Localization of the Binding Site for Transforming Growth Factor-\(\beta\) in Human \(\alpha_2\)-Macroglobulin to a 20-kDa Peptide That Also Contains the Bait Region*

(Received for publication, October 21, 1997, and in revised form, March 9, 1998)

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\(\alpha_2\)-Macroglobulin (\(\alpha_2\)M) functions as a major carrier of transforming growth factor-\(\beta\) (TGF-\(\beta\)) in vivo. The goal of this investigation was to characterize the TGF-\(\beta\)-binding site in \(\alpha_2\)M. Human \(\alpha_2\)M, which was reduced and denatured to generate 180-kDa subunits, bound TGF-\(\beta1\), TGF-\(\beta2\), and NGF-\(\beta\) in ligand blotting experiments. Cytokine binding was not detected with bovine serum albumin that had been reduced and alkylated, and only minimal binding was detected with purified murinoglo-\(\gamma\)ulin. To localize the TGF-\(\beta\)-binding site in \(\alpha_2\)M, five cDNA fragments, collectively encoding amino acids 122–1302, were expressed as glutathione S-transferase (GST) fusion proteins. In ligand blotting experiments, TGF-\(\beta2\) bound only to the fusion protein (FP3) that includes amino acids 614–797. FP3 bound \(^{125}\)I-TGF-\(\beta1\) and \(^{125}\)I-TGF-\(\beta2\) in solution, preventing the binding of these growth factors to immobilized \(\alpha_2\)M-methionyl (\(\alpha_2\)M-MA). The IC\(_{50}\) values were 33 ± 5 and 26 ± 6 \(\mu\)M for TGF-\(\beta1\) and TGF-\(\beta2\), respectively; these values were comparable with or lower than those determined with native \(\alpha_2\)M or \(\alpha_2\)M-MA. A GST fusion protein that includes amino acids 798–1082 of \(\alpha_2\)M (FP4) and purified GST did not inhibit the binding of TGF-\(\beta\)- to immobilized \(\alpha_2\)M-MA. FP3 (0.2 \(\mu\)M) neutralized the activity of TGF-\(\beta1\) and TGF-\(\beta2\) in fetal bovine heart endothelial cell proliferation assays; FP4 was inactive in this assay. FP3 also increased NO synthesis by RAW 264.7 cells, mimicking an \(\alpha_2\)M activity that has been attributed to the neutralization of endogenously synthesized TGF-\(\beta\). Thus, we have isolated a peptide corresponding to 13% of the \(\alpha_2\)M sequence that binds TGF-\(\beta\) and neutralizes the activity of TGF-\(\beta\) in two separate biological assays.

Human \(\alpha_2\)-macroglobulin (\(\alpha_2\)M) \(^1\) is a 718-kDa glycoprotein

* This work was supported in part by National Institutes of Health Grant CA-53462. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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† The abbreviations used are: \(\alpha_2\)M, \(\alpha_2\)-macroglobulin; \(\alpha_2\)M-MA, \(\alpha_2\)M-methionyl; MUG, murinoglo-\(\gamma\)ulin; TGF-\(\beta\), transforming growth factor-\(\beta\); NGF-\(\beta\), nerve growth factor-\(\beta\); NO, nitric oxide; LRP, low density lipoprotein receptor-related protein; FBS, fetal bovine serum; IAM, iodacetamide; DTT, dithiothreitol; BSA, bovine serum albumin; PVDF, polyvinylidene fluoride; GST, glutathione S-transferase; PBS, phosphate-buffered saline; SFM, serum-free medium; FP1–FP5, GST fusion proteins 1–5; DMEM, Dulbecco’s modified Eagle’s medium; PAGE, polyacrylamide gel electrophoresis; FP LC, fast protein liquid chromatography; FBHE cell, fetal bovine heart endothelial cell.

that was originally characterized as a broad spectrum proteinase inhibitor (1). The structure of \(\alpha_2\)M consists of four identical subunits, each with 1451 amino acids (2). The subunits are linked into dimers by disulfide bonds and into intact homotetramers by noncovalent interactions (3, 4). Proteinases react with \(\alpha_2\)M by cleaving any of a number of susceptible peptide bonds in the “bait region,” which includes amino acids 666–706 (1, 3, 5). Bait region cleavage causes \(\alpha_2\)M to undergo a major conformational change, which effectively “traps” the attacking proteinase in a complex that is nondissociable, even when the proteinase and the inhibitor are not covalently linked (1, 6–8). Conformational change also reveals binding sites for the \(\alpha_2\)M receptor/low density lipoprotein receptor-related protein (LRP) (9). These binding sites have been localized to 18-kDa peptides at the C terminus of each \(\alpha_2\)M subunit; Lys-1370 and Lys-1374 play particularly important roles (10–13).

Like the complement components, C3 and C4, each \(\alpha_2\)M subunit contains a novel thiol ester bond, which is formed from the side chains of Cys-949 and Glu-952 (14–16). The thiol esters may be instrumental in determining the conformational state of \(\alpha_2\)M (17, 18). When \(\alpha_2\)M reacts with a proteinase, the thiol esters emerge from within hydrophobic, solvent-restricted clefts and are cleaved by nucleophiles or H\(_2\)O (14, 18). Small primary amines, such as methylamine, penetrate the hydrophobic clefts and react with \(\alpha_2\)M thiol esters independently of proteinases, inducing an equivalent or nearly equivalent conformational change (6, 7).

In addition to its activity as a proteinase inhibitor, \(\alpha_2\)M functions as a major carrier and regulator of certain cytokines, including isoforms of the transforming growth factor-\(\beta\) (TGF-\(\beta\)) family. O’Connor-McCourt and Wakefield (19) first identified \(\alpha_2\)M as a physiologically significant carrier of TGF-\(\beta\) in human serum (19). Their studies demonstrated that nearly all of the TGF-\(\beta1\) in serum is associated with \(\alpha_2\)M and that the bound TGF-\(\beta1\) is inactive. Huang et al. (20) confirmed the role of \(\alpha_2\)M as a TGF-\(\beta\)-carrier and demonstrated that the TGF-\(\beta\)-binding activity of \(\alpha_2\)M depends on its conformational state.

More recent studies have demonstrated the function of \(\alpha_2\)M as a TGF-\(\beta\)-carrier in animal model systems. When radioiodinated TGF-\(\beta1\) is injected intravascularly in mice, the cytokine is cleared rapidly at first; however, this is followed by a slow clearance phase, during which time the TGF-\(\beta\)- is almost entirely \(\alpha_2\)M-associated (21–23). In cell culture systems, \(\alpha_2\)M neutralizes both exogenously added and endogenously synthesized TGF-\(\beta\) (24–28). Neutralization of endogenously synthesized TGF-\(\beta\) results in altered gene expression, including greatly increased expression of inducible nitric-oxide synthase by murine macrophages and increased expression of platelet-derived growth factor \(\alpha\)-receptor by vascular smooth muscle cells (27, 28). \(\alpha_2\)M gene knockout mice demonstrate increased tolerance to endotoxin challenge (29); this characteristic is
most likely explained by the enhanced function of TGF-β as an immunosuppressant, in the absence of αM (30). The function of αM, as a significant modulator of TGF-β activity in vivo and in vitro has prompted us to elucidate the αM-TGF-β interaction on a molecular level.

Binding of TGF-β to αM is initially noncovalent and reversible; however, the complex can become covalently stabilized as a result of thiol-disulfide exchange (23). The latter reaction is observed primarily with conformationally altered αM, since native αM lacks free thiol groups (23, 31, 32). We have used a number of complementary methods to determine equilibrium dissociation constants (Kp) for the interaction of TGF-β with αM (23, 31, 33). The Kp values for the binding of TGF-β1 and TGF-β2 to native αM are 300 and 10 nM, respectively; the Kp values for the binding of TGF-β1 and TGF-β2 to methylamine-modified αM (αM-MA) are 80 and 10 nM, respectively. These binding constants accurately predict the ability of αM to neutralize TGF-β in cell culture systems (26, 30, 34, 35).

The mechanism by which αM binds cytokines remains unclear. Early studies, suggesting a prominent role for the thiol-ester-derived Cys-residues, were not confirmed for TGF-β1 and TGF-β2 (32). When αM-MA was treated with papain to release the 18-kDa peptide from the αM, a change in mobility was observed on SDS-PAGE (41, 42). Monomeric αM was prepared by exposing the native form of the protein to a high concentration of DTT (2 mM) under nondenaturing conditions, as described by Moncino et al. (43). Incompletely dissociated αM was separated from the monomers by FPLC on Superose-6. Monomeric αM, which is prepared as described, does not reassociate at 22 °C (44).

Preparation of Constructs Encoding GST-αM-Peptide Fusion Proteins—The human αM cDNA in pAT153/Poul/l8 (pAT-αM) was obtained from the ATCC (16). Restriction digest analysis revealed an additional ScaI cleavage site, which was not predicted by the published sequence (16), due to a single base substitution at nucleotide 2431 (C → T). To generate a construct encoding GST-αM peptide fusion protein-1 (FP1), a fragment from pAT-αM that encodes amino acids 122–415 was excised with BstXI, blunt-ended with T4 DNA polymerase, and ligated into pGEX-3X at the Smal site. The construct encoding FP2 was prepared by digesting pAT-αM with EcoRI and Nael, to yield a partial cDNA encoding amino acids 364–712, which was further digested with ScaI, to generate a cDNA encoding amino acids 364–613. This fragment was blunt-ended and ligated into pGEX-3X at the SmaI site.

Constructs encoding FP3 and FP4 were prepared by isolating cDNAs, from a ScaI digest of pAT-αM, corresponding to amino acids 614–798 and 798–1082, respectively. These cDNAs were blunt-ended and ligated into the SmaI site of pGEX-2T. The construct encoding FP5 was prepared by digesting pAT-αM with XbaI and PstI. A resulting cDNA, which encodes amino acids 1053–1302, was blunt-ended and ligated into pGEX-2T at the Smal site. Restriction digest analysis of the five constructs confirmed that the αM cDNA inserts were in the correct orientation. Fig. 1 shows the relationship of the five peptides to the intact structure of αM.

Purification of GST-αM-Peptide Fusion Proteins—BL21 cells harboring pGEX-αM-peptide expression constructs were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) and harvested in 50 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, pH 8.0. Nearly pure fusion protein preparations were generated by treating bacterial suspensions with 1 mg/ml lysozyme for 15 min on ice. The suspensions were sonicated and subjected to centrifugation at 12,000 × g for 10 min. All five fusion proteins remained in the insoluble fraction. These fractions were subjected to centrifugation at 22,000 × g for 2 h, sonicated, and subjected to a second centrifugation step. The fusion proteins, which again remained in the insoluble fractions, were solubilized by sonication in 2.0% SDS. To block free sulphydryls, each fusion protein was reacted with 1 mM iodoacetamide (IAM) for 25 °C. Equivalent amounts of each protein (5 μg) were incubated in 2% SDS, in the presence or absence of 1 mM DTT, for 30 min at 37 °C. To block free sulphydryls, some samples were treated with 5 μg IAM for 2 h at 25 °C. Equivalent amounts of each protein (5 μg) were subjected to affinity chromatography on glutathione-Sepharose 4B. FP3 and FP4, which eluted from the column, were dialyzed against 1.5% (v/v) sarcosyl and 5 mM DTT. The FP3 and FP4, which solubilized in the sarcosyl, were passed sequentially through 18- and 25-gauge needles and subjected to centrifugation at 12,000 × g. The supernatants, which contained the fusion proteins, were treated with Triton X-100 (2% v/v) to sequester the sarcosyl and subsequently subjected to affinity chromatography on glutathione-Sepharose 4B and 798–1082, respectively. These cDNAs were blunt-ended and ligated into the SmaI site of pGEX-2T. The construct encoding FP5 was prepared by digesting pAT-αM with XbaI and PstI. A resulting cDNA, which encodes amino acids 1053–1302, was blunt-ended and ligated into pGEX-2T at the SmaI site. Restriction digest analysis of the five constructs confirmed that the αM cDNA inserts were in the correct orientation. Fig. 1 shows the relationship of the five peptides to the intact structure of αM.

MATERIALS AND METHODS

Reagents and Proteins—TGF-β2 was purchased from Genzyme (Cambridge, MA). TGF-β1 was from R & D Systems (Minneapolis, MN). Nerve growth factor-β (NGF-β) was purified from male mouse submaxillary glands by the method of Darling and Shooter (38). Methyamine chloride, T, iodoacetamide (IAM), diithiothreitol (DTT), isopropylthio-β-galactoside, N-actyl glucosamine, glutathione S-transferase (GST), glutathione, anti-GST IgG fraction of antiserum, and bovine serum albumin (BSA) were from Sigma. Nd13 was from Amersham Pharmacia Biotech. pGEX-3X, pGEX-2T, and prepackaged glutathione-Sepharose-4B columns were from Amersham Pharmacia Biotech. Immulon 2 microtiter plates were from Dynatech Laboratories (Chantilly, VA). Polyvinylidene fluoride (PVDF) and nitrocellulose membranes were from Millipore Corp. IODO-GEN was from Pierce. RPMI 1640, Dulbecco’s modified Eagle’s medium (DMEM), and Trypsin-EDTA were from Life Technologies, Inc. Fetal bovine serum (FBS) was from HyClone Laboratories. Acidic growth factor and basic fibroblast growth factor were from Promega.

α-Macroglobulins and Related Derivatives—Human αM was purified from plasma by the method of Imber and Pizzo (39). Murinoglobulin (MUG) was purified from the plasma of CD-1 female mice as described previously (30). SDS-PAGE analysis of purified MUG revealed a single band with an apparent mass of 180 kDa. αM-MA was prepared by dialyzing human αM against 200 mM methylamine-HCl in 50 mM Tris-HCl, pH 8.2, for 12 h at 22 °C followed by extensive dialysis against 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), at 4 °C. Complete modification of native αM by methylamine was confirmed by loss of trypsin binding activity (greater than 96%) (40) and by the characteristic increase in electrophoretic mobility, when analyzed by nondenaturing PAGE (41, 42). Monomeric αM was prepared by exposing the native form of the protein to a high concentration of DTT (2 mM) under nondenaturing conditions, as described by Moncino et al. (43). Incompletely dissociated αM was separated from the monomers by FPLC on Superose-6. Monomeric αM, which is prepared as described, does not reassociate at 22 °C (44).
using the method recommended by the manufacturer. To determine whether TGF-β binding to FP3 is noncovalent and specific, membranes containing immobilized FP3 (0.5 μg) were incubated with 125I-TGF-β1 (0.25 nm) or 125I-TGF-β2 (0.25 nm) in the presence of unlabeled TGF-β1 (200 nm), unlabeled TGF-β2 (200 nm), or solution phase FP3 (1.0 μM). After washing the membranes with PBS-T, bound radioligands were detected by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

Western Blot Analysis—GST-α2M peptide fusion proteins were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes. The membranes were blocked with 5% milk in PBS-T for 12 h at 4°C, incubated with a polyclonal antibody that recognizes GST and then with peroxidase-conjugated goat anti-rabbit IgG. Binding of secondary antibody was detected by enhanced chemiluminescence (Amer sham Pharmacia Biotech).

Binding of 125I-TGF-β2 to FP3 and FP4 as Determined by FPLC—125I-TGF-β2 (0.5 nm) was incubated with FP3 or FP4 (0.5 μm) in PBS for 30 min at 37°C. The FP3 and FP4 were purified by glutathione affinity chromatography, treated with IAME, and free of detergents. 125I-TGF-β2-fusion protein complexes were separated from free 125I-TGF-β2 by FPLC on prepacked Superose-12 columns. The flow rate was 0.4 ml/min. Elution of FP3 or FP4 was detected by monitoring the absorbance at 280 nm. 125I-TGF-β2 was detected in elution fractions using a γ-counter. To calibrate the FPLC, the following proteins were subjected to chromatography on the same column: soybean trypsin inhibitor (Mr, ~21,500, Vm of 14.1 ml), ovalbumin (Mr, ~45,000, Vm of 12.9 ml), BSA (Mr, ~66,000, Vm of 12.1 ml), and BSA dimer (Mr, ~132,000, Vm of 10.9 ml).

RESULTS

Ligand Blot Analysis of 125I-TGF-β Binding to α2M—Native α2M, α2M-MA, and BSA were denatured in SDS (with or without reducing), subjected to SDS-PAGE, and electrotransferred to PVDF membranes. Some samples were treated with IAM prior to electrophoresis. The membranes were stained with Coomassie Blue, demonstrating nearly equivalent electrotransfer of the three proteins (results not shown). Unreduced α2M migrated as a single band with an apparent mass of 360 kDa, as expected; reduced α2M migrated as a single major band with an apparent mass of 180 kDa (3). Methylamine treatment did not alter the mobility of α2M (14, 15, 48).

125I-TGF-β2 bound to native α2M and α2M-MA, which were immobilized on PVDF membranes (Fig. 1). 125I-TGF-β2 binding was unchanged when the α2M was treated with DTT or with IAM prior to electrophoresis. 125I-TGF-β2 also bound to
IAM-treated samples are marked with a plus sign by PhosphorImager analysis. The membranes were blocked and incubated with 5 mM IAM for 2 h at 25 °C. DTT- or IAM-treated samples are marked with a plus sign in the respective rows. All samples were subjected to SDS-PAGE and electrotransferred to PVDF membranes. The membranes were blocked and incubated with the indicated cytokines for 2 h at 25 °C. Cytokine binding was detected by PhosphorImager analysis.

BSA; however, this interaction was observed only after DTT treatment and was eliminated by treating the BSA with IAM. Thus, binding of 125I-TGF-β2 to reduced BSA probably involves free sulfhydryl groups that are not available in the native BSA structure. The ability of isolated αM subunits to bind 125I-TGF-β2, by an IAM-insensitive mechanism, suggests that the ligand blotting system accurately models the interaction of TGF-β with nondenatured αM and that αM quaternary structure is not necessary for this interaction.

To further assess the growth factor binding activity of isolated αM subunits in the ligand blotting system, studies were performed with 125I-TGF-β1 and 125I-NGF-β. Two cytokines bind to non-denatured αM with similar affinity (31). As shown in Fig. 2, 125I-TGF-β1 and 125I-NGF-β both bound to immobilized αM by an IAM-insensitive mechanism. Reduc-tant-treated BSA also bound 125I-TGF-β1 and 125I-NGF-β; however, this interaction was eliminated when the BSA was treated with IAM.

Ligand Blot Analysis of the Binding of 125I-TGF-β2 to MUG—MUG is a monomeric murine homologue of human αM. Although tetrameric murine αM, in its native form, and human αM bind TGF-β1 and TGF-β2 similarly, MUG does not bind either TGF-β1 isof orm with significant affinity (Kd ~ 0.8 M) (30). Thus, we compared the binding of 125I-TGF-β2 to human αM and MUG, as another test of the validity of the ligand blotting method. As shown in Fig. 3, only trace levels of 125I-TGF-β2 bound to MUG, and the amount of binding was decreased when the MUG was treated with IAM. These results support the hypothesis that ligand blotting is a valid method for the analysis of cytokine binding to αM-macroglobulins. Apparently, MUG does not contain a cryptic TGF-β-binding site that is exposed by SDS treatment.

TGF-β2-Binding to GST-αM Peptide Fusion Proteins—The five fusion proteins were subjected to SDS-PAGE and electrotransferred to PVDF. The electrophoretic mobility of the major Coomassie-stained band, in each preparation, indicated a molecular mass that was identical to the mass of the monomeric fusion protein predicted by the cDNA sequence (Fig. 4). Western blot analysis with a GST-specific antibody confirmed that the major band in each lane was a GST fusion protein. The low mobility bands also bound GST-specific antibody and thus most likely represent SDS-insensitive fusion protein aggregates. In ligand blotting experiments, only FP3 bound 125I-TGF-β2. Since all five fusion proteins were IAM-treated, free sulfhydryl groups in FP3 did not account for the 125I-TGF-β2 binding. FP1, FP2, FP4, and purified GST (not shown) did not bind 125I-TGF-β2.

In separate ligand blotting experiments, affinity-purified FP3 and FP3 that was stored in SDS bound TGF-β2 equivalently (results not shown). Thus, the two preparations were interchangeable when analyzed by this method. In order to demonstrate that 125I-TGF-β2 binding to FP3 is noncovalent and specific, 125I-TGF-β was incubated with PVDF-immobilized FP3 in the presence of excess solution phase FP3 or unlabeled TGF-β. FP3 (1 μM) in solution inhibited the binding of 125I-TGF-β1 and 125I-TGF-β2 to immobilized FP3 by 97 ± 3 and 92 ± 5%, respectively. Unlabeled TGF-β1 (0.2 μM) inhibited 125I-TGF-β1 binding to immobilized FP3 by 72 ± 8%; unlabeled TGF-β2 (0.2 μM) inhibited 125I-TGF-β2 binding to immobilized FP3 by 90 ± 4%.

Binding of 125I-TGF-β2 to FP3 in Solution—125I-TGF-β2 (0.5 nM) was incubated with FP3 or FP4 (0.5 μM) in solution, in the absence of detergents. Free and fusion protein-associated 125I-TGF-β2 were separated by FPLC on Superose-12. We previously demonstrated that free TGF-β interacts substantially with Superose and thus is recovered slowly at volumes that...
42% of the 125I-TGF-β2 (49). Substantial amounts of radioactivity co-eluted with FP3, corresponding to apparent masses of 95- and 107-kDa. Other to immobilized a M-peptide fusion proteins. The five fusion proteins (FP1–FP5) were subjected to SDS-PAGE and electrotransferred to PVDF or nitrocellulose membranes. PVDF membranes were stained with Coomassie Blue. Western blot analysis was performed with an anti-GST IgG with 125I-TGF-β2–binding was detected by PhosphorImager analysis. Ligand blot analysis was performed with an anti-GST IgG with 125I-TGF-β2. After incubation for 2 h, 125I-TGF-β2-binding was detected by PhosphorImager analysis.

FIG. 4. Binding of 125I-TGF-β2 to GST-a M-peptide fusion proteins. The five fusion proteins (FP1–FP5) were subjected to SDS-PAGE and electrotransferred to PVDF or nitrocellulose membranes. PVDF membranes were stained with Coomassie Blue. Western blot analysis was performed with an anti-GST IgG fraction of antiserum. Ligand blot analysis was performed with 125I-TGF-β2. After incubation for 2 h, 125I-TGF-β2-binding was detected by PhosphorImager analysis.

FIG. 5. Binding of 125I-TGF-β2 to purified FP3 as determined by FPLC. FP3 and FP4 were incubated with 125I-TGF-β2 for 30 min at 37 °C and then subjected to FPLC on a Superose-12 column. Radioactivity recovery in each fraction is plotted as a percentage of the originally loaded radioactivity. The solid tracings show the absorbance of the eluate at 280 nm as a function of time.
was included in the medium. By contrast, FP3 nearly completely inhibited the activities of both TGF-β1 and TGF-β2, increasing [3H]thymidine incorporation to within 3 and 6% of the control values.

_Fig. 7._ NO synthesis by RAW 264.7 cells treated with FP3. RAW 264.7 cells were treated with α2-M-MA or FP3 in SFM. The control (C) was incubated in SFM (no α2-M-MA or FP3). After 24 h, nitrite levels in the conditioned media were measured. The presented results represent the mean ± S.E. (n = 4).

| Agent added | [3H]Thymidine incorporation (% of control) |
|-------------|------------------------------------------|
| TGF-β1      | 31 ± 3                                   |
| TGF-β2      | 43 ± 2                                   |
| FP3 + TGF-β1| 97 ± 5                                   |
| FP4 + TGF-β1| 31 ± 4                                   |
| FP3 + TGF-β2| 94 ± 6                                   |
| FP4 + TGF-β2| 58 ± 6                                   |

**DISCUSSION**

The TGF-β family of cytokines regulates diverse processes including cellular growth, differentiation, wound healing, and inflammation (for review, see Refs. 50 and 51). At the cellular level, TGF-β response is mediated by or regulated by a variety of receptors and binding proteins, including the type I and type II receptors, which are serine/threonine kinases, β-glycan, and endoglin. TGF-β activity is also regulated by processes that alter delivery of the active cytokine to the cell surface. For example, TGF-β is secreted as a large latent complex that includes latency-associated peptide and a second gene product, latent TGF-β-binding protein (52–54). Conversion of latent TGF-β into active 25-kDa homodimer requires dissociation of latency-associated peptide and latent TGF-β-binding protein in reactions that may be mediated by proteinases (55), thrombospondin (56), the mannose 6-phosphate/insulin-like growth factor-II receptor (57) and acidic microenvironments (58). Once activated, the 25-kDa form of TGF-β may bind to α2M, once again forming a complex that is unavailable for receptor binding.

The fate of α2M-associated TGF-β depends on the α2M con-
formation. Native α2M, which is the predominant form of α2M present in the plasma and probably in most extravascular microenvironments, binds TGF-β reversibly and noncovalently (23, 31, 32). Thus, native α2M may buffer tissues against rapid changes in TGF-β levels by binding or slowly releasing the cytokine in response to the free TGF-β concentration. Based on the Kᵦ value, we predict that approximately 95% of the TGF-β1 in plasma is α2M-associated under equilibrium conditions, even though TGF-β1 binds to native α2M with lower affinity than TGF-β2 (31). Conversion of α2M into the transformed conformation, which probably occurs most frequently at sites of inflammation due to the increase in cellular proteinase secretion, alters the mechanisms by which TGF-β is regulated. First, transformed α2M has free Cys residues and thus undergoes thiol-disulfide exchange with TGF-β (23, 31, 32), eliminating the potential for release of active cytokine. Second, α2M-protease complexes bind to the endocytic receptor, LRP; bound TGF-β is internalized with the α2M-protease complex and probably delivered to lysosomes (22, 36).

The goal of the present investigation was to identify the binding site for TGF-β in α2M. Our original ligand blotting experiments, with human α2M, demonstrated that intact quaternary structure is not necessary for TGF-β binding. Treatment of α2M with IAM did not inhibit TGF-β-binding, indicating that free Cys residues, which arose either as a result of thiol aminolysis or DTT treatment, are not involved. We also studied the binding of TGF-β to purified MUG by ligand blotting, since nondenatured MUG, unlike human α2M and tetrameric murine α2M, does not bind TGF-β with significant affinity (30). MUG bound only trace levels of TGF-β in ligand blotting studies, supporting our hypothesis that ligand blotting provides a valid model of TGF-β-α-macroglobulin interactions that occur under nondenaturing conditions. The inability of TGF-β and NGF-β to bind to reduced and alkylated BSA further supports the use of ligand blotting as a valid model system.

When the majority of the α2M cDNA was expressed in a series of five GST fusion proteins, TGF-β-binding was localized exclusively to FP3. The other four fusion proteins and purified GST did not bind TGF-β. Selective binding of TGF-β to affinity-purified FP3, and not to FP4, was demonstrated by FPLC and by radioligand-binding competition assay. FP3 was more effective than native α2M or α2M-MA at inhibiting TGF-β1 binding to immobilized α2M-MA. This result is intriguing for at least three reasons. First, in comparing FP3 and intact α2M, we used the concentrations of intact α2M tetramer and FP3 monomer, although our FPLC results suggested that FP3 is a noncovalent dimer. If, instead, we had based the IC₅₀ values on the concentration of the α2M “subunit,” then the difference between FP3 and intact α2M would have been 4-fold greater. Second, the experimentally determined IC₅₀ values accurately estimate the Kᵦ only if one molecule of competitor is sufficient to completely prevent TGF-β-binding to immobilized α2M-MA; otherwise, the Kᵦ is lower than the IC₅₀. Although it is possible that two copies of FP3 or α2M are required to neutralize TGF-β, given the homodimeric structure of TGF-β, this possibility is considered less likely with intact α2M, due to its large size and complex structure, as discussed below. Also, as discussed below, our studies suggest that tetrameric α2M may bind more than one molecule of TGF-β. Finally, we cannot be certain that FP3 adopts a secondary and tertiary structure that is optimal for TGF-β binding. Taken together, these results suggest that a specific sequence in FP3 binds TGF-β with relatively high affinity. The equivalent sequence may be partially masked within intact α2M, accounting for the observed decrease in TGF-β binding affinity. The masking of the TGF-β-binding site in intact α2M may also explain why α2M conformational change markedly alters TGF-β binding affinity (31).

Human α2M and bovine α2M bind TGF-β2 with increased affinity compared with TGF-β1 (24, 31), explaining why TGF-β1 is preferentially active in certain cell culture assays that require serum-supplemented medium (24–26). Danielpour and Sporn (24) provided evidence that the α-macroglobulins from rabbit also preferentially bind TGF-β2. By contrast, murine α2M binds TGF-β1 and TGF-β2 with equivalent affinity (30). In this study, we demonstrated that TGF-β1 and TGF-β2 bind to FP3 with equivalent affinity as well. This result suggests that the isoform specificity in TGF-β binding to certain α-macroglobulins may be due to the ability of TGF-β2 to preferentially access the FP3-binding site in the intact α-macroglobulin. When the structural constraints of intact α2M are eliminated, as in FP3, isoform specificity in TGF-β binding is no longer observed. We do not understand why the binding site for TGF-β2 may be “less masked” in the structure of intact human α2M compared with the binding site for TGF-β1; however, NMR and x-ray crystallography studies have demonstrated the presence of small differences in the overall shape and structure of TGF-β1 and TGF-β2 (59–61).

In addition to the TGF-β-binding site, FP3 also contains the α2M bait region. Models have been developed regarding the location of the bait region within the complex three-dimensional structure of α2M based on electron microscopy (62, 63); the x-ray crystal structure, which has been solved at 10-Å resolution (64); NMR and EPR spectroscopy studies (65, 66); and fluorescence resonance energy transfer studies (67). The overall structure of α2M resembles a hollow cylinder with a two-compartment central cavity. In α2M-protease complexes, the proteinases occupy the central cavities. The bait regions are located within the central cavities, toward the center of the α2M structure, and within 11–17 Å of the Cys residues (Cys-949) that form the thiol ester bonds (64). If, in fact, the bait region and the TGF-β-binding site are equivalent or overlapping, then the TGF-β-binding site may be accessible only from within the α2M central cavity. TGF-β-specific antibodies fail to recognize α2M-associated TGF-β (19, 24), supporting the hypothesis that TGF-β occupies the central cavity; however, it is not clear whether the α2M, which was studied in the antibody experiments, was in the native or conformationally altered form. Thus, the location of the FP3-binding site for TGF-β, within intact α2M, remains unresolved. The bait region is known as an area of extreme sequence variability among α-macroglobulins from different species (5). Since TGF-β-binding is conserved among many α-macroglobulins, with the exception of MUG (30, 31, 34), one can argue that the bait region and TGF-β-binding site are unlikely to be equivalent. Further studies will be necessary to determine the relationship between these two important functional regions.

The stoichiometry of cytokine binding to α2M has been estimated at 1:1 or 2:1 (19, 68). Our results suggest that the binding site contained within a single α2M subunit may be sufficient to bind TGF-β. Thus, an estimate of four cytokine-binding sites per α2M does not seem unreasonable. Limitations in the number of cytokine-binding sites in intact α2M may result from steric hindrance. If α2M-associated cytokines occupy the central cavity, then the number of cytokines that bind may be limited by the available cavity space. Of equal importance is the possibility that a high affinity complex between α2M and TGF-β requires that the cytokine engage two equivalent copies of FP3 on different subunits. Kᵦ values, determined by the α2M-MA immobilization method and by our previously described BS₃-cross-linking method (31, 33), assume a single cytokine-binding site per α2M tetramer. If there are two
independent binding sites, then the $K_d$ for each site would be increased by a factor of 2; however, our reported binding constants may still be most useful for predicting the cytokine-neutralizing activity of $\alpha_M$ in biological assays.

In FBHE cell proliferation assays, we demonstrated that FP3 not only binds TGF-$\beta_1$ and TGF-$\beta_2$ but also neutralizes the activities of these cytokines. When added to RAW 264.7 cell cultures, FP3 promoted the accumulation of nitrite more efficiently than $\alpha_M$-MA. Since we previously demonstrated that the induction of NO synthesis by TGF-$\beta_1$ is due to its increased binding affinity for TGF-$\beta_1$, we hypothesized that the increased potency of FP3 may be due to its increased binding affinity for TGF-$\beta_1$. To test this hypothesis, we measured the secretion of TGF-$\beta_1$ and TGF-$\beta_2$ by RAW 264.7 cells using isoform-specific enzyme-linked immunosorbent assays. In medium that was conditioned for 24 h, the concentrations of active and total (active plus latent) TGF-$\beta_1$ were 2 and 10 pM, respectively. The concentrations of active and total TGF-$\beta_2$ were 1 and 4 pM, respectively. The active TGF-$\beta$ levels reported here are only slightly lower than those determined previously using an endothelial cell growth assay (27). More importantly, the enzyme-linked immunosorbent assays confirm that RAW 264.7 cells express both TGF-$\beta$ isoforms but higher levels of TGF-$\beta_1$, supporting the hypothesis that the increased potency of FP3 reflects its increased capacity to neutralize TGF-$\beta_1$.

In summary, we have identified a single peptide from the structure of $\alpha_M$ that contains the binding site for TGF-$\beta_1$ and TGF-$\beta_2$. The high affinity of FP3 for both TGF-$\beta$ isoforms and the substantial potency of FP3 in two TGF-$\beta$ neutralization assays suggests that the TGF-$\beta$-binding sequence may be partially masked in intact $\alpha_M$. Like TGF-$\beta_1$ and TGF-$\beta_2$, NGF-$\beta$ bound to dissociated $\alpha_M$ subunits, suggesting that intact quaternary structure and the resulting $\alpha_M$ central cavity or trap is not necessary. However, at this time, we have not determined whether the NGF-$\beta$-binding site or the binding site for any other cytokine is contained within FP3. The fusion proteins generated in this study will represent excellent templates for defining other cytokine-binding sites in $\alpha_M$ and for further refinement of the TGF-$\beta$-binding sequence.

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