its receptors may involve steric constraints. A similar mechanism is less likely to be operational with the small peptides, P3 and P3.7. Instead, the ability of these peptides to inhibit affinity labeling of the TGF-β receptors suggests that P3 and P3.7 interact directly with the receptor-binding site in TGF-β or with an overlapping sequence.

α_2M-Derived Peptides Counteract TGF-β Activity. To test whether α_2M-derived peptides antagonize the activity of TGF-β, we examined FBHE cell proliferation. When FBHE cells were pulse-exposed to TGF-β1 (50 pM) for 1 h, proliferation was decreased by 61 ± 8%, as determined by [^{3}H]thymidine incorporation (Figure 6). When P3 (15 μM) was added together with TGF-β1, during the pulse-exposure period, the activity of the TGF-β1 was completely neutral-

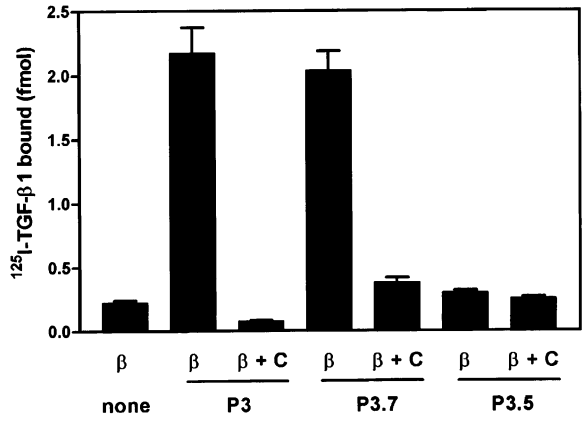

FIGURE 7: Direct binding of TGF-β1 to synthetic peptides. P3, P3.5, and P3.7 (4 μg/100 μL) were immobilized in 96-well microtiter plates, which were then blocked with PBS-T. None indicates PBS-T-blocked wells without peptides. ^{125}I-TGF-β1 (0.1 nM) was incubated with the immobilized peptides alone (β) or in the presence of the corresponding peptide (3.0 μM) as the competitor in solution (β + C). Bound ^{125}I-TGF-β1 was recovered in 0.1 M NaOH, 2% SDS, and quantitated in a γ-counter.

ized. P3.7 caused partial reversal of the TGF-β response ($p < 0.05$), whereas P3.5 had no significant effect. None of the three peptides affected FBHE proliferation in the absence of TGF-β1. These activity assays are consistent with the receptor affinity-labeling results and show that P3 is an effective antagonist of TGF-β activity.

TGF-β Binds Directly to P3 and P3.7. Because P3 and P3.7 were derived from the sequence of α_2M, we hypothesized that these peptides bind directly to TGF-β1 and thereby inhibit the interaction of TGF-β1 with α_2M and TGF-β receptors. It is also possible that the peptides inhibit the same interactions by binding to either α_2M or the receptors. To test whether the peptides bind directly to TGF-β, P3 and P3.7 were immobilized in microtiter plates. ^{125}I-TGF-β1 bound to immobilized P3 and P3.7 but not to immobilized P3.5 or to control microtiter wells that were blocked with PBS-T (Figure 7). Binding of ^{125}I-TGF-β1 to immobilized P3 and to P3.7 was inhibited by including an excess of the equivalent peptide in solution, indicating that the interactions are specific.

DISCUSSION

The α_2M subunit includes well-defined regions that contribute to the overall function of the protein. The bait region (amino acids 666−706) and thiol ester bond (amino acids 949 and 952) function in proteinase-binding (*47*−*49*). A sequence, which is adjacent to the bait region (amino acids 718−733), may be responsible for growth factor-binding (*32*, *39*, *45*). The recognition sequence for LRP-1 is centered in an α helix that includes amino acids 1370−1374 (*50*−*53*). Finally, a distinct site, which mediates the binding of β-amyloid peptide, is located near the C-terminus but separate from the LRP-1-recognition sequence (*54*). Thus, the α_2M subunit may be viewed as a compartmentalized structure; however, much of the function of intact α_2M is dependent on its unusual quaternary structure. For example, in the transformed conformation, two separate subunits of α_2M may engage separate molecules of LRP-1 (*55*). Furthermore, by trapping an attacking proteinase within its hollow core, intact tetrameric α_2M may form a nondissociable proteinase−inhibitor complex without a covalent linkage (*1*, *4*, *5*).

The first goal of the present investigation was to identify amino acids in the α_2M growth factor-binding sequence that are important for TGF-β-binding (*32*, *39*). The timeliness of the goal was heightened by the recent report of the crystal structure of the TβRII ectodomain−TGF-β3 complex (*40*). The TGF-β3-binding site in TβRII consists of a hydrophobic ridge and acidic residues that pair with Arg^{25} and Arg^{94} in TGF-β3 (*40*). The amino acids at the receptor-binding interface are completely conserved in TGF-β1 and TGF-β3. We hypothesized that the same basic amino acids in TGF-β1 may interact with Glu^{714} and Asp^{719} in the α_2M-derived peptides and that a hydrophobic binding interface may also be involved.

To test the role of hydrophobic residues in the TGF-β-P3 interaction, we screened a series of overlapping eight amino acid synthetic peptides. To study the role of acidic amino acids, we mutated the fusion protein, FP3, instead of synthesizing new peptides, because charge reversal in synthetic peptides may generate a structure that interacts with the opposite side of the same protein-binding interface. Our results suggest that a hydrophobic binding surface is probably involved because all of the peptides that bound TGF-β1 included at least one of two hydrophobic tripeptide sequences (WIW or VVV). Although mutation of FP3 did not eliminate binding, the binding affinity for TGF-β was significantly reduced. Thus, our results support a model in which similar structural properties are important for the interaction of TGF-β with the α_2M-derived peptides and TβRII.

On the basis of our biochemical data, we undertook studies to determine whether the α_2M-derived peptides target the TGF-β active site. TGF-β1 bound directly to both immobilized P3 and P3.7, suggesting that the peptides have the potential to inhibit multiple TGF-β interactions. In affinity labeling experiments, P3 and P3.7 inhibited the interaction of TGF-β1 with both major TGF-β receptors. The peptides also significantly decreased the amount of TGF-β that was noncovalently associated with the cell surface. Thus, the peptides antagonize interactions of TGF-β with its receptors and other possible cell-surface binding sites without demonstrable selectivity. These results suggest that the same site in TGF-β mediates all cell-surface interactions and that this site is blocked by P3 and other related α_2M-derived peptides. The increased efficacy of P3 in the receptor affinity-labeling experiments, compared with P3.7, despite the fact that P3.7 binds to TGF-β with higher affinity, argues for a model in which the receptor-binding site and the α_2M-peptide-binding site, in TGF-β, are overlapping but nonidentical. In this case, the slightly larger size of P3 may allow it to more effectively block the receptor-binding sequence in TGF-β.

Because the α_2M-derived peptides inhibited the binding of TGF-β to its receptors, we anticipated that the peptides would also inhibit TGF-β activity, and this result was obtained. In FBHE cell proliferation assays, P3 was again more effective than P3.7, suggesting that inhibition of TGF-β activity is directly related to inhibition of receptor-binding. In the absence of exogenously added TGF-β, the peptides had no effect on endothelial cell growth. Although our unpublished studies suggest that P3 may interact with other growth factors, as we have reported for the parent protein α_2M (*30*), the absence of an effect on endothelial cell growth

under our control conditions argues for some degree of specificity in the function of P3.

TGF-β is a pleiotropic growth factor (*12*). Thus, it is difficult to predict how neutralizing TGF-β may affect physiology in vivo. There may be specific conditions under which one activity of TGF-β predominates and neutralizing the activity of TGF-β may be advantageous. For example, in breast cancer, TGF-β has been identified as a major immunosuppressive agent released by the neoplastic cells (*56*, *57*). Similarly, TGF-β may adversely affect the immune response to other cancers (*58*, *59*). The peptides identified here represent new reagents to test the function of TGF-β in various forms of pathophysiology and determine the efficacy of neutralizing TGF-β in vivo.

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