α2-Macroglobulin Is a Binding Protein for Basic Fibroblast Growth Factor*

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After incubation with human serum or plasma, 125I-bFGF (basic fibroblast growth factor; molecular mass 18.5 kDa) exhibits molecular mass forms greater than 200 kDa as determined by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiography. These high molecular mass forms of bFGF are immunoprecipitable with antisera raised against α2-macroglobulin (α2M). Purified α2M and 125I-bFGF form a covalent complex in a specific, saturable manner. Excess unlabeled bFGF competes with 125I-bFGF for complex formation. Complex formation is complete after 4 h and is inhibited by pretreating α2M with dithiothreitol, iodoacetamide, iodoacetic acid, and N-ethylmaleimide. The complex is resistant to acidic conditions and desaturants such as urea. Heparin, which binds bFGF, has no effect on bFGF's interactions with other molecules is scarce. Therefore, the interaction of availability is extremely interesting. Although the existence of extracellular bFGF has been claimed in vitro (10), the mechanism of release of bFGF from cells remains unclear. bFGF appears to lack a classical signal sequence (11), and most cells that produce bFGF release only miniscule amounts (12, 13). Furthermore, with the exception of the in vitro interactions between bFGF and heparin, heparan sulfate proteoglycans, and its cellular receptor, information regarding bFGF's interactions with other molecules is sparse. Therefore, we have sought to understand the extracellular environment encountered by bFGF more fully by identifying other proteins that interact with bFGF.

While binding proteins for growth factors have been isolated from tissues (e.g. epidermal growth factor (14) and nerve growth factor (15) binding proteins from the mouse submaxillary gland), several growth factor binding proteins have been isolated from serum or plasma. Insulin-like growth factors (16-18), insulin (19), epidermal growth factor (20), nerve growth factor (21), PDGF (22, 23), and TGF-β (24, 25), all interact with at least one serum- or plasma-derived protein. In this paper, we describe the binding of bFGF to a serum protein which we have identified as α2M. We also describe the effect of α2M on the biological activities of bFGF.

EXPERIMENTAL PROCEDURES

Materials

Na125I (17 Ci/mg) was purchased from Du Pont-New England Nuclear. Urea (U-15) was purchased from Fisher. Protein A-Sepharose, phosphorylase b (cross-linked), bacitracin, methylamine HCl, iodoacetamide, iodoacetic acid, dithiothreitol, plasmin (2 units/mg), aprotinin, and N-ethylmaleimide were obtained from Sigma. Rabbit anti-human α2M antisera were purchased from Calbiochem. Lyophilized trypsin (192 units/mg) was purchased from Worthington. Gelatin was purchased from Kodak. Preincubated high molecular weight markers were obtained from Bethesda Research Laboratories. DSS and IODO-GEN were bought from Pierce Chemical Co. Sepharose and Sephadex G-200 were purchased from Pharmacia LKB Biotechnology Inc. a-MEM and DMEM were obtained from Flow Laboratories, McLean, VA. Human PDGF was purchased from Col...
laboratory Research, Inc., Bedford, MA. Bovine brain aFGF and porcine platelet TGF-β were obtained from R & D Systems, Minneapolis, MN. Recombinant bFGF was a generous gift from Synergen, Inc., Boulder, CO. Outdated human plasma and serum were obtained from the New York University Medical Center Transfusion Service.

**Methods**

**Cell Culture**—BCE cells were isolated from adrenal cortices of recently slaughtered veal cattle by the method of Folkman et al. (26). Initial isolates were grown in α-MEM containing 10% (v/v) calf serum and supplemented with medium conditioned by mouse sarcoma 180 cells as described (27). Once established, the cultures were grown in α-MEM containing 5% (v/v) calf serum and no conditioning factor. BCE cells were grown in DMEM with 10% (v/v) calf serum.

**Iodinations**—bFGF was iodinated by the method of Moscatelli (28). Specific activity (expressed as counts/min/fmol) was calculated as described (28). No loss in biological activity of bFGF after iodination was observed as determined by the ability of bFGF to induce PA production in BCE cells (27).

**Purification of a2M**—a2M was isolated as described previously (29, 30). Nondenaturing gel electrophoresis of trypsin-treated and untreated purified a2M revealed that approximately 50% was in the electrophoretically "fast" form.

**Formation of 125I-bFGF-a2M Complexes**—Complex formation was carried out in a reaction mixture containing 10 μg of purified a2M or 20 μg of human serum or plasma, 0.15% gelatin, and 10 ng of 125I-bFGF (specific activity no less than 400 cpm/fmol) in PBS at 37°C for 4 h unless otherwise noted. Nonspecific binding was determined by including controls that contained a 500-fold molar excess of unlabeled bFGF. Other effectors of complex formation such as other growth factors and reductants were added at the initiation of the reaction. Complex formation was quantitated by SDS-PAGE (31), using a 3-16% gradient of acrylamide and nonreducing conditions, followed by autoradiography. Radioactive bands of interest were either excised and counted in a γ scintillation counter (Packard model 5210) or scanned by densitometry and the peaks cut out and exposed for autoradiography. In addition, faint bands were seen at positions corresponding to 55 and 44 kDa. These may represent aggregates of bFGF, since they were not seen with all bFGF preparations and were not investigated further. Since a2M has been shown to bind several growth factors (21-25) and has a molecular mass greater than 200 kDa, the ability of antibodies against a2M to immunoprecipitate the high molecular mass forms of 125I-bFGF was tested. All of the high molecular weight radioactive bands were immunoprecipitated with anti-a2M antibodies coupled to protein A-Sepharose (Fig. 1, lanes 4-6), whereas they were not immunoprecipitated with nonimmune γ-globulin coupled to protein A-Sepharose.

**RESULTS**

**Identification of a2M as a bFGF Binding Protein**—In initial experiments, 20 μl of human serum or plasma was incubated with 125I-bFGF for 4 h at 37°C. After nonreducing SDS-PAGE followed by autoradiography, three high molecular mass forms of 125I-bFGF (greater than 200 kDa) were detected (Fig. 1, lanes 1-3). In addition, faint bands were seen at positions corresponding to 55 and 44 kDa. These may represent aggregates of bFGF, since they were not seen with all bFGF preparations and were not investigated further. Since a2M has been shown to bind several growth factors (21-25) and has a molecular mass greater than 200 kDa, the ability of antibodies against a2M to immunoprecipitate the high molecular mass forms of 125I-bFGF was tested. All of the high molecular weight radioactive bands were immunoprecipitated with anti-a2M antibodies coupled to protein A-Sepharose (Fig. 1, lanes 4-6), whereas they were not immunoprecipitated with nonimmune γ-globulin coupled to protein A-Sepharose.

![Figure 1](image-url)

**FIG. 1. Identification of a2M as a bFGF binding protein.** 20 μl of outdated human serum (lanes 1, 4, and 7) or human plasma (lanes 2 and 5; patient 1; lanes 3 and 6; patient 2) was incubated with 125I-bFGF for 4 h at 37°C. In lanes 1-3, the reaction was stopped with 4X nonreducing Laemmli buffer. In lanes 4-6, identical samples to those in lanes 1-3 were immunoprecipitated after 4 h with anti-a2M antibodies coupled to protein A-Sepharose. In lane 7, 20 μl of serum was carefully removed, and aliquots of the γ-globulin coupled to protein A-Sepharose. After pelleting and washing the protein A-Sepharose beads, 4X nonreducing Laemmli buffer was added. Samples were loaded onto a 3-16% SDS gel, and the gel was dried and exposed for autoradiography.
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The small amount of free bFGF immunoprecipitated was probably the result of nonspecific binding of bFGF to protein A-Sepharose. To investigate the interaction between bFGF and α2M further, purified α2M was utilized in all subsequent experiments.

Characterization of the bFGF-α2M Bond—As shown in Fig. 2, the binding between purified α2M and 125I-bFGF is saturable with respect to time. The point of saturation appears to be reached at 4 h. In general, 20–60% of the added 125I-bFGF bound to α2M, depending on the age of the 125I-bFGF. Furthermore, the binding was specific, as unlabeled bFGF routinely competed for 80–90% of the binding with 125I-bFGF (data not shown).

The next set of experiments was designed to determine the stability of the α2M-bFGF complex. Fig. 3 shows that when the bifunctional cross-linking reagent DSS is added to the incubation mixture (lane 1), an increased amount of 125I-bFGF (relative to the total radioactivity) is found bound to α2M, and the migration of the radioactive bands is altered. The increase in radioactivity associated with the cross-linked complex (43.2% versus 18.7% (control, lane 2) of the total radioactivity) may indicate that some of the bFGF bound to α2M is sensitive to SDS-PAGE. However, attempts to visualize bFGF-α2M complexes on non-SDS polyacrylamide gels were unsuccessful. The change in migration of the predominant high molecular mass band, from M, 580,000 in the control sample to greater than M, 584,000 in the cross-linked sample, seems to indicate that the disulfide-linked subunits of α2M (M, 185,000 each) may be cross-linked to retain the native molecular weight of the α2M molecule (M, 725,000). Other combinations, such as dimerization of the intact α2M, may account for the radioactive bands that barely enter the gel.

Because acid treatment increases the yield of placental bFGF during purification (32), studies were performed to determine whether the increased yield is due to the release of bFGF from bFGF-α2M complexes. The ability of 1 M acetic acid and 8 M urea to dissociate the complex was investigated. Fig. 4, lanes 2 and 3, show that 1 M acetic acid and 8 M urea, when incubated with the complex for 0.5 h, did not dissociate the complex (lane 1, control). Next, the effect of reduction on dissociating the complex was studied. Fig. 4, lanes 6 and 7, demonstrate that the migration of the reduced sample (lane 7) was altered when compared to the nonreduced sample (lane 6). The faint band detected at M, 210,000 in lane 7 indicates that 125I-bFGF binds to the 185-kD subunit chain of α2M, since this subunit arises when α2M is denatured and reduced. When the radioactive bands of each lane were excised and counted in a γ counter, it was evident that 51% of the radioactivity in the control sample was removed by reduction. Thus, 49% of the 125I-bFGF appears to be bound to the α2M in a non-sulfhydryl-mediated covalent manner, whereas 51% is bound by a bond sensitive to reduction.

Inhibitors of Complex Formation—Methylamine, an agent that blocks protease binding to α2M, and iodoacetamide, an agent that blocks free sulfhydryl groups, were tested for their ability to inhibit complex formation. When 20 mM methylamine was added to the incubation mixture (Fig. 4, lane 4), there was no inhibition of complex formation as compared to a control sample (lane 1). In contrast, when 5 mM iodoacetamide was added, complex formation was inhibited (lane 5). Complex formation was also inhibited by a reductant, dithiothreitol (100 mM) (data not shown). Heparin, which has high affinity for bFGF, did not inhibit complex formation (data not shown). Together, these data demonstrate the importance of free sulfhydryl groups in the formation of the complex.

A similar inhibition of complex formation was observed if the α2M was pretreated for 1 h at 37 °C with reducing agents (5 mM iodoacetamide, 5 mM iodoacetic acid, and 5 mM N-
FIG. 4. Inhibition and dissociation of α2M-125I-bFGF complex. After complex formation, acetic acid (final concentration 1 M) (lane 2), urea (final concentration 8 M) (lane 3), or incubation buffer (lane 1) was added for 0.5 h. The samples were analyzed by nonreducing SDS-PAGE followed by autoradiography. Lanes 4 and 5 reveal that 20 mM methylamine had no effect on complex formation (lane 4), whereas 5 mM iodoacetamide (lane 5) inhibited complex formation. Lanes 6 and 7 (from a different SDS gel that had a larger separating gel) show the effect of denaturation followed by reduction on the dissociability of the α2M-125I-bFGF complex. In lane 6 (control), complex formation is carried out as above. In lane 7, the complex is denatured with urea (final concentration 8 M) as described, followed by reduction with Laemmli buffer containing β-mercaptoethanol and boiling.

ethylmaleimide) (data not shown). Pretreatment with 200 mM methanolamine, however, increased the amount of complex formation 2-fold.

The fact that the intensity of the radioactive bands associated with the α2M-bFGF complex was greater when the α2M was pretreated with methanolamine indicated that bFGF may have a preference for binding to the electrophoretically fast form of α2M. This form is induced by the cleavage of thioester bonds in α2M upon binding of methanolamine. By comparing the migration of fast α2M (induced by trypsin) and the 125I-bFGF-α2M complex on nondenaturing gel electrophoresis, we were able to show that the α2M associated with the 125I-bFGF-α2M complex was only in the fast form (data not shown). Because a similar conformational change is induced in α2M when it reacts with proteases, purified α2M (50% already in the fast form) was pretreated with plasmin or trypsin and the ability of the protease-α2M complex to bind 125I-bFGF was assessed. Fig. 5A shows that complex formation was not inhibited by plasmin pretreatment of α2M. Incubation of α2M for 1 h at 37 °C (lane 1) and pretreatment of α2M with a 10-fold molar excess of aprotinin (lane 3) had little or no effect on complex formation. Pretreating α2M with plasmin (3:1 plasmin:α2M molar ratio) for 1 h at 37 °C, followed by aprotinin (10:1 aprotinin:α2M molar ratio) also had no effect on complex formation (lane 2). These results were confirmed by isolating the α2M-plasmin complex by chromatography on a Sephadex G-200 column. Fractions containing the plasmin-α2M complex were concentrated, and aliquots were added to 125I-bFGF to allow complex formation. The samples were analyzed as before (data not shown). Fig. 5B illustrates that if α2M was incubated for 1 h at 37 °C (lane 1) or pretreated with aprotinin (lane 3) for 1 h at 37 °C, complex formation was not affected. Trypsin pretreatment of α2M followed by aprotinin resulted in some inhibition of 125I-bFGF binding (lane 2). Whether this inhibition is related to its ability to compete for bFGF binding or its proteolytic properties is unclear. Although the bFGF binding behavior of α2M is affected differently by plasmin and trypsin, these data imply that different binding sites for proteases and bFGF exist on α2M. In addition, the differences in quantity of bFGF bound to the fast forms of α2M induced by methylamine, plasmin, or trypsin indicate that these fast forms of α2M may differ in their ability to bind bFGF.

Competition with Other Growth Factors for Complex Formation—Because other growth factors bind to α2M (21–25), the efficiency with which other growth factors compete with 125I-bFGF for binding to α2M was assessed. 500-fold molar excesses of either bFGF, aFGF, PDGF, or TGF-β were added to incubation mixtures containing 125I-bFGF. The control sample lacked any competitor. The data illustrated in Fig. 6 demonstrate the varying degrees of competition exhibited by bFGF, aFGF, PDGF, and TGF-β. Competition for binding of 125I-bFGF to α2M was significant with bFGF, aFGF, and TGF-β, which may imply a common binding site for these ligands on α2M. This was not unexpected for aFGF, since bFGF and...
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TGF-β

FIG. 6. Competition with other growth factors for binding of 125I-bFGF to α2M. Each sample of 10 ng 125I-bFGF, 10 μg α2M, and a 500-fold molar excess of the indicated growth factor. (The control sample had no competitor added.) The reactions were carried out for 4 h and stopped with 4 × Laemmli buffer. Nonreducing SDS-PAGE was followed by autoradiography. The bands corresponding to the 125I-bFGF-α2M complex were scanned by densitometry and weighed, and the weight was expressed as a percentage of the weight of the 125I-bFGF-α2M complex in the control sample.

α2FGF are highly homologous. The fact that α2FGF did not compete as well as bFGF was probably due to the fact that the commercial preparation of α2FGF was impure. PDGF did not compete for binding. This was not due to the inability of the PDGF to bind α2M, since iodinated PDGF bound to α2M (data not shown). Consistent with this data is the demonstration by Huang et al. (23) that fibroblast growth factors did not compete for binding of 125I-PDGF to α2M.

Stimulation of PA Activity in BCE Cells—In order to compare the biological abilities of the bound and free forms of bFGF, 125I-bFGF-α2M complexes were isolated using heparin-Sepharose chromatography (see “Methods”). Equal amounts of radioactivity of each form (the complexed 125I-bFGF and the free 125I-bFGF) were added to BCE cells, and PA levels were measured 18 h later. Fig. 7 shows that free bFGF, at a concentration of 7 ng/ml, stimulated PA activity 5-fold over control cultures. bFGF complexed to α2M stimulated PA activity 0.5-fold over control cultures at this concentration. At higher concentrations, an increased stimulation was observed. However, at the highest concentration of 125I-bFGF-α2M complex tested (18 ng/ml), only a 2-fold stimulation was observed. This may reflect the contribution of small amounts of contaminating free bFGF that may either renature during the course of the assay or may not be covalently bound to α2M.

Binding of 125I-bFGF-α2M to BHK-21 Cells—Once it had been established that the complexed bFGF had very little PA stimulatory activity, studies were performed to determine whether bFGF bound to α2M could recognize bFGF binding sites on BHK-21 cells. A binding study was conducted as described by Moscatelli (28). 125I-bFGF-α2M complexes were isolated. Equal amounts of radioactivity of free and complexed 125I-bFGF (equivalent to 20 ng/ml) were added to the cells for 2 h at 4 °C. Samples with a 500-fold excess unlabeled bFGF were included to account for nonspecific binding. After 2 h, the incubation medium was removed, and the cells were washed three times with PBS. A 2 M NaCl rinse was performed to remove any radioactivity that was bound to the low affinity sites. As seen in Fig. 8, the complexed 125I-bFGF was unable to bind (only 2.0% of that observed for free bFGF) to the low affinity sites thought to be heparan sulfate proteoglycans. This is consistent with earlier data demonstrating that the complex did not bind to heparin-Sepharose (see “Methods”). High affinity binding (lane 3, free 125I-bFGF; lane 4, complexed 125I-bFGF) reflects the radioactivity that remained cell associated after the 2 M NaCl wash.
binding sites for proteases and bFGF. This implies bFGF even though proteases may be bound covalently or noncovalently. a2M binds bFGF in at least three ways, similar to the concentration-dependent binding of trypsin to a2M (34).

DISCUSSION

By demonstrating that bFGF will bind to a serum and plasma protein that can be immunoprecipitated with antiserum against a2M and by demonstrating that bFGF can bind to purified a2M in vitro, we have shown that a2M is a binding protein for bFGF. When we first identified a2M as a binding protein for bFGF (Fig. 1), we found three a2M-associated 125I-bFGF species that differed in molecular mass by as much as 300 or 400 kDa. Since SDS causes a2M to dissociate into disulfide-linked monomers, the molecular mass differences seen between these species may represent incomplete denaturation of native or polymerized a2M by SDS.

Our biochemical studies indicate that covalent bond formation occurs slowly between bFGF and a2M, over the course of 4 h, and is dependent on the availability of sulfhydryl groups on a2M. This is supported by the inhibitory effect of reagents that react with sulfhydryl groups (iodoacetamide, etc.) and the potentiating effect of methylene, which reacts with a2M and results in the appearance of free sulfhydryl groups (33). However, not all of the bFGF is bound to a2M through disulfide bonds, since only 51% of the radioactivity associated with the complex was removed by denaturation followed by reduction. The fact that free sulfhydryl groups are necessary for bond formation yet only account for 51% of the bFGF bound to a2M is consistent with a sequential means of binding where disulfide bonds are formed first, followed by a redundant covalent linkage. However, the time course of bond formation does not seem biphasic (Fig. 2). Interestingly, the redundant-resistant linkage between bFGF and a2M is reminiscent of the manner in which proteases covalently bind to a2M.

Although we have described two means of bFGF binding to a2M (redundant-resistant and redundant-sensitive covalent bonding), bFGF may also bind to a2M in a noncovalent manner. We were unable to use other nondenaturing means to quantitate complex formation, as 125I-bFGF precipitated when gel filtration was performed and migrated anomalously under conditions of nondenaturating gel electrophoresis. However, we were able to show an increase in complex formation using the assay system of SDS-PAGE/autoradiography when DSS was added to the incubation mixture (Fig. 3). Furthermore, when DSS was added to isolated 125I-bFGF, a2M complexes, virtually all of the 125I-bFGF that migrated originally at M, 18,000 was found associated with a2M (data not shown). In both experiments, since there was no evidence of nonspecific cross-linking (e.g. formation of multimers of 125I-bFGF or 125I-bFGF-gelatin complexes), we believe that some of the M, 18,000 125I-bFGF visualized before complex isolation and almost all of the M, 18,000 125I-bFGF visualized after complex isolation is associated with a2M in a noncovalent manner that is sensitive to SDS-PAGE. Thus, a2M may bind bFGF in at least three ways, similar to the concentration-dependent binding of trypsin to a2M (34).

Since a2M is known to exist in at least two forms, a native form and an electrophoretically fast form which represents a sprung "trap" when a protease or metalloenzyme binds (35), we assessed the ability of protease-treated a2M to bind bFGF and found that bFGF could bind to either plasmin- or trypsin-a2M complexes (Fig. 5). Thus a2M is likely to have different binding sites for proteases and bFGF. This implies bFGF must have access to free sulfhydryl groups within the trap even though proteases may be bound covalently or noncovalently. a2M may also have different binding sites for specific growth factors. Of the growth factors tested, only bFGF, aFGF, and TGF-β competed for binding of 125I-bFGF to a2M. The competition exhibited by TGF-β was unexpected, since TGF-β binds to a2M in a reversible manner. The lack of competition by PDGF was expected (22). The fact that sites may exist for specific growth factors on a2M may help define the biological relevance of each growth factor's binding to a2M.

Finally, can our data be related to the extracellular environment encountered by bFGF? From the irreversible nature of the bond between bFGF and a2M and the diminished ability of a2M-bound bFGF to recognize either high or low affinity bFGF binding sites, a2M does not appear to act as a carrier protein for bFGF. If this reaction were physiologically relevant, a2M may serve to inactivate and/or clear bFGF that might be released into the circulation. This is supported by the selectivity of 125I-bFGF for the fast form of a2M, which is rapidly cleared from circulation (36). Were bFGF to bind to protease-a2M complexes in vivo, it would be expected that the a2M-protease-bFGF complex would be cleared quickly from the circulation through binding and internalization through the a2M receptor. Furthermore, if bFGF is susceptible to proteolytic cleavage when bound to protease-a2M complexes, binding and internalization through the a2M receptor may not be necessary to limit the activity of bFGF in vivo. In this scenario, a2M may simply serve as an intermediary between bFGF and an inactivating protease. In either case, the role for a2M as a limiter of growth factor activity is not new, as it has been proposed for PDGF and TGF-β (23-25). Our evidence with bFGF underscores the need to consider a2M not only as a ubiquitous proteinase inhibitor but also as a protein that may inactivate the distant effects of potent growth factors released in vivo.

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REFERENCES

