Characterization of the interaction between α_2-macroglobulin and fibroblast growth factor-2: the role of hydrophobic interactions

Smitha MATHEW*, Sanja ARANDJELOVIC†, Wayne F. BEYER*, Steven L. GONIAS† and Salvatore V. PIZZO*[1]

*Department of Pathology, Box 3712, Duke University Medical Center, Durham, NC 27710, U.S.A., and †Departments of Pathology, Biochemistry and Molecular Genetics, University of Virginia School of Medicine, Charlottesville, VA 22908, U.S.A.

Basic fibroblast growth factor (FGF-2) is important in development, wound healing and angiogenesis. The human plasma proteinase inhibitor α_2-macroglobulin (α_2M) binds to and regulates the biological activity of various growth factors, including FGF-2. FGF-2 binds specifically and saturably to native α_2M and conformationally modified α_2M (α_2M*); however, the K_D for FGF-2 binding to α_2M* is 10-fold lower. This study investigates the biochemical nature of the interaction between FGF-2 and α_2M* and localizes a possible FGF-2 binding site in the α_2M subunit. FGF-2 binding to α_2M* was not affected by shifts in pH between 6.5 and 10; however, increasing temperature decreased the K_D for this interaction. The binding affinity of FGF-2 for α_2M* also increased with increasing ionic strength. These results are consistent with the hypothesis that hydrophobic interactions predominate in promoting FGF-2 association with α_2M*. Consistent with this hypothesis, FGF-2 bound to a glutathione S-transferase fusion protein containing amino acids 591–774 of the α_2M subunit (FP3) and to a hydrophobic 16-amino-acid peptide (amino acids 718–733) within FP3. Specific binding of FGF-2 to the 16-amino-acid peptide was inhibited by excess transforming growth factor-β1. When the 16-amino-acid peptide was chemically modified to neutralize the only two charged amino acids, FGF-2-binding activity was unaffected, supporting the predominant role of hydrophobic interactions. FGF-2 presentation to signalling receptors is influenced by growth factor binding to heparan sulphate proteoglycans (HSPGs), which is electrostatic in nature. Our results demonstrate that the interactions of FGF-2 with α_2M* and HSPGs are biochemically distinct, suggesting that different FGF-2 sequences are involved.

Key words: α_2-macroglobulin, basic fibroblast growth factor, growth factor, protease, transforming growth factor-β.

INTRODUCTION

The fibroblast growth factor (FGF) family of heparin-binding proteins consists of 23 members [1–3]. Acidic FGF (FGF-1) and basic FGF (FGF-2) are the two prototypic members of this family. FGFs initiate cell signalling by binding to at least five different high-affinity transmembrane receptor tyrosine kinases [4,5]. As a result of the signalling responses, FGFs stimulate proliferation of cells of mesodermal origin, including fibroblasts, astrocytes, smooth muscle cells, melanocytes and endothelial cells [1,6]. FGFs also are important in other physiological processes in the adult and developing embryo, including neuronal development, cellular differentiation, wound healing, tissue repair, neovascularization and angiogenesis [1,7–10].

FGF-1 and FGF-2 lack signal sequences [2,11]; nevertheless, they are efficiently secreted by cells through novel endoplasmic reticulum/Golgi-independent exocytosis pathways [12,13]. Once FGFs are present in the pericellular space, they bind to heparan sulphate proteoglycans (HSPGs) present on the cell surface and the extracellular matrix [14]. This interaction localizes FGFs to the pericellular spaces, providing a reservoir of growth factor; moreover, HSPGs also protect FGFs from proteolytic cleavage [14]. This protective mechanism prolongs the half-life of extracellular FGFs and may alter delivery to the FGF receptors, which is critical in determining cellular response [15].

α_2-Macroglobulin (α_2M) is a 718-kDa homotetrameric protein present in human plasma at a concentration of 2–5 μM. The intact protein is a broad-specificity proteinase inhibitor that reacts with proteinases from all four mechanistic classes [16]. Upon reaction with α_2M, the proteinase attacks a susceptible peptide bond in the so-called 'bait region', causing α_2M to undergo a conformational change which entraps the proteinase within its hollow core [16]. α_2M conformational change renders internal β-cysteinyl-γ-glutamyl thiol esters (one per subunit) susceptible to nucleophilic attack [16]. ε-Amino groups, in lysine residues from the entrapped proteinase, may bind directly to the thiol esters and thus become covalently bound to the α_2M at this site [16]. Small nucleophiles such as NH_3 or methylamine react with the α_2M thiol esters, in the absence of a proteinase, and induce an equivalent α_2M conformational change [16], which exposes receptor-recognition sites on each subunit. Conformationally modified α_2M (α_2M*; also known as activated or receptor-recognized α_2M) binds to two cell-surface receptors: the low-density-lipoprotein-receptor-related protein ('LRP') and the α_2M signalling receptor [17–19].

In addition to proteinases, α_2M binds a variety of cytokines and growth factors, including transforming growth factor-β (TGF-β), tumour necrosis factor-α ('TNF-α'), interleukin 1β ('IL-1β'), platelet-derived growth factor-BB (PDGF-BB), nerve growth factor-β ('NGF') and vascular endothelial growth factor ('VEGF') [20,21]. These interactions do not induce α_2M conformational change; however, in many cases the affinity of the interaction is highly dependent on the α_2M conformation. A candidate binding site for TGF-β1 and PDGF-BB has been localized near the centre of the α_2M subunit [22], but it is not likely that the same binding site is responsible for all α_2M–growth-factor interactions [22,23]. Currently, the nature of the interactions

Abbreviations used: FGF, fibroblast growth factor; HSPG, heparan sulphate proteoglycan; α_2M, α_2-macroglobulin; α_2M*, conformationally modified α_2M; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; GST, glutathione S-transferase.

[1] To whom correspondence should be addressed (e-mail pizzo001@mc.duke.edu).

that promote growth factor binding to α_2M remains incompletely understood.

Binding of FGF-2 to human α_2M was first demonstrated by Dennis et al. [24]. We demonstrated that only select members of the FGF gene family bind to α_2M [25]. FGF-1, FGF-2, FGF-4 and FGF-6 bind α_2M, whereas FGF-5, FGF-7, FGF-9 and FGF-10 do not [25]. α_2M* binds FGFs with significantly higher affinity than native α_2M [25]. While there is evidence that α_2M regulates the activity of growth factors, such as TGF-β1, by a straightforward mechanism that involves reversible binding and inhibition of receptor interactions [26,27], in the case of FGF-2 a more complicated pattern of activity regulation has emerged. α_2M* inhibits FGF-2-induced proliferation of fetal bovine heart endothelial cells ('FBHEs') and human umbilical vein endothelial cells ('HUVECs') in a dose-dependent manner; however, α_2M* does not inhibit FGF-2-induced endothelial cell tubule formation in Matrigel® or in type I collagen gels [25]. We hypothesized that these results are due to the ability of FGF-2 to distribute between solution phase (α_2M*) and immobilized carrier macromolecules (HSPGs) with differing effects on activity. Furthermore, we hypothesized that α_2M* may not be able to access matrix-associated growth factors, perhaps due to its large size or due to interaction with a common region of FGF-2 that also binds to extracellular matrix components.

X-ray-crystallographic and site-directed mutagenesis studies have suggested that the binding of FGF-2 to HSPGs is driven by electrostatic interactions [28–30]. Because of the possibility that HSPGs and α_2M* form a system to co-regulate FGF-2 activity, we sought to elucidate the properties of the FGF-2–α_2M* interaction. Our results indicate that hydrophobic rather than electrostatic interactions are responsible for the FGF-2–α_2M* interaction. A glutathione S-transferase (GST) fusion protein, which contains amino acids 591–774 of the human α_2M subunit (FP3), and a 16-amino-acid peptide contained within FP3, both bound FGF-2, which is significant since this fusion protein and peptide have been shown previously to bind TGF-β1 [31,32]. The 16-amino-acid peptide is notable for its high content of hydrophobic residues and acidic residues; however, for FGF-2 binding, the acidic residues were not critical. These results demonstrate that distinct mechanisms govern the interaction of FGF-2 with HSPGs and α_2M*.

EXPERIMENTAL

Materials

^{125}I-labelled Bolton–Hunter reagent was purchased from NEN Life Science Products (Boston, MA, U.S.A.). 5,5′-Dithiobis-(2-nitrobenzoic acid), methylamine/HCl, gelatin and BSA were purchased from Sigma (St. Louis, MO, U.S.A.). Electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Human α_2M was purified from plasma according to a protocol described previously [33]. The concentration of α_2M was determined using an $A_{280}^{(1\%/1\,cm)}$ value of 8.93 and assuming a molecular mass of 718 kDa [34]. α_2M was modified with methylamine to form α_2M* as described previously [35]. The fusion protein FP3 (amino acids 591–774) was expressed in *Escherichia coli* BL-21 cells and purified by chromatography on glutathione–Sepharose, as described previously [31]. Recombinant carrier-free FGF-2, TGF-β1 and FGF-1 were purchased from R&D Systems (Minneapolis, MN, U.S.A.). FGF-2 was labelled with ^{125}I-labelled Bolton–Hunter reagent according to the manufacturer's recommended protocol. ^{125}I-FGF-2 was used within 2 weeks of labelling. All other reagents were of the highest quality commercially available.

Specific binding of ^{125}I-FGF-2 to immobilized α_2M*

Specific binding of ^{125}I-FGF-2 to α_2M* was determined as described previously [25]. In brief, 96-well plates were coated with 1 μg of α_2M* by incubation in 20 mM Hepes/150 mM NaCl, pH 7.5, for 4 h at 25 °C. The wells were blocked overnight with the same buffer containing 0.1 % (v/v) Tween-20. The total amount of α_2M* in the wells was determined in concurrent experiments with ^{125}I-α_2M*. ^{125}I-FGF-2 (6 nM) was added to the wells in the presence or absence of a 1000-fold molar excess of unlabelled FGF-2 and allowed to incubate at 37 °C for 24 h. As a control for background, wells that were not coated with α_2M*, but only blocked overnight with 20 mM Hepes/150 mM NaCl, pH 7.5, containing 0.1 % (v/v) Tween-20, were incubated with ^{125}I-FGF-2 (6 nM) at 37 °C for 24 h. The wells were then washed three times with 20 mM Hepes/150 mM NaCl, pH 7.5, containing 0.1 % (v/v) Tween-20 to remove unbound ^{125}I-FGF-2. The radioactivity associated with each well was determined by gamma counting. The radioactivity associated with the background wells was insignificant, but nonetheless these values were subtracted from the radioactivity associated from the experimental wells prior to further quantitative analysis.

Effects of pH, temperature and ionic strength on the binding of FGF-2 to α_2M*

Binding of ^{125}I-FGF-2 to α_2M* was determined as a function of pH, temperature and ionic strength. To assess the effects of pH, ^{125}I-FGF-2 (35 nM) was incubated, in solution, with 6–3800 nM α_2M*, for 24 h at 37 °C in 20 mM Hepes/150 mM NaCl at pH 6.5, 7.5, 8.5, 9.0 and 10.0. To determine the effects of temperature and ionic strength, ^{125}I-FGF-2 (35 nM) was incubated with 10–2600 nM α_2M* for 24 h in 20 mM Hepes/150 mM NaCl, pH 7.5, at 4, 22 and 37 °C, or with 4–1800 nM α_2M* for 24 h at 37 °C in 20 mM Hepes with 0, 150 or 300 mM NaCl, pH 7.5. Following incubation, the solutions were subjected immediately to non-denaturing PAGE on 5 % slab gels, using a Tris/glycine buffer system (pH 8.3). The gels were stained with Coomassie Brilliant Blue to identify the α_2M* and then dried and subjected to PhosphorImager™ analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.). The radioactivity associated with α_2M* was quantified using ImageQuant™ analysis software (version 3.3; Molecular Dynamics). In control experiments, we determined that free ^{125}I-FGF-2 does not migrate near the α_2M in the non-denaturing PAGE system. As a further control for loading, each sample was subjected to SDS/PAGE, and recovery of ^{125}I-FGF-2 was determined by PhosphorImager™ analysis. Apparent equilibrium disassociation constants (K_D) were determined by direct fit of the PhosphorImager™ data, using a one-site binding model in SigmaPlot™ software (version 4.0; Jandel Scientific, San Rafael, CA, U.S.A.). Specifically, we plotted concentrations of free α_2M* at equilibrium against the amount of ^{125}I-FGF-2 bound at each of the conditions we tested. Free α_2M* was determined by correcting the total concentration for the amount that was ^{125}I-FGF-2-associated, assuming 1:1 stoichiometric binding. Scatchard analysis was also performed to determine the B_{max} values and to confirm the K_D values obtained from SigmaPlot™ analysis for FGF-2 binding to α_2M*.

Inhibition of ^{125}I-FGF-2 binding to α_2M* by FP3

^{125}I-FGF-2 (7 nM) was incubated with α_2M* (0.1 μM) alone or in the presence of FP3 (0.1–0.8 μM) in PBS for 1 h at 37 °C. The samples were then subjected to non-denaturing PAGE. The gels were then dried and subjected to PhosphorImager™ analysis. The

radioactivity associated with the α_2M* band was quantified using ImageQuant™. The radioactivity associated with the α_2M* in the absence of FP3 was defined as 100 %.

Synthesis and esterification of the 16-amino-acid peptide derived from human α_2M (P3)

The 16-amino-acid peptide, designated P3 (WDLVVVNSAGV-AEVGV), corresponding to amino acids 718–733 of the human α_2M sequence, was synthesized by Research Genetics, Invitrogen Corporation (Huntsville, AL, U.S.A.). The N-terminus was acetylated and the C-terminus was amidated. Reversed-phase HPLC utilizing a porous column (16 mm × 100 mm) was used to purify the peptide. The amino acid sequence and the purity of P3 was confirmed employing electrospray MS. For use as a negative control, an 8-amino-acid peptide, designated P3.5 (EVGVTVPD), was synthesized and modified at the N- and C-termini as described above.

To neutralize the two anionic amino acids in P3, the peptide was esterified using the methanol/HCl procedure as described by Means and Feeney [36]. Briefly, the synthetic peptide dissolved in DMSO was mixed with a 17-fold molar excess of cold methanol. HCl was added to the mixture to a final concentration of 0.1 M. The synthetic peptide/methanol mixture was incubated at 4 °C for 48 h. The esterification reaction was then stopped by flooding the reaction mixture with a large volume of ice-cold water. In order to remove the methanol and HCl, the sample was dried using a Speedvac. The synthetic peptide was then resuspended in water and dried using the Speedvac three times. The amino acid sequence and the purity of the esterified P3 were confirmed employing matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS. Reversed-phase HPLC utilizing an ABI Spheri 5 PTH column (220 mm × 2.1 mm) was used to purify the esterified P3 from the unmodified P3.

Binding of ^{125}I-FGF-2 to P3 or esterified P3 polypeptide

We coated 96-well plates with 1 μg of P3, esterified P3 or P3.5 by incubation in 20 mM Hepes/150 mM NaCl, pH 7.5, for 4 h at 25 °C. The coated wells were blocked overnight with 20 mM Hepes/150 mM NaCl, pH 7.5, containing 0.1 % (v/v) Tween-20. ^{125}I-FGF-2 (6 nM) was incubated with the immobilized peptides in the presence or absence of a 1000-fold molar excess of unlabelled FGF-2, a 100-fold molar excess of TGF-β1 or a 1000-fold molar excess of FGF-1, for 24 h at 37 °C. ^{125}I-FGF-2 (6 nM) was incubated with the immobilized esterified P3 only in the presence or absence of a 1000-fold molar excess of FGF-2 for 24 h at 37 °C. As a control for background, wells that were not coated with any peptide, but only blocked overnight with 20 mM Hepes/150 mM NaCl, pH 7.5, containing 0.1 % (v/v) Tween-20, were incubated with ^{125}I-FGF-2 (6 nM) at 37 °C for 24 h. The wells were then washed three times with 20 mM Hepes/150 mM NaCl, pH 7.5, containing 0.1 % (v/v) Tween-20 to remove unbound ^{125}I-FGF-2. The radioactivity associated with each well was determined by gamma counting. The radioactivity associated with the background wells was insignificant, but this value was nonetheless subtracted from the radioactivity associated from the experimental wells prior to further quantitative analysis.

RESULTS

Specific binding of ^{125}I-FGF-2 to immobilized α_2M*

Because FGF-2 binds selectively to α_2M* and with substantially lower affinity to native α_2M [21,25], we focused our efforts on α_2M*. To demonstrate specific binding of FGF-2 to α_2M*, we performed *in vitro* binding experiments utilizing ^{125}I-FGF-2 and immobilized α_2M* in microtitre wells. Immobilized α_2M* was incubated with ^{125}I-FGF-2 in either the presence or absence of 1000-fold molar excess of FGF-2. ^{125}I-FGF-2 bound to the immobilized α_2M* and 85 ± 4 % of the binding was inhibited by unlabelled FGF-2 (Figure 1). These data indicate that binding of FGF-2 to α_2M* is specific, confirming our previously published data [25].

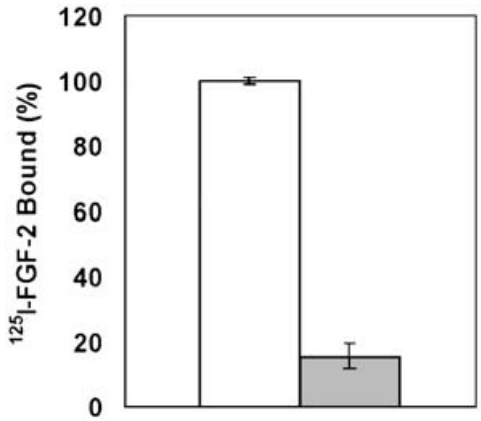

Figure 1 Specific binding of FGF-2 to immobilized α_2M*

Wells coated with 1 μg of α_2M* were incubated with ^{125}I-FGF-2 (6 nM) in the presence or absence of a 1000-fold molar excess of unlabelled FGF-2. Following incubation, the wells were washed and the radioactivity associated with each well was quantified in a gamma counter. Binding of ^{125}I-FGF-2 to α_2M* in the absence of unlabelled FGF-2 was defined as 100 %. Each bar represents the mean ± S.E.M. from six independent experiments.

Figure 2 Effect of pH on the binding of FGF-2 to α_2M*

^{125}I-FGF-2 (35 nM) was incubated with various concentrations of α_2M*, ranging from 6 to 3800 nM (with the highest concentration on the left), for 2 h at 37 °C and pH 6.5, 7.5, 8.5, 9.0 and 10.0, followed by non-denaturing PAGE. A PhosphorImager™ was used to quantify the radioactivity associated with the α_2M* relative to the radioactivity of the ^{125}I-FGF-2 alone, which was defined as 100 %. These PhosphorImager™ scans, which show the radioactivity associated with the α_2M* band as a result of ^{125}I-FGF-2 binding, are representative of three different experiments performed at each pH condition.

Effect of pH on the binding of ^{125}I-FGF-2 to α_2M*

In the present study, we sought to determine whether pH influences the binding of FGF-2 to α_2M*. ^{125}I-FGF-2 was incubated with varying concentrations of α_2M* at pH 6.5, 7.5, 8.5, 9.0 and 10.0 for 24 h at 37 °C. In this pH range, we anticipated titration of histidine residues and some basic residues (arginine and lysine), depending on the local microenvironment. Binding was determined by non-denaturing PAGE and PhosphorImager™ analysis. A representative experiment is shown in Figure 2. The measured equilibrium dissociation constant (K_D) for the binding of ^{125}I-FGF-2 to α_2M* at pH 7.5 was 62 ± 18 nM

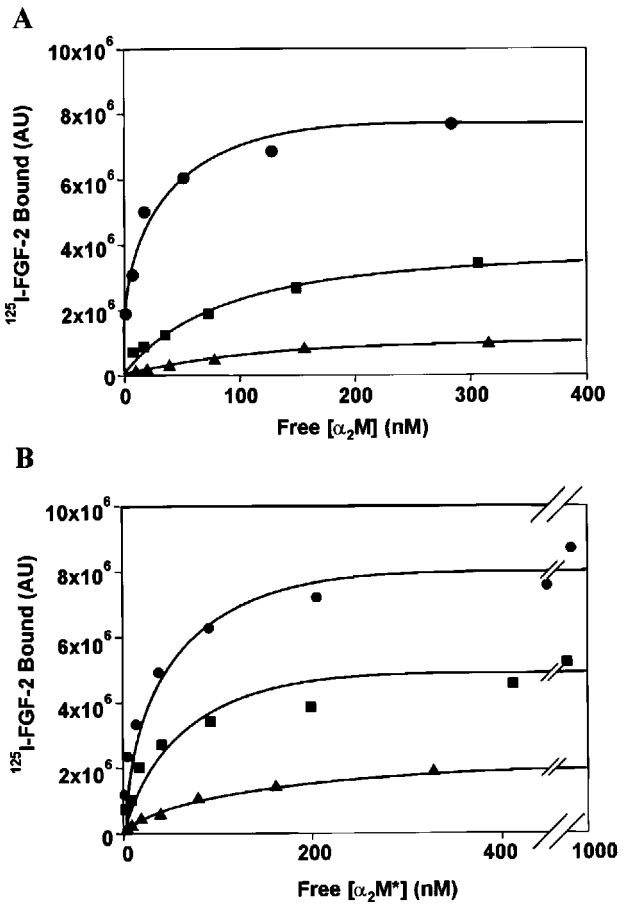

Figure 3 Effect of temperature and ionic strength on the binding of FGF-2 to α_2M*

(**A**) ^{125}I-FGF-2 (35 nM) was incubated with varying concentrations of α_2M*, ranging from 10 to 2600 nM, for 24 h at 4 °C (▲), 22 °C (■) and 37 °C (●), pH 7.5, followed by non-denaturing PAGE. (**B**) ^{125}I-FGF-2 (35 nM) was incubated with varying concentrations of α_2M*, ranging from 4 to 1800 nM, for 24 h at 37 °C in 0 mM (▲), 150 mM (■) or 300 mM (●) NaCl, pH 7.5, followed by non-denaturing PAGE. Radioactivity associated with the α_2M* band was quantified on the PhosphorImager™ and represented by arbitrary units (AU). Binding and lines that represent the best least-square fit ($R^2 > 0.95$) are plotted. The data are representative of at least three independent experiments. Tables 1 and 2 summarize the K_D and B_{max} values derived from these binding experiments.

(mean ± S.E.M., $n = 9$). In the pH range 6.5–10.0 significant variation in the affinity of FGF-2 binding to α_2M* was not observed (results not shown). Some FGF-2–α_2M* complex may have dissociated during electrophoresis; however, this should not have substantially affected the apparent K_D values. These results suggest that the binding of FGF-2 to α_2M* is not mediated by electrostatic interactions involving amino acids that are titrated in the pH range of 6.5–10.0.

Effect of temperature and ionic strength on the binding of ^{125}I-FGF-2 to α_2M*

We next performed experiments to determine the effect of temperature on the binding of FGF-2 to α_2M*. ^{125}I-FGF-2 was incubated with varying concentrations of α_2M* for 24 h at 4, 22 and 37 °C at pH 7.5 and representative binding curves generated from these experiments are shown in Figure 3(A). Scatchard analysis suggested a single major class of binding sites. As shown in Table 1, as the temperature was increased from 4 to 37 °C, the K_D for the binding of FGF-2 to α_2M* decreased more than 5-fold. Increasing temperature was also associated with an increase in the B_{max}. In our system, the B_{max} value reflects the fraction of ^{125}I-FGF-2 that is available to bind α_2M*. The decrease in B_{max} at low temperature suggests that some FGF-2 may have been unavailable to bind α_2M*, probably due to self-association. The more important parameter in this system is the K_D, which reflects temperature-dependent stabilization of the FGF-2–α_2M* complex. It has been proposed that hydrophobic interactions increase in magnitude with increasing temperature, whereas other types of interaction may decrease in strength [37,38]. Thus our results are consistent with a model in which hydrophobic interactions play an important role in the FGF-2–α_2M* interaction.

Table 1 Equilibrium dissociation constants and B_{max} values for ^{125}I-FGF-2 binding to α_2M* at different temperatures

Binding was performed as described in the Experimental section. The binding of ^{125}I-FGF-2 to α_2M* was quantified by measuring the α_2M*-associated radioactivity by PhosphorImager™ analysis. K_D values were determined by direct fit of the PhosphorImager™ counts to a one-site binding model using SigmaPlot™ software. B_{max} values were determined by using Scatchard analysis. Each K_D and B_{max} value represents the mean ± S.E.M. from at least three independent experiments, except for 37 °C, where $n = 9$.

Temperature (°C)	K_D (nM)	B_{max} (nM)
4	430 ± 29	0.024 ± 0.0028
22	202 ± 43	0.19 ± 0.016
37	62 ± 18	1.65 ± 0.195

Table 2 Equilibrium dissociation constants and B_{max} values for ^{125}I-FGF-2 binding to α_2M* at different ionic strengths

Details are as for Table 1. Each K_D and B_{max} value represents the mean ± S.E.M. from at least three independent experiments, except for 150 mM NaCl, where $n = 9$.

NaCl concentration (mM)	K_D (nM)	B_{max} (nM)
0	197 ± 37	0.124 ± 0.013
150	62 ± 18	1.65 ± 0.195
300	34 ± 9	1.87 ± 0.746

To support this hypothesis further, we determined the effects of increasing ionic strength on the binding of FGF-2 to α_2M*. Increasing ionic strength resulting from a salt such as NaCl strengthens hydrophobic interactions by decreasing the solubility of non-polar molecules in a polar solvent. ^{125}I-FGF-2 was incubated with varying concentrations of α_2M* for 24 h at 37 °C in buffer containing 0, 150 or 300 mM NaCl at pH 7.5. Binding curves generated from these experiments conducted at increasing ionic strength were similar to the binding curves obtained for the binding of ^{125}I-FGF-2 to α_2M* at increasing temperatures (Figure 3B). The B_{max} values for binding of α_2M* to FGF-2 increased 15-fold as the salt concentration was increased from 0 to 300 mM NaCl (Table 2). Furthermore, as the NaCl concentration increased from 0 to 300 mM, the K_D value decreased 4-fold, indicating that binding of FGF-2 to α_2M* is of higher affinity at increased ionic strength (Table 2). This further supports our hypothesis that hydrophobic interactions primarily mediate the binding of FGF-2 to α_2M* since increasing ionic strength promotes hydrophobic interactions.

Inhibition of ^{125}I-FGF-2 binding to α_2M* by FP3

FGF-2 competes directly with TGF-β1 for binding to both α_2M and α_2M*, suggesting that the two growth factors may interact

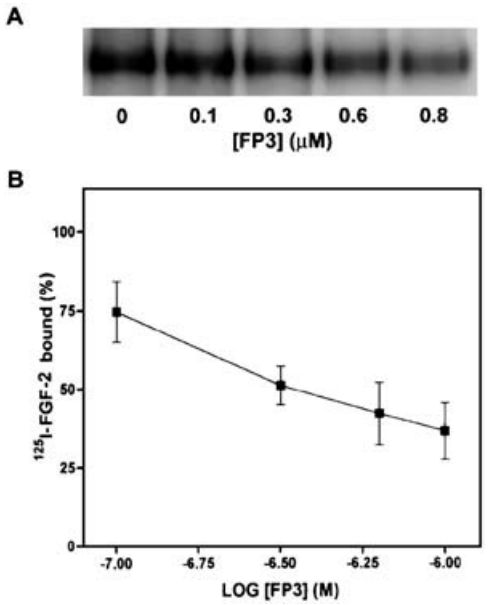

Figure 4 Inhibition of ^{125}I-FGF-2 binding to α_2M* by FP3

^{125}I-FGF-2 (7 nM) was incubated with α_2M* (0.1 μM) alone or with α_2M* (0.1 μM) and FP3 (0.1–0.8 μM) for 1 h at 37 °C followed by non-denaturing PAGE. (**A**) PhosphorImager™ scan showing the radioactivity associated with the α_2M* band as result of ^{125}I-FGF-2 binding in the presence of increasing concentrations of FP3. (**B**) The radioactivity associated with the α_2M* band was quantified by PhosphorImager™. The radioactivity associated with the α_2M* in the absence of FP3 was defined as 100 % to determine the percentage of ^{125}I-FGF-2 that bound to α_2M* in the presence of increasing concentrations of FP3. Each point represents the mean ± S.E.M. from at least three independent experiments.

with a common binding site on α_2M [24,25]. A candidate binding site for TGF-β1 has been localized to amino acids 591–774 of the human α_2M subunit, which are contained within the GST fusion protein FP3 [22]. To determine whether FGF-2 binds to FP3, binding experiments were performed in which ^{125}I-FGF-2 was incubated with α_2M* and increasing concentrations of FP3. In solutions that contained FP3, binding of ^{125}I-FGF-2 to α_2M* was significantly inhibited (Figure 4A). In the presence of 0.8 μM FP3, ^{125}I-FGF-2 binding to α_2M* was inhibited by 64 ± 9 % (Figure 4B). These results demonstrate that FP3 contains a binding site for FGF-2, which may be identical with or overlapping with the TGF-β1 binding site.

The binding of ^{125}I-FGF-2 to the 16-amino-acid peptide derived from human α_2M (P3)

A candidate TGF-β1 binding site on human α_2M has been localized to a 16-amino-acid peptide, P3, corresponding to amino acids 718–733, within FP3 [32]. Having demonstrated that FGF-2 binds directly to FP3, our goal was to determine whether an FGF-2 binding site could be further localized to this 16-amino-acid peptide, P3. In these experiments, ^{125}I-FGF-2 was incubated with P3, which was immobilized in microtitre wells, in the presence or absence of 1000-fold molar excess of unlabelled FGF-2, a 100-fold molar excess of TGF-β1, or a 1000-fold molar excess of FGF-1. Binding of ^{125}I-FGF-2 to P3 was specific, since excess unlabelled FGF-2 inhibited binding by 85 ± 6 % (Figure 5). Furthermore, ^{125}I-FGF-2 binding to P3 was inhibited by 62 ± 9 % as a result of direct competition by TGF-β1 (Figure 5). By contrast, a 1000-fold molar excess of FGF-1 did not inhibit the binding of ^{125}I-FGF-2 to P3 (Figure 5). As a negative control,

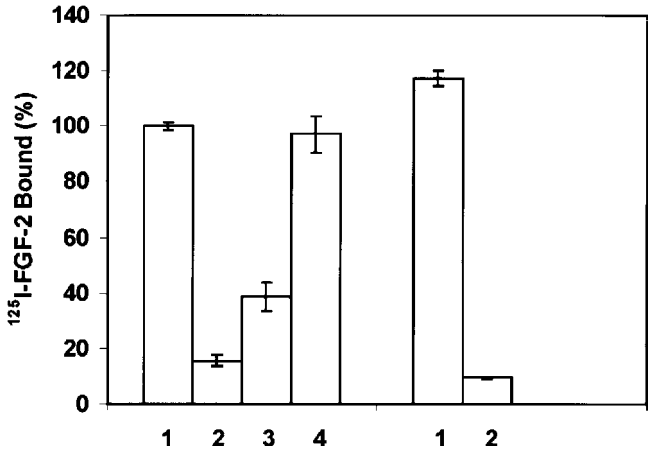

Figure 5 Competition by FGF-2, TGF-β1 and FGF-1 for ^{125}I-FGF-2 binding to P3 and esterified P3

Wells coated with 1 μg of either P3 (left-hand bars 1–4) or esterified P3 (right-hand bars 1 and 2) were incubated with ^{125}I-FGF-2 (6 nM) in the absence (bars 1) or presence of a molar excess of unlabelled growth factors for 24 h at 37 °C (bars 2–4). The unlabelled growth factors were as follows: 1000-fold molar excess of FGF-2 (bars 2), 100-fold molar excess of TGF-β1 (bar 3) and 1000-fold molar excess of FGF-1 (bar 4). Following incubation, the wells were washed and the radioactivity associated with each well was quantified in a gamma counter. Binding of ^{125}I-FGF-2 to P3 in the absence of unlabelled growth factor was defined as 100 %. Each bar represents the mean ± S.E.M. from at least three independent experiments, except for binding to P3 done in the absence of growth factors and done in the presence of excess FGF-2, where $n = 10$.

P3.5 was immobilized in microtitre wells and incubated with ^{125}I-FGF-2. Significant binding of ^{125}I-FGF-2 to P3.5 was not observed (results not shown).

P3 is remarkable for its high content of hydrophobic amino acids and its acidic residues [32]. To determine whether the acidic residues in P3 are necessary for binding to FGF-2, P3 was esterified utilizing the methanol/HCl procedure. A homogeneous sample of the reversed-phase HPLC-purified esterified P3 was immobilized in microtitre plates and incubated with ^{125}I-FGF-2 in either the presence or absence of a 1000-fold molar excess of FGF-2. ^{125}I-FGF-2 bound to the esterified P3, and 91 ± 1 % of the binding was inhibited by a 1000-fold molar excess of FGF-2, demonstrating the specific nature of this interaction (Figure 5). Our results suggest that the acidic residues present in the highly hydrophobic 16-amino-acid peptide are not essential for binding to FGF-2.

DISCUSSION

In the present study we characterized the binding of FGF-2 to α_2M* and localized a candidate FGF-2-binding site in human α_2M*. *In vitro* binding experiments performed with α_2M* and ^{125}I-FGF-2 in the presence of excess unlabelled FGF-2 demonstrated that the binding of FGF-2 to α_2M* is specific. Next, we varied parameters such as pH, temperature and ionic strength. The range of pH from 6.5 to 10.0 did not affect the binding of FGF-2 to α_2M*, nor did it affect the K_D for the binding of FGF-2 to α_2M*. FGF-2 has a pI of 9.6, whereas α_2M* has a pI of 5.3 [4,39]. Varying the pH alters the ionization states of amino acid side chains, which in turn changes protein charge distributions. Since the range of pH from 6.5 to 10.0 had no effect on the binding affinity of FGF-2 for α_2M*, it seemed likely that the binding of FGF-2 to α_2M* is not mediated by electrostatic interactions from amino acids that are titrated in the wide pH range we tested.

We next considered the role of hydrophobic interactions. Temperature is known to have a direct effect on hydrophobic interactions [37]. Water molecules organize into highly ordered hydrogen-bonded arrays around non-polar surfaces at lower temperatures. The ability of water to organize into ordered arrays and solvate hydrophobic surfaces diminishes as the temperature increases, which in turn promotes the interaction between two hydrophobic surfaces [37,40]. When we varied the temperature at which the *in vitro* binding studies were performed, we observed that, in contrast with the pH studies, temperature did have a significant effect on the binding of FGF-2 to α_2M^*. Increasing the temperature significantly increased the amount of FGF-2 that bound to α_2M^* and decreased the K_D values for the binding of FGF-2 to α_2M^*. This indicates that the binding of FGF-2 to α_2M^* is of higher affinity at higher temperatures which is consistent with the observed temperature-sensitive nature of hydrophobic interactions.

To support further the hypothesis that FGF-2 interacts with α_2M^* via a mechanism that is primarily hydrophobic in nature, we varied the ionic strength during the *in vitro* binding experiments. An increase in ionic strength with a salt such as NaCl strengthens hydrophobic interactions by making it more unfavourable to immobilize water on the hydrophobic surface. Increasing the ionic strength caused an increase in the amount of FGF-2 that bound to α_2M^*. As reflected by the K_D values derived for the binding of FGF-2 to α_2M^*, the binding affinity of FGF-2 for α_2M^* increased significantly in the presence of salt. Taken together, the results of the pH, temperature and salt studies suggest that the binding of FGF-2 to α_2M^* is mediated primarily by hydrophobic rather than electrostatic interactions.

We previously reported that TGF-β1 competes for the binding of FGF-2 to both α_2M and α_2M^*, suggesting that FGF-2 and TGF-β1 bind to either identical or overlapping regions on α_2M [25]. Out of the six GST fusion proteins that collectively include amino acids 99–1451 of human α_2M, it has been demonstrated that TGF-β1 binds specifically to FP3, which consists of amino acids 591–774 [22]. In this study, we demonstrated that FP3 also binds FGF-2. Furthermore, we demonstrated that the 16-amino-acid peptide, P3, which corresponds to a sequence in FP3, also binds FGF-2, which is remarkable because P3 binds TGF-β1 and PDGF-BB [32]. The P3 sequence that binds FGF-2, TGF-β1 and PDGF-BB lies just C-terminal to the bait region, separated only by 12 residues [41,42]. Models that have been developed for the three-dimensional structure of α_2M through data obtained from electron microscopy [43], NMR [44], low-resolution X-ray crystallography [45] and fluorescence resonance energy transfer studies [46] have proposed that the bait region is located within the central cavity of the intact α_2M tetramer. In fact, TGF-β-specific antibodies fail to recognize intact α_2M-associated TGF-β [47,48]. It has also been demonstrated that α_2M^*–proteinase complexes, in which 2 mol of proteinase are bound per mol of α_2M, do not bind TGF-β [49]. These data support the hypothesis that TGF-β is binding inside the central cavity of α_2M where the P3 sequence is located. It is therefore possible that, like TGF-β, FGF-2 binds inside the central α_2M cavity.

In competition experiments in which P3 was incubated with ^{125}I-FGF-2 in the presence or absence of excess growth factors, FGF-1 did not compete with FGF-2 for binding to P3. Among the 23 members that currently make up the FGF family of proteins, FGF-1 shares the highest degree of sequence homology with FGF-2 [50]. We have shown that FGF-1 does bind to both α_2M and α_2M^* with measurable affinity, although FGF-1 binds to α_2M and α_2M^* with lower affinity than does FGF-2 [25]. Considering the fact that FGF-1 and FGF-2 are two closely related proteins that exhibit a similar range of biological activities, we initially expected that the two proteins would compete with each other for binding to α_2M. Despite their high degree of sequence homology, FGF-1 and FGF-2 do differ in some of their physical and chemical properties, such as pI values, and perhaps these differences affect their interaction with human α_2M.

P3 includes a high proportion of hydrophobic amino acids. In fact, 10 out of the 16 amino acid residues are hydrophobic, and, of the remaining six amino acid residues, two are negatively charged, two are hydrophilic and two are neutral. In light of our results suggesting that the binding of FGF-2 to α_2M^* is mediated primarily by hydrophobic interactions, we executed a series of experiments to determine the characteristics of P3 that are critical for FGF-2 binding.

The negative charges of the D and E residues were neutralized by an esterification reaction to determine the importance of these charged residues for binding to ^{125}I-FGF-2. Esterification of P3 did not inhibit the ability of ^{125}I-FGF-2 to bind to the peptide. Binding of ^{125}I-FGF-2 to the esterified P3 was specific. This demonstrates that the two negatively charged residues in the peptide sequence are not essential for the binding to FGF-2. Instead, the hydrophobic amino acids play a more critical role.

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