α₂-Macroglobulin: a New Component in the Insulin-like Growth Factor/Insulin-like Growth Factor Binding Protein-1 Axis*

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Insulin-like growth factors (IGFs) are crucial for many aspects of development, growth, and metabolism yet control of their activity by IGF-binding proteins (IGFBPs) remains controversial. The effect of IGFBP-1 depends on its phosphorylation status; phosphorylated IGFBP-1 inhibits IGF actions whereas the nonphosphorylated isoform is stimulatory. In order to understand this phenomenon, we purified phosphorylated IGFBP-1 from normal human plasma by immunoaffinity chromatography. Unexpectedly, the resulting preparation enhanced IGF-stimulated 3T3-L1 fibroblast proliferation, due to the presence of a co-purified protein of ~700 kDa. Matrix-assisted laser desorption ionization-mass spectrometry and Western immunoblotting analysis identified this co-purified protein as α₂-macroglobulin (α₂M). Anti-α₂M antibodies co-immunoprecipitated IGFBP-1 from human plasma and from 125I-IGFBP-1/α₂M complexes formed in vitro. The 125I-IGFBP-1/α₂M association could be inhibited with excess unlabeled IGFBP-1. Surface plasmon resonance analysis indicated that α₂M preferentially associates with the phosphorylated isoform of IGFBP-1 and that when complexed to α₂M, IGFBP-1 can still bind IGF-I. These findings have functional significance since α₂M protects IGFBP-1 from proteolysis and abrogates the inhibitory effect of phosphorylated IGFBP-1 on IGF-I stimulation of 3T3-L1 cell proliferation. We conclude that α₂M is a binding protein of IGFBP-1 which modifies IGF/IGFBP-1 actions resulting in enhanced IGF effects. In line with its role in regulating the clearance and activity of other growth factors, we predict that α₂M has a novel and important role in controlling the transport and biological activity of IGFs.

The insulin-like growth factors (IGFs) have a key role in the metabolism, development, growth, and maintenance of many tissues and organs (1). IGF bioavailability is controlled by a number of binding proteins (IGFBPs) and one of these, IGFBP-1, is capable of either inhibiting (2–4) or potentiating (5–9) IGF activity at the cellular level. IGFBP-1 inhibits IGF actions by competing with the type 1 IGF receptor for IGF binding, however, the mechanism by which IGFBP-1 enhances IGF activity is less certain. Early work to address this phenomenon resulted in the isolation of two IGFBP-1 isoforms from amniotic fluid, which had similar physicochemical properties but markedly different effects on IGF activity (5). IGFBP-1 association with the cell surface was suggested as an explanation of these findings since only the stimulatory isoform was found to bind to cell membranes. It is now known that IGFBP-1 binds to α₂M via its RGD site and disruption of this interaction leads to inhibition of the subsequent cellular response (6). Polymerization of IGFBP-1 was also postulated as a mechanism for enhancing IGF action (7) and this has been confirmed recently (8).

Many studies have also focused on the influence of phosphorylation in relation to IGFBP-1 effects on IGF activity. The inhibitory isoform purified by Busby et al. (5) was subsequently shown to be phosphorylated (9) whereas the stimulatory preparation contained nonphosphorylated IGFBP-1. Highly phosphorylated IGFBP-1, which is the only form found in plasma (10) has a high affinity for IGF-I (9, 11) and can therefore inhibit IGF-I actions by sequestering it from cell surface receptors. Nonphosphorylated IGFBP-1, which has a relatively low affinity for IGF (9, 11) is thought to allow more IGF/IGF receptor interactions and this hypothesis has been supported by numerous in vitro studies (9, 12, 14). However, it is unclear whether in vivo alteration of IGFBP-1 phosphorylation status represents an important mechanism for regulating IGF bioavailability in the non-pregnant adult, since non- and lesser phosphorylated isoforms of IGFBP-1 are only present at high concentrations during pregnancy (10, 15).

In the light of the above findings, we were surprised to observe that phosphorylated IGFBP-1 purified from plasma could enhance IGF-I stimulated cell proliferation. Further biochemical analysis led to the discovery that IGFBP-1 in plasma is associated with the homotetrameric glycoprotein α₂-macroglobulin (α₂M). This paper describes our characterization of the α₂M/IGFBP-1 association and its functional impact on the IGF axis.

MATERIALS AND METHODS

Purification of IGFBP-1 from Plasma—Immunoaffinity chromatography was used to isolate IGFBP-1 from normal human plasma. Monoclonal antibody 6303 (a kind gift of Medix Biochemica, Kauniainen, Finland) was coupled to Sephacryl S-300 (16) at 1 mg/ml to form the immunoaffinity matrix. A 10-ml column was equilibrated for 24 h at

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‡ The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; α₂M, α₂-macroglobulin; PBS, phosphate-buffered saline; MALDI, matrix-assisted laser desorption ionization; PAGE, polyacrylamide gel electrophoresis; LRP, low-density lipoprotein receptor related protein.

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Cross-linked affinity labeled complexes were incubated overnight at 4 °C by the application of PBS, 0.25% bovine serum albumin, 0.1% Tween 20 at a flow rate of 5 ml/h.

250 ml of plasma was recirculated through the column for 72 h at a flow rate of 3.75 ml/h, the column was washed with 100 ml of Tris buffer, pH 8.0 (50 mM Tris, 0.5 mM NaCl, 0.1% Tween 20), and then the bound peptides from the application of hydrogen peroxide (10 × 1-ml fractions were collected into tubes containing 200 μl of 1× Tris, pH 9.0, and analyzed for IGFBP-1 by radioimmunoassay (10). Fractions containing >100 ng/μl IGFBP-1 were pooled and concentrated by centrifugation through Centricon 10 filters (Amicon, Stonehouse, Gloucestershire, UK).

Biochemical Characterization of IGFBP-1 Phosphorylation Status—IGFBP-1 phosphorylation status was determined using our previously described method of immunoprecipitation followed by n-octylglucoside electrophoresis and Western ligand blotting (10). Samples were incubated with anti-IGFBP-1 (6303) antibody at 4 °C overnight and then anti-mouse IgG antibody (Sac Cel; IDS, Tyne & Wear, United Kingdom) was added for 1 h at room temperature. Bound antibody was separated by centrifugation for 10 min and the precipitated proteins were washed once with 1× PBS and added to 35 μl of 200 mM CaCl₂ for 16 h at 37 °C. Following addition of 2 × SDS loading buffer, samples were subjected to 10% SDS-PAGE followed by autoradiography.

Cell Culture—3T3-L1 fibroblasts (American Type Culture Collection [Manassas, VA]) and mouse embryo fibroblasts (generously provided by Professor Willnow, Berlin, Germany) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 5 μg/ml gentamicin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂.

Activation of α₅M—α₅M was activated by reaction with 300 μM methylamine as previously reported (21). Tris borate native electrophoresis (22) demonstrated differing mobility between the activated and native isoforms indicating that conformational change and thus activation was confirmed. The ratio of activated α₅M and α₅I-activated α₅M (iodinated with chloramine T to a specific activity of 0.4 μCi/μg) in mouse embryo fibroblasts expressing the LRP receptor which only recognizes α₅M in the activated form. Briefly, cells plated at 2 × 10⁵ were serum starved for 3 h before the addition of 3 × 10⁻⁵M IGF-I or 10⁻⁵M-activated α₅M. After 10–360 min incubation at 37 °C, cells were washed twice with PBS and then incubated for 4 min at 25 °C with EDTA, trypsin, 0.2 mg/ml proteinase K. The resulting cell suspension was centrifuged at 13,000 rpm; membrane bound versus incorporated ¹²⁵I-IGF-I was determined by counting the supernatant and solubilized cell pellet, respectively.

RESULTS

Effect of IGFBP-1 Purified from Plasma on IGF-I Stimulated [³H]Thymidine Uptake by 3T3-L1 Fibroblasts—IGFBP-1 (10 ng/ml) caused a 1.5-fold increase (p < 0.005) in [³H]thymidine uptake by 3T3-L1 fibroblasts in mid-log growth (Fig. 1). This was unaffected by nonphosphorylated IGFBP-1 added at a 1:1 molar ratio (40 ng/ml) and/or 100 mM sodium α₅M. 20 h later, [³H]-thymidine was added to a final concentration of 0.25 μCi/ml and after a further 4 h, cells were washed twice with PBS and once with 10% trichloroacetic acid. The cells were incubated with 10% trichloroacetic acid for 2 h at 4 °C and solubilized with 0.1 M NaOH and counted on a β-counter using Opiphase HiSafe liquid scintillant.
Fig. 1. A, the effect of IGF-I on nonphosphorylated (np) IGFBP-1 or phosphorylated (p) IGFBP-1 on [3H]thymidine uptake by 3T3-L1 fibroblasts. IGF-I (10 ng/ml) ± 40 ng/ml np- or pIGFBP-1 was incubated with serum-starved cells for 20 h before the addition of 0.25 μCi/ml [3H]thymidine for a further 4 h. Uptake is expressed as percentage increase over control and is shown as the mean (± S.D.) of three experiments performed in triplicate. B, the effect of 3T3-L1 cells on the phosphorylation status of IGFBP-1. 40 ng/ml of a preparation of phosphorylated IGFBP-1 purified from normal human plasma was incubated with 3T3-L1 fibroblasts or BeWo choriocarcinoma cells. After 24 h medium was harvested, immunoprecipitated with anti-IGFBP-1 monoclonal antibody 6303, and subjected to n-octylglucoside electrophoresis and Western ligand blotting with [125I]-IGFBP-1. The phosphorylation pattern of recombinant IGFBP-1 and IGFBP-1 from normal plasma, amniotic fluid (AF) and decidual endometrial cells (decidua CM) is shown for comparison.

Protein Co-purified with IGFBP-1 Is Identified as α2-Macroglobulin—These results suggested that a protein which had co-purified with IGFBP-1 might be enhancing IGF-I action on target cells. Indeed, increased IGF-I stimulated [3H]thymidine uptake was observed in response to a plasma-derived preparation that had been depleted of IGFBP-1 by immunoprecipitation (data not shown). Proteins in the IGFBP-1 preparation were therefore isolated from silver-stained SDS-polyacrylamide gels, treated with trypsin, and subjected to MALDI-MS analysis. The predominant contaminating component was identified using the Pro-Found data base as α2-macroglobulin.

IGFBP-1/α2M Are Associated in Plasma—Western immunoblotting with an anti-human α2M antibody confirmed the presence of α2M in the IGFBP-1 preparation purified from plasma (Fig. 2A). Immunoprecipitates of human plasma with an anti-human IGFBP-1 antibody, contained a high molecular weight protein that co-migrated with human α2M, providing further proof of the association between IGFBP-1 and α2M (Fig. 2B).

Characterization of IGFBP-1/α2M Complexes Formed in Vitro—IGFBP-1/α2M binding in solution was assessed by incubating [125I]-IGFBP-1 with human plasma (10 μl) for 4 h, fixing the resulting complexes with the cross-linking agent BS3, and SDS-PAGE analysis both before and after immunoprecipitation with an anti-α2M antibody. Fig. 3A demonstrates that [125I]-IGFBP-1 can associate with a high molecular weight species in plasma which can be immunoprecipitated with an antibody to α2M. This complex co-migrated with the labeled species seen as the result of [125I]-IGFBP-1 incubation with α2M (10 μg). The high molecular weight [125I]-IGFBP-1/α2M binding protein and the [125I]-IGFBP-1/α2M complexes could also be precipitated by an antibody to IGFBP-1 (Fig. 3B) although not by protein A-Sepharose CL-4B alone. The radioactive species migrating to ~30 kDa represents uncomplexed [125I]-IGFBP-1. [125I]-IGFBP-1 also formed high molecular weight complexes with activated α2M (data not shown).

Specificity of IGFBP-1/α2M association was confirmed by competition studies in which [125I]-IGFBP-1 was cross-linked to α2M in the presence of increasing concentrations of unlabeled IGFBP-1. Fig. 3C shows that excess unlabeled IGFBP-1 (10–1000-fold) decreased binding of [125I]-IGFBP-1 to α2M.

Surface Plasmon Resonance Analysis of IGFBP-1/α2M Association—Surface plasmon resonance was used to investigate further the association of IGFBP-1 and α2M; using standard amine coupling procedures, phosphorylated or nonphosphorylated IGFBP-1 was immobilized on a BIAcore sensor chip CM5 and then exposed to α2M. The sensogram depicted in Fig. 4A shows binding of α2M to phosphorylated IGFBP-1. α2M was applied at a concentration sufficient to saturate the immobilized IGFBP-1, based on a 1:1 binding ratio. After binding α2M, the chip was further exposed to IGF-I and Fig. 4A shows that IGFBP-1 can still bind IGF-I in the presence of α2M. Nonphosphorylated IGFBP-1, however, does not bind to α2M (Fig. 4B) although IGF-I binding of this isoform is evident.

α2M Protects IGFBP-1 from Proteolysis—Since α2M is a recognized protease inhibitor, one physiological consequence of
the IGFBP-1/α₂M association could be protection of IGFBP-1 from proteolysis. ¹²⁵I-IGFBP-1 was incubated with 1 μg of chymotrypsin (23) in the presence of α₂M (0–10 μg). Fig. 5 demonstrates that 1 μg of chymotrypsin is sufficient to proteolysis ¹²⁵I-IGFBP-1 completely since there is no evidence of intact or partially fragmented IGFBP-1 following incubation for 16 h. However, in the presence of 5 μg of α₂M, radiolabeled proteins of molecular weights corresponding to IGFBP-1 frag-
ments are apparent, suggesting that chymotrypsin activity is reduced by a low concentration of α2M. IGFBP-1 proteolysis was completely abolished by the presence of 10 μg of α2M; here the majority of 125I-IGFBP-1 was detected in high molecular weight complexes which co-migrate with the radiolabeled species observed when IGFBP-1 and α2M are incubated in the absence of chymotrypsin. This suggests that in this instance, α2M protects against proteolysis by associating with the substrate (IGFBP-1) rather than the protease.

Effect of α2M on IGF-I-stimulated 3H/Thymidine Uptake—
α2M influences the action of other growth factors and therefore the effect of α2M on IGF-I-stimulated 3H/thymidine uptake by 3T3-L1 fibroblasts was investigated to determine whether α2M could be responsible for the enhanced stimulation observed in the presence of plasma-derived IGFBP-1. Conversion of α2M into the activated isofrom, which is recognized by the α2M receptor LRP (Fig. 6A (ii)), was achieved by reaction with methyleneamine. Activated α2M (100 μg/ml) but not the native isoform (100 μg/ml), was able to enhance IGF-I-stimulated 3H/thymidine uptake 2-fold (p < 0.001; Fig. 6A (ii)). Importantly, α2M could also abrogate the inhibitory effect of IGF-I-1; an HPLC-purified preparation of phosphorylated IGFBP-1 could reduce IGF action (Fig. 6B, p < 0.01), however, when 100 μg/ml α2M was also included in the incubation, IGF-I-stimulated 3H/thymidine uptake (p < 0.01) was enhanced. Activated α2M (200 μg/ml) also had an independent effect on mitogenesis (p < 0.05), whereas phosphorylated IGFBP-1 (40 ng/ml) alone had no effect (Fig. 6B).

DISCUSSION

Analysis of the highly phosphorylated IGFBP-1 found in the circulation of non-pregnant adults has demonstrated association with α2M-macroglobulin, another plasma protein. α2M can enhance IGF-I-stimulated proliferation of fibroblasts, even in the presence of phosphorylated IGFBP-1, however, when α2M is absent, phosphorylated IGFBP-1 is inhibitory.

Highly phosphorylated IGFBP-1 has a high affinity for IGF-I (9, 11) and can therefore sequester IGF-I from its cell surface receptors. In order for IGFBP-1 enhancement of IGF action to occur, it is generally thought that IGFBP-1 must be either in the nonphosphorylated isofrom, which has a lower affinity for IGF-I (5), or associated with cell membranes (6, 24). We found that increased IGF action in the presence of a preparation of IGFBP-1 purified from plasma was not the result of dephosphorylation by 3T3-L1 cells. BeWo choriocarcinoma cells, however, are able to dephosphorylate IGFBP-1 (23) and this isoform of IGFBP-1 is normally only detected in the human during pregnancy (10), raising the possibility that altering IGFBP-1 phosphorylation status may predominantly be a mechanism for regulating IGF bioavailability in pregnancy.

An early report from Clemmons and Gardner (25) suggested that a factor present in plasma was necessary for IGFBP-1 to potentiate IGF stimulated smooth muscle cell DNA synthesis. This group discerned the factor to be a macromolecule and excluded mitogens such as platelet-derived growth factor, epidermal growth factor, and fibroblast growth factor and the carrier proteins transferrin, albumin, and fibronectin, although its identity remained elusive. These studies led us to suspect that our purified preparation of plasma IGFBP-1 contained another plasma component that had co-purified on the anti-IGFBP-1 monoclonal antibody immunoaffinity column by virtue of its association with IGFBP-1 in plasma.

Radioimmunoassay of the IGFBP-1 preparation refuted our initial assumption that IGF-I was the contaminating protein and so we then used a combination of MALDI-TOF mass spectrometry and co-immunoprecipitation/Western blot studies, to identify the co-purified factor as α2-macroglobulin.

Early size exclusion chromatographic studies of plasma did not detect the association between IGFBP-1 and α2M. Several explanations for this are tenable. First, α2M is a large protein (~700,000) which would have been outside the molecular weight range of most chromatographic studies. Appearance of material within the void volume could have been discounted as being due to protein solubility/aggregation. Second, the association of IGFBP-1 with α2M may be low affinity resulting in dissociation of the complex on size exclusion chromatography; these studies indicate a kD of 2.75 × 10^-3 s^-1, which supports this hypothesis. Third it is possible that some IGFBP-1 antibodies cannot recognize the peptide when bound to α2M. Furthermore, initial chromatographic studies of other IGFBPs did not demonstrate associations with plasma proteins and yet it has recently been recognized that this is the case. For example, IGFBP-3 specifically binds to lactoferrin (26), transferrin (27),
α2M binds IGFBP-1 and enhances IGF action

α₂M binds IGFBP-1 and Enhances IGF Action

α₂M is a homoetrameric glycoprotein that circulates at concentrations of 2–4 mg/ml (30). Each subunit contains multiple reactive sites suggesting that α₂M has diversified functions as a binding, carrier, and targeting protein and it may therefore be important for several aspects of IGFBP-1 function.

α₂M is well known as a protease inhibitor and has the unique ability of being able to inhibit proteinases from all four mechanistic classes (30). Protease cleavage of native α₂M results in a conformational change to form activated-α₂M which traps the protease so that it is sterically hindered from access to substrate. Nonproteolytic peptides trapped by α₂M become largely protected from exogenous proteinases and our own results show that when associated with α₂M, IGFBP-1 is protected from proteolysis by chymotrypsin. Thus in plasma, α₂M may be acting as a chaperone to IGFBP-1 and this may explain why there are no reports of IGFBP-1 fragments in the circulation despite the fact that IGFBP-1 has many of the cleavage motifs displayed by other circulating IGFBPs which are proteolysed.

Another physiological consequence of IGFBP-1/α₂M association may be in regulating IGF activity. α₂M is known to associate with several other growth factors including fibroblast growth factor (31), vascular endothelial growth factor (32), epidermal growth factor (33), transforming growth factor-β1 (34), and platelet-derived growth factor (35) to synergistically enhance their action on cell proliferation (36). We have found that α₂M also enhances IGF-I-stimulated mitogenesis and importantly, that α₂M can also influence how IGFBP-1 modulates this effect. In our initial studies using the IGFBP-1 preparation purified from plasma, IGF-I stimulated [³H]thymidine uptake by 3T3-L1 fibroblasts was seemingly enhanced by phosphorylated IGFBP-1 and the preparation also appeared to have an independent mitogenic effect. However, we have now demonstrated that this effect was more likely due to the presence of α₂M.

The mechanism behind α₂M enhancement of IGF-I activity is unclear, although it probably involves one of the two α₂M receptors, LRP/α₂MR, or the recently described α₂M signaling receptor (α₂MSR). The LRP/α₂MR is a member of the low density lipoprotein receptor superfamily (37–39) which recognizes both free and growth factor-associated α₂M once it has been activated by proteases or amines such as methamphetamine (40–42). Thus α₂M, through IGFBP-1, could serve to increase the concentration of IGF-I in the local environment of the cell; dissociation at or near the cell surface could release IGF-I to interact with its cell surface receptor. Our preparation of native α₂M was unable to enhance IGF-I activity, whereas activated α₂M did enhance IGF actions. These data strongly suggest that such synergy was due to α₂M/receptor interactions and not the presence of an additional α₂M-associated mitogen such as platelet-derived growth factor. Alternatively, the LRP/α₂MR scavenges α₂M complexes resulting in their rapid clearance from the circulation and thus internalization of IGFBP-1 along with α₂M could alleviate the inhibitory effect on paracrine or autocrine IGF-I, or, if both IGF-I and IGFBP-1 were internalized, their dissociation within an endocytic compartment could influence signal transduction in a manner analogous to that of epidermal growth factor (43–45).

A further mechanism by which α₂M may regulate cellular growth involves a second α₂M receptor (46), which also recognizes only activated α₂M. α₂M interaction with this receptor increases phosphatidylinositol 3-kinase activity (47) and elevates p21Ras-GTP (48), which may explain our finding that α₂M acts as an independent mitogen. However, signaling through the type 1 IGF receptor also involves activation of the phosphatidylinositol 3-kinase and Ras/Raf pathways (1) and so it is possible that α₂M could enhance endogenous and exogenous IGF-I actions as a result of synergy between their intracellular signaling components.

In summary, we have demonstrated that α₂M is a binding protein of IGFBP-1 which modifies IGFBP-1/IGF interaction. This represents a novel and potentially important mechanism for controlling the transport and biological activity of IGFs since the effect of IGFBP-1 on IGF activity depend not only on IGFBP-1 phosphorylation status, but also on whether IGFBP-1
is bound to α2M and the complement of α2M receptors at the cell surface.

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