Plasminogen binds the heparin-binding domain of insulin-like growth factor-binding protein-3

PHIL G. CAMPBELL,1 SUSAN K. DURHAM,2 ADISAK SUWANICHKUL,2 JAMES D. HAYES,1 AND DAVID R. POWELL2

1Orthopaedic Research Laboratory, Allegheny University of Health Sciences, Pittsburgh, Pennsylvania 15212; and 2Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

Campbell, Phil G., Susan K. Durham, Adisak Suwanichkul, James D. Hayes, and David R. Powell. Plasminogen binds the heparin-binding domain of insulin-like growth factor-binding protein-3. Am. J. Physiol. 275 (Endocrinol. Metab. 38): E321–E331, 1998.—Limited proteolysis lowers affinity of insulin-like growth factor (IGF)-binding protein (IGFBP)-3 for bound IGFs, resulting in greater IGF bioavailability. Plasmin is one of many proteases that cleave IGFBP-3, and the plasmin system may regulate IGFBP-3 proteolysis and IGF bioavailability in cultured cells in vitro. A role for the plasmin system in IGFBP-3 proteolysis in vivo is suggested by data presented here showing that IGFBP-3 binds plasminogen (Pg; Glu-Pg) with a dissociation constant (Kd) ranging from 1.43 to 3.12 nM. IGF-I and Glu-Pg do not compete for IGFBP-3 binding; instead, the binary IGFBP-3/Glu-Pg complex binds IGF-I with high affinity (Kd = 0.47 nM) to form a ternary complex. Competitive binding studies suggest that the kringle 1, 4, and 5 domains of Glu-Pg and the heparin-binding domain of IGFBP-3 participate in forming the IGFBP-3/Glu-Pg complex, and other studies show that Glu-Pg in this complex is activated at a normal rate by tissue Pg activator. Importantly, IGFBP-3/Glu-Pg complexes were detected in both human citrate plasma and serum, indicating that these complexes exist in vivo. Binding of IGFBP-3 to Glu-Pg in vivo suggests how Glu-Pg activation can specifically lead to IGFBP-3 proteolysis with subsequent release of IGFs to local target tissues.

MATERIALS AND METHODS

Materials. Recombinant human IGF-I was purchased from GroPep (Adelaide, Australia). Recombinant human IGFBP-5 was produced in a baculovirus expression system and purified by affinity chromatography and reverse-phase (RP)-HPLC (1). IGFBP-1 was purified from human amniotic fluid by affinity chromatography and reverse-phase (RP)-HPLC (6); quantitation was by RIA using a kit from Diagnostic Systems Laboratories (DSL; Webster, TX). Glu-Pg was purified from fresh-frozen human plasma with the use of Lys-Sepharose in the presence of 1 mM benzamidine, 50 µg/ml trypsin inhibitor, and 20 KIU/ml aprotinin (9). Commercial vendors provided Lys-Pg (Enzyme Research Laboratories, South Bend, IN), mini-Pg (which consists of Glu-Pg kringle domain 5 and the B chain containing the catalytic portion of Glu-Pg) and PgK-4 (which consists of Glu-Pg kringle domain 4) (American Diagnostics, Greenwich, CT), PgK-1–3 (which consists of Glu-Pg kringle domains 1–3), α2-antiplasmin and fibrinogen (Sigma, St. Louis, MO), plasmin substrate S2251 (Chromogenics-Pharma, and tissue Pg activator (tPA; Genentech, South San Francisco, CA). IGFBP-5 peptides IGFBP-5-(138—152),...
IGFBP-5 (130–142), and IGFBP-5 HBD (Table 1) were kindly provided by Dr. Dennis Andress (Univ. of Washington, Seattle, WA). The IGFBP-3 HBD peptide (Table 1) was synthesized by Genemed Synthesis (South San Francisco, CA). Human serum and plasma were obtained from outdated blood bank supplies or from laboratory personnel.

IGFBP-3 mutagenesis. A full-length IGFBP-3 cDNA was isolated from a human placental cDNA library (14); it spanned from 47 to 1,239 bp of the human IGFBP-3 cDNA sequence reported by Wood et al. (40) and had the same nucleotide sequence as the published cDNA. This IGFBP-3 cDNA was inserted into the M13-based vector M13mp18 at the EcoRI site and was mutated by the Kunkel method with the use of synthetic oligonucleotides and the Muta-Gene kit (Bio-Rad) (36). The initial mutation used oligonucleotide 5'-CAGACGATGAGGCTTCCCACG-3' to introduce a HindIII site at base pairs +877 to +882, immediately 3' to the IGFBP-3 translation stop codon. The oligonucleotide 5'-CAGGTGCCTTCATGACCGGGGACGCTT-3' was then used to create IGFBP-3HBD (Table 1). The sequence of each mutation was confirmed by DNA sequence analysis using Sequenase (US Biochemicals, Cleveland, OH) in the dideoxy chain termination method (36).

IGFBP-3 expression and purification. The expression vector pKG3226 contains the human β-actin promoter, SV40 polyadenylation signal and neomycin phosphotransferase resistance gene (34). IGFBP-3 and IGFBP-3HBD cDNAs were introduced into pKG3226 at the EcoRI (5') and HindIII (3) sites. The resulting expression vectors pKG-BP3 and pKG-BP3HBD were stably transfected into Chinese hamster ovary (CHO)-K1 cells. Briefly, 3 × 10^6 CHO-K1 cells/60-mm plate were maintained for 24 h in McCoy's 5A medium (GIBCO BRL, Gaithersburg, MD) containing 2.5% FCS and 6% MEM medium (GIBCO BRL) and 8 µl Lipofectin (GIBCO BRL, Gaithersburg, MD) in the dideoxy chain termination method (36).

IGFBP-3 expression and purification. The expression vector pKG3226 contains the human β-actin promoter, SV40 polyadenylation signal and neomycin phosphotransferase resistance gene (34). IGFBP-3 and IGFBP-3HBD cDNAs were introduced into pKG3226 at the EcoRI (5') and HindIII (3) sites. The resulting expression vectors pKG-BP3 and pKG-BP3HBD were stably transfected into Chinese hamster ovary (CHO)-K1 cells. Briefly, 3 × 10^6 CHO-K1 cells/60-mm plate were maintained for 24 h in McCoy's 5A medium (GIBCO BRL, Gaithersburg, MD) containing 2.5% FCS and 7.5% calf serum (complete medium). Five micrograms of pKG-BP3 or pKG-BP3HBD plasmid were mixed with optimized MEM medium (GIBCO BRL) and 8 µl Lipofectin (GIBCO BRL), and the mixture was then incubated with the CHO-K1 cells for 6 h at 37°C. The DNA/Lipofectin mixture was then removed from the CHO-K1 cells; after incubation overnight in complete medium, these cells were then incubated in complete medium containing 1 mg/ml gentamicin (G-418)/ml. Surviving cells formed individual clones; ~60 pKG-BP3 and pKG-BP3HBD clones were transferred into individual wells of 96-well plates. Cells were incubated in serum-free McCoy's 5A medium and then screened for IGFBP-3 expression by immunoblot (see MATERIALS AND METHODS for process of IGFBP-3 expression and purification).

Solid-phase plate-binding assay. Binding of IGFBP-3 to Glu-Pg was characterized using an immobilized Glu-Pg-based assay system (7). Optimal binding conditions, Glu-Pg coating concentration, plate type, and incubation parameters were determined and are presented in the following assay protocol. Ninety-six-well immunologic plates (Polsorb, NUNC, Fisher Scientific, Pittsburgh, PA) were coated with 5 µg/ml Glu-Pg in 0.1 M Na2CO3, pH 9.8, overnight at 4°C. The plates are rinsed with 200 µl of 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl and blocked with 200 µl of 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 80, 1% BSA, and 0.02% NaN3, pH 7.5, for 1 h at 37°C. Plates are rinsed twice with 200 µl of 20 mM Na2CO3, pH 9.8, overnight at 4°C. The plates are rinsed with 200 µl of 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl and blocked with 200 µl of 10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 80, 1% BSA, and 0.02% NaN3, pH 7.5, for 1 h at 37°C. Plates are rinsed twice with 200 µl of 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl and once with 200 µl of 30 mM Tris-acetate, pH 7.4, 10 mM sodium phosphate, 0.1% Tween 20, 0.02% NaN3 (assay buffer). IGFBP-3 and IGF-I were iodinated by the chloramine T method to a specific activity of ~150 µCi/µg protein (8). [125I]-labeled IGFBP-3 [50,000 counts/min (cpm)] or [125I]-I GF-I (50,000 cpm) was incubated with various concentrations of IGFBP-3, IGFBP-3HBD, IGF-I-1, IGF-I-2, IGFBP-3 HBD peptide, IGFBP-5 HBD peptide, IGF-I, Glu-Pg, mini-Pg, Lys-Pg, Pgk-1–3, Pgk-4, Arg, e-aminocaproic acid (e-ACA), α2-antiplasmin, or heparin in 100 µl of assay buffer; unless otherwise stated, incubations were for 1 h at 37°C. Unbound radioactivity was removed by rinsing the wells twice with 200 µl RADIOACTIVITY.
µl of ice-cold assay buffer. Bound radioactivity was solubilized with 200 µl of 1 N NaOH, transferred to 12 × 75 mm glass test tubes, and counted for radioactivity.

Fluid-phase plate-binding assay. Ninety-six-well immunologic plates (MaxiSorb, NUNC, Fisher Scientific) were coated with 5 µg/ml rabbit anti-human Glu-Pg antibody (Boehringer Mannheim Biochemicals, Indianapolis, IN) overnight at 4°C or for 3 h at 22°C in 100 µl of 0.1 M Na₂CO₃, pH 9.18. Plates were rinsed with PBS and blocked as described in the solid-phase plate assay protocol. In a separate uncoated 96-well test plate (Sarstedt), various concentrations of Glu-Pg, IGFBP-3, 125I-IGFBP-3, and/or human plasma or serum preparations were incubated in a total volume of 200 µl of assay buffer for 1 h at 37°C; this allows all reagents to associate in the fluid phase. At the end of the incubation period, 100 µl of reagents were transferred to the blocked and rinsed 96 wells that were coated with the anti-Glu-Pg antibody. After 30 min at 23°C, unbound radioactivity was removed by rinsing wells twice with assay buffer. Bound radioactivity, representing IGFBP-3/Glu-Pg complexes, was released by incubating wells with 200 µl of 1 M acetic acid for 10 min at 23°C. Acid washes were transferred to 12 × 75 mm glass test tubes and counted for radioactivity. Radioactivity bound in the absence of added Glu-Pg served as an estimate of nonspecific binding.

125I-IGFBP-3 ligand blotting. Pure Glu-Pg (10 µg) or IGFBP-1 (10 µg) was electrophoresed on a 12% SDS-polyacrylamide gel under nonreducing conditions. These proteins were then transferred to a nitrocellulose membrane as described previously (6). The membrane was washed for 30 min with 3% Nonidet P-40 in Tris-buffered saline (TBS), blocked for 2 h at 4°C in 0.5% BSA in TBS, washed for 10 min with 0.1% Tween 20 in TBS, and then incubated overnight with ~7 × 106 cpm 125I-IGFBP-3 in 0.1% Tween 20-TBS. After being washed and dried, film was exposed to filters overnight.

Copurification of IGFBP-3, ALS, and Glu-Pg from human plasma. Six milligrams of either goat anti-human IGFBP-3 antibody (a kind gift from DSL) or nonimmune goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were covalently linked to 2 ml of Affi-Gel 10 beads (Bio-Rad) and then poured into separate columns. Citrate human plasma (150 ml) mixed with 150 ml of 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl was then run across each column. After being washed with 600 ml of 10 mM sodium phosphate, pH 7.5, and 150 mM NaCl, each column was step-wise eluted with 100 ml of 50 mM sodium phosphate, pH 7.5, containing either 0.3, 0.6, or 1.0 M NaCl, followed by a final elution using 20 ml of 1% acetic acid. Aliquots (100 µl) of each elution fraction from the goat anti-human IGFBP-3 antibody column or goat nonimmune IgG column served to establish nonspecific binding.

RESULTS

125I-IGFBP-3 binds immobilized Glu-Pg. 125I-IGFBP-3 bound specifically to Glu-Pg immobilized on immunocap-
IGF-I/IGFBP-3 complex binds immobilized Glu-Pg. Coincubation of 125I-IGF-I and increasing amounts of unlabeled IGFBP-3 in plates coated with Glu-Pg resulted in a steady increase in 125I-IGF-I binding to these plates (Fig. 3A). Similar results were noted when increasing amounts of unlabeled IGFBP-3 were incubated with immobilized Glu-Pg before the addition of 125I-IGF-I (Fig. 3B). In each of these experiments, very little 125I-IGF-I bound to the Glu-Pg-coated plates in the absence of IGFBP-3, and coincubation of 125I-IGF-I with 100 ng/ml IGFBP-3 did not result in specific 125I-IGF-I binding if plates were not coated with Glu-Pg; these results suggest that 125I-IGF-I was binding to Glu-Pg-bound IGFBP-3. To determine the affinity of IGF for IGFBP-3 bound to immobilized Glu-Pg, increasing amounts of unlabeled IGF-I were coincubated with 125I-IGF-I in the presence of the IGFBP-3/Glu-Pg complex (Fig. 3C); Scatchard analysis of these data shows that IGF-I bound the immobilized IGFBP-3/Glu-Pg complex with \( K_d = 0.47 \pm 0.12 \text{nM} \) (mean \( \pm \text{SE of 2 independent experiments} \)). Finally, coincubation of unlabeled IGF-I with 125I-IGFBP-3 did not affect the ability of 125I-IGFBP-3 to bind immobilized Glu-Pg (Fig. 3D); this provides indirect evidence that Glu-Pg binds equally well to IGFBP-3 and the IGFBP-3/IGF-I complex.

IGFBP-3 HBD participates in Glu-Pg binding. Because 1) the IGFBP-5 HBD participates in binding to Glu-Pg (7), 2) IGFBP-3 has a homologous HBD (5), and 3) IGFBP-5 competes efficiently with 125I-IGFBP-3 for Glu-Pg binding, it seemed likely that the IGFBP-3 HBD participates in Glu-Pg binding. To test this hypothesis, heparin and synthetic 18-amino acid peptides encoding the IGFBP-3 and IGFBP-5 HBDs were evaluated for their ability to compete with 125I-IGFBP-3 for Glu-Pg binding. As shown in Fig. 4A, heparin clearly interfered in a dose-dependent manner with binding of 125I-IGFBP-3 to immobilized Glu-Pg. As shown in Fig. 4B, both the IGFBP-3 and IGFBP-5 HBD peptides competed efficiently with 125I-IGFBP-3 for Glu-Pg binding, but the IGFBP-5-(138—152) peptide and the Lys- and Arg-rich IGFBP-5-(130—142) peptide did not; the inhibitory effect of increasing amounts of the IGFBP-3 HBD peptide was not observed.
HBD peptide on \textsuperscript{125}I-IGFBP-3 binding is shown in Fig. 4C. In addition, IGFBP-3\textsubscript{mHBD}, a full-length IGFBP-3 protein mutated in the HBD region (see Table 1), had an approximately fivefold lower affinity for Glu-Pg than did native IGFBP-3 (Fig. 4D).

Multiple Pg domains participate in IGFBP-3 binding. The compound \(\alpha\)-ACA binds with high affinity to Lys binding sites present in Glu-Pg kringle domains 1, 4, and 5 (10), whereas Arg and the Arg analog \(p\)-aminobenzamidine bind specifically to Glu-Pg kringle domain 5 (38). The ability of \(\alpha\)-ACA (Fig. 5A), Arg (Fig. 5A), and \(p\)-aminobenzamidine (data not shown) to compete with \textsuperscript{125}I-IGFBP-3 for Glu-Pg binding suggests that Glu-Pg kringle domain 5 and possibly Glu-Pg kringle domains 1 and 4 participate in IGFBP-3 binding.

Figure 5B examines the ability of Glu-Pg and a variety of Glu-Pg fragments to compete with immobilized Glu-Pg for \textsuperscript{125}I-IGFBP-3 binding. Lys-Pg (the plasmin-cleaved form of Glu-Pg, which contains kringle domains 1–5) competed more efficiently than Glu-Pg for \textsuperscript{125}I-IGFBP-3 binding. All other fragments tested, including PgK-1–3 (which consists of Glu-Pg kringle domains 1–3), PgK-4 (which consists of Glu-Pg kringle domain 4), and mini-Pg (which consists of Glu-Pg kringle domain 5 and the B chain containing the catalytic portion of Glu-Pg) also competed with immobilized Glu-Pg for \textsuperscript{125}I-IGFBP-3 binding in a dose-dependent manner. The PgK-1 domain was further implicated by studies showing that \(\alpha_2\)-antiplasmin, which specifically binds Glu-Pg kringle domain 1 (39), competed with \textsuperscript{125}I-IGFBP-3 for binding to Glu-Pg (data not shown).

IGFBP-3, Pg, and ALS forms co-purify from human citrate plasma. Human citrate plasma was run over columns containing Affi-Gel 10 beads covalently bound to either goat anti-human IGFBP-3 antibody or to a nonimmune (control) goat IgG; after being washed, the columns were eluted with a step-wise NaCl gradient followed by a final elution using 1% acetic acid. India ink staining of the elution fractions showed that multiple proteins bound to the anti-IGFBP-3 antibody column in the presence of high NaCl concentrations and that most of these proteins were eluted from the column only in the presence of acetic acid; in contrast, very little protein bound to the goat IgG column (Fig. 6A). Immunoblotting showed that Glu-Pg, ALS, and IGFBP-3 forms each bound to the anti-IGFBP-3 antibody column but not to the nonimmune (control) goat IgG column (Fig. 6B–D). The Glu-Pg immunoblot (Fig. 6B) suggested that most of the proteins eluting in the acetic acid fractions were Pg forms. The largest form migrated at a molecular mass (\(M_r\)) slightly less than \(\sim 97\) kDa; in other experiments, this form comigrated with purified Glu-Pg, which has an \(M_r\) of 92 kDa (data not shown). A second form, which migrated slightly more slowly than ALS, has an \(M_r\) consistent with that of Lys-Pg (84 kDa). The anti-Glu-Pg antibody also recognized third and fourth forms, which migrated at \(\sim 60\) and \(\sim 25\) kDa, consistent with the \(M_r\) of plasmin heavy and light chains, respectively. The characteristic ALS doublet at \(\sim 85\) kDa was released from the anti-
IGFBP-3 antibody column primarily in the NaCl elution fractions but also to a lesser extent in the acetic acid fractions (Fig. 6C). The IGFBP-3 immunoblot (Fig. 6D) showed that all IGFBP-3 forms remained on the anti-IGFBP-3 antibody column in the presence of 1 M NaCl and were removed from the column only in the presence of acetic acid. The largest IGFBP-3 forms were the characteristic doublet of intact, differentially

![Diagram](http://alpendo.physiology.org.org)

**Fig. 5.** Determination of IGFBP-3 binding sites on Glu-Pg. In each study, ~50,000 cpm ¹²⁵I-IGFBP-3 were incubated with or without competitors in Glu-Pg-coated immunocapture plates. Nonspecific binding was determined in presence of 100 µg heparin. A: increasing concentrations of either Arg or ε-aminocaproic acid (ε-ACA) were coincubated with ¹²⁵I-IGFBP-3. Data are presented as percentages of nontreatment (no added competitor) control values; each data point is mean ± SE of 3 independent determinations performed in duplicate. B: increasing concentrations of Glu-Pg, Lys-Pg, PkK-1–3, PkK-4, or mini-Pg were coincubated with ¹²⁵I-IGFBP-3 (see MATERIALS AND METHODS for explanation of PkK-1–3, PkK-4, and mini-Pg). Data are presented as percentages of nontreatment (no added competitor) control values; each data point is mean ± SE of duplicate determinations.

![Diagram](http://alpendo.physiology.org.org)

**Fig. 4.** IGFBP-3 heparin-binding domain (HBD) participates in binding to Glu-Pg. In each study, ~50,000 cpm ¹²⁵I-IGFBP-3 were incubated with or without competitors in Glu-Pg-coated immunocapture plates. Nonspecific binding was determined in presence of 100 µg heparin, which was subtracted from total binding to derive specific binding. B0 was determined in absence of any competing ligand. A: increasing amounts of heparin were coincubated with ¹²⁵I-IGFBP-3. Each data point is mean of duplicate determinations. B: 1 µg/ml IGFBP-3 HBD peptide (BP3HBD), IGFBP-5 HBD peptide (BP5HBD), IGFBP-5-(138—152), or IGFBP-5-(130—142) was coincubated with ¹²⁵I-IGFBP-3. Data are presented as percentages of nontreatment (no added competitor) control values; each data point is mean ± SE of triplicate determinations. C: increasing amounts of IGFBP-3 HBD peptide were coincubated with ¹²⁵I-IGFBP-3. Each data point is mean ± SE of duplicate determinations. D: increasing amounts of native IGFBP-3 or IGFBP-3mHBD (mutation) were coincubated with ¹²⁵I-IGFBP-3. Each data point is mean ± SE of duplicate determinations.
glycosylated IGFBP-3, which migrated at ~50 kDa (Fig. 6D); in other experiments, this doublet comigrated with intact, glycosylated human IGFBP-3 expressed in CHO-K1 cells (data not shown). In addition, an abundant ~29-kDa IGFBP-3 form was present in the acetic acid fractions; this is almost certainly an IGFBP-3 fragment commonly found in the circulation (16). Control immunoblots on fresh human plasma, which was treated immediately with protease inhibitors (3 mM EDTA, 100 µg/ml soybean trypsin inhibitor, 3 mM p-benzamidine, and 100 U/ml aprotinin), showed that Pg and IGFBP-3 fragmentation occurred before plasma was passed over the affinity column and may well reflect the distribution of Pg and IGFBP-3 forms in the circulation (data not shown).

125I-IGFBP-3 binds Pg forms in the fluid phase. As shown in Fig. 7A, wells coated with anti-Glu-Pg antibodies were able to bind 125I-IGFBP-3 that had been preincubated with Glu-Pg in the fluid phase. In these studies, the binding of 125I-IGFBP-3 to Glu-Pg was dose dependent and saturable (Fig. 7B), with $K_d = 3.12 \pm 0.65$ nM (mean ± SE of 2 independent experiments). In additional studies, 125I-IGFBP-3 also bound Lys-Pg, PgK-1–3, PgK-4, and mini-Pg in the fluid phase (data not shown). 125I-IGFBP-3 was also found to complex with Pg forms present in serum or citrate plasma but not in heparin plasma (Fig. 7C); this suggested that IGFBP-3/Pg complexes may well form in the fluid phase in vivo.

IGFBP-3/Pg complexes are present in human plasma and serum. Figure 8A shows that Glu-Pg/IGFBP-3 complexes formed in vitro can be detected by a double-antibody sandwich RIA using antibodies to Glu-Pg and IGFBP-3. This assay was then used to demonstrate the presence of endogenous IGFBP-3/Pg complexes in human citrate plasma and, to a lesser extent, in human serum (Fig. 8B).

IGFBP-3 does not affect the tPA-catalyzed activation of Glu-Pg. As shown in Table 2, the ability of tPA to activate Glu-Pg was enhanced by fibrinogen. In contrast, IGFBP-3 appeared to have no effect on the tPA-catalyzed conversion of Glu-Pg to plasmin.

Figure 9 confirms that in the presence of physiological plasma levels of IGFBP-3, IGFs, Glu-Pg, and tPA, IGFBP-3 is proteolyzed when Glu-Pg is activated to plasmin. Significant but incomplete proteolysis occurred with 5 ng/ml tPA (circulating concentration during normal, resting state), and complete proteolysis
occurred with 50 ng/ml tPA (circulating concentration during pathological conditions such as sepsis).

**DISCUSSION**

Complex formation between IGFBP-3 and Pg forms was shown by several experimental approaches, including solid- and fluid-phase plate-binding assays, ligand blotting, copurification of IGFBP-3 and Pg forms from human citrate plasma using an anti-IGFBP-3 antibody affinity column, and detection of IGFBP-3/Pg complexes in human serum and citrate plasma using anti-Glu-Pg and anti-IGFBP-3 antibodies in a double-antibody sandwich RIA. These last two approaches strongly suggest that IGFBP-3/Pg complexes are present in the circulation in vivo. Similar affinity techniques have been used to identify other proteins that bind Pg (11, 25).

The binding affinity of IGFBP-3 for Glu-Pg is comparable to or higher than the binding affinity of other extracellular and plasma proteins known to interact
Table 2. Initial rate of tPA-catalyzed activation of Glu-Pg in presence of IGFBP-3

<table>
<thead>
<tr>
<th>Protein</th>
<th>Rate</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>39.1 ± 1.6</td>
<td>0.934</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>38.2 ± 1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>59.7 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

Data represent means ± SE of 4 separate experiments. Plasminogen (Pg; Glu-Pg, 400 nM) was incubated with either IGFBP-3 (100 nM) or fibrinogen (100 nM) for 1 h at 37°C in assay buffer. Plasminogen substrate S2251 was added at final concentration of 0.2 mM. Activation reaction was initiated by adding 0.77 nM tissue Pg activator (tPA). Substrate hydrolysis was monitored at 37°C over 10 min at an absorbance of 405 nm. Initial reaction velocities were determined and are expressed in μM/min. Mean IGFBP-3 and fibrinogen values were compared to no addition (control) values using ANOVA followed by Tukey’s post hoc test.

with Pg, IGFBP-3 binds immobilized Glu-Pg with a K_d = 1.43 nM and binds to Glu-Pg in fluid phase with a K_d = 3.12 nM. These values are comparable to the binding affinities of fibrinogen and collagen type IV for Glu-Pg (35); are up to 100-fold greater than the binding affinities of fibronectin, osteonectin, thrombospondin, and laminin for Glu-Pg (15, 21, 31, 32); and are between 100- and 1,000-fold greater than the binding affinities of procarboxypeptidase B, vitronectin, and histidine-rich glycoprotein for Glu-Pg (25, 29, 37). In addition, the binding affinity of Glu-Pg for IGFBP-3 is 2,800- and 30,000-fold greater than the affinity of Glu-Pg for α_2-antiplasmin and fibrin, respectively (39).

IGFBP-3 appears to interact with Lys binding sites within Glu-Pg kringle domains. These same Lys binding sites mediate the interaction of Glu-Pg with a number of other plasma proteins, including fibrin, α_2-antiplasmin, histidine-rich glycoprotein, tetranectin, osteonectin, and procarboxypeptidase B (10, 11, 21, 25, 37, 39). The ability of Glu-Pg kringle domains 1–3 (PgK-1–3), PgK-4, mini-Pg, α_2-antiplasmin, Arg, and the Lys analog ε-ACA to each inhibit IGFBP-3 binding to Glu-Pg suggests that IGFBP-3 interacts with the PgK-1, PgK-4, and PgK-5 regions of Glu-Pg (10, 11, 15, 21, 25, 37, 39). The high affinity of IGFBP-3 for Glu-Pg may be explained by the interaction of IGFBP-3 with multiple Glu-Pg kringle domains.

Glu-Pg appears to bind to a specific HBD of IGFBP-3 that is rich in Lys groups. Another Lys-rich peptide, IGFBP-5 (130–142), fails to inhibit Glu-Pg binding to IGFBP-3, suggesting that the particular spatial orientation of Lys groups in the HBD is required for binding. The sequence and location of the IGFBP-3 HBD is conserved in two of the other five IGFBPs, IGFBP-5 and IGFBP-6 (5), and IGFBP-5 has been reported previously to bind Glu-Pg (7). Studies presented here show that IGFBP-5 has an approximately fivefold greater affinity for Glu-Pg than does IGFBP-3 and suggest that this binding requires the IGFBP-5 HBD. In contrast, the IGFBP-3 HBD sequence is not conserved in IGFBP-1, IGFBP-1 does not bind appreciably to Glu-Pg, and partial replacement of the IGFBP-3 HBD sequence with homologous sequence from IGFBP-1 (creating IGFBP-3mHBD) results in a fivefold loss of affinity for Glu-Pg. Thus the HBD may play an important role in IGFBP-3 cleavage by providing preferential access to Glu-Pg. The possibility that this may be a more generalized mechanism is suggested by the observation that prekallikrein also binds the IGFBP-3 HBD (data not shown).

Formation of IGFBP-3 and Glu-Pg into a binary complex does not prevent the additional binding of IGF-I to IGFBP-3, thus forming a ternary complex. The binding affinity (K_d value) estimated for IGF-I binding to IGFBP-3 in the IGFBP-3/Glu-Pg binary complex is ~0.47 nM. This K_d value is in the range reported previously for IGF-I/IGFBP-3 binding (0.03 to 0.5 nM) and is close to the value of 0.23 nM recently reported by biosensor measurement (20). Thus binding of Glu-Pg to IGFBP-3 does not appear to have a major inhibitory effect on the ability of IGFBP-3 to bind IGF-I.

The binding affinity of IGFBP-3 for Glu-Pg is comparable to the K_d = 2.0 nM binding affinity of the IGFBP-3/IGF-I binary complex for ALS (24). These results suggest that IGFBP-3/Glu-Pg complexes should form in the circulation, a hypothesis confirmed by data presented in this report. However, the affinity of Glu-Pg for numerous other proteins in the circulation and on the endothelial cell surface may limit the Glu-Pg pool available to interact with IGFBP-3. For example, plasma
levels of ~1.5 µM for histidine-rich glycoprotein and ~1 µM for α2-antiplasmin suggest that these proteins may bind up to ~50 and ~15%, respectively, of circulating Glu-Pg (25). In contrast, ALS has not been reported to bind other proteins and may bind exclusively to IGFBP-3. Evidence that IGFBP-3 is present in a ternary serum complex with IGFs and ALS is based in part on gel filtration studies performed at neutral pH; here, IGFBP-3 is found almost exclusively in the ~150-kDa serum fractions. However, Glu-Pg has a similar Mr to ALS; thus a portion of the IGFBP-3 circulating in the ~150-kDa fractions could be complexed to Glu-Pg. Although the binary IGFBP-3/Glu-Pg complex binds IGF-I, IGFBP-3/Glu-Pg should not form a ternary complex with ALS, since both ALS (3) and Glu-Pg bind IGFBP-3 through the HBD.

Although citrate plasma and serum were used to demonstrate that IGFBP-3/Glu-Pg complexes exist in vivo, it is unclear how much IGFBP-3/Glu-Pg complex is present in the circulation. More of this complex was detected in citrate plasma, which contains essentially all circulating proteins, than in serum, which is depleted of proteins involved in clot formation. Nevertheless, IGFBP-3/Glu-Pg complexes were clearly present in serum. In addition, others have attempted to purify ALS from rat serum using an affinity column in which serum. In addition, others have attempted to purify ALS from rat serum using an affinity column in which in serum. In addition, others have attempted to purify ALS from rat serum using an affinity column in which

We thank Heide Eash and Richard Ting for assistance in conducting the solid- and fluid-phase plate-binding assays.

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Award R01-DK-38773 (to D. R. Powell).

Address for reprint requests: D. R. Powell, Texas Children's Hospital, Feigin Center, MC# 3-2482, 6621 Fannin, Houston, TX 77030.

Received 3 November 1997; accepted in final form 29 April 1998.

REFERENCES


15. DePol, T., P. Bacon-Baguley, S. Dendra-Frankacz, M. T. Cederholm, and D. A. Walz. Thrombospondin interaction with...


