Insulin-like Growth Factor-binding Protein-3 Binds Fibrinogen and Fibrin

(Received for publication, March 19, 1999, and in revised form, July 15, 1999)

Phil G. Campbell‡, Susan K. Durham.§, James D. Hayes‡, Adisak Suwanichkul¶, and David R. Powell¶¶

From the ¶Institute for Complex Engineered Systems, Carnegie Mellon University, Pittsburgh, Pennsylvania 15212 and the §Department of Pediatrics, Baylor College of Medicine, Houston, Texas 77030

Following tissue injury, a fibrin network formed at the wound site serves as a scaffold supporting the early migration of stromal cells needed for wound healing. Growth factors such as insulin-like growth factor-I (IGF-I) concentrate in wounds to stimulate stromal cell function and proliferation. The ability of IGF-binding proteins (IGFBPs) such as IGFBP-3 to reduce the rate of IGF-I clearance from wounds suggests that IGFBP-3 might bind directly to fibrinogen/fibrin. Studies presented here show that IGFBP-3 does indeed bind to fibrinogen and fibrin immobilized on immunocapture plates, with $K_d$ values of 0.67 and 0.70 nM, respectively, and competitive binding studies suggest that the IGFBP-3 heparin binding domain may participate in this binding. IGF-I does not compete for IGFBP-3 binding; instead, IGF-I binds immobilized IGFBP-3-fibrinogen and IGFBP-3-fibrin complexes with affinity similar to that of IGF-I for the type I IGF receptor. In the presence of plasminogen, most IGFBP-3 binds directly to fibrinogen, although 35–40% of the IGFBP-3 binds to fibrinogen-bound plasminogen. IGFBP-3 also binds specifically to native fibrin clots, and addition of exogenous IGFBP-3 increases IGF-I binding. These studies suggest that IGF-I can concentrate at wound sites by binding to fibrin-immobilized IGFBP-3, and that the lower IGF affinity of fibrin-bound IGFBP-3 allows IGF-I release to type I IGF receptors of stromal cells migrating into the fibrin clot.

Insulin-like growth factor-binding protein-3 (IGFBP-3), one of 6 structurally related IGFBPs that bind IGF peptides with high affinity, is a 29-kDa protein found in body fluids as multiple ~40–50-kDa forms due to differential glycosylation (1–3). IGFBP-3 is the most abundant serum IGFBP during extracellular life, where it circulates with one IGF peptide and a single acid-labile subunit in a ~150-kDa ternary complex (1, 4, 5). Expression of IGFBP-3 by many tissues suggests that it is also available locally to modulate the autocrine/paracrine actions of the IGF peptides (1).

IGF-I is a 7.6-kDa protein with mitogenic, metabolic, differentiative, chemotactic, and anti-apoptotic effects (1, 6, 7). Since IGFBPs such as IGFBP-3 have higher affinity for IGF-I than does the type I IGF receptor, it is not surprising that IGFBP-3 can inhibit IGF action (1, 8). However, IGFBP-3's bound to extracellular matrix may have lower IGF affinity (9–11). By binding an 18-amino acid heparin binding domain (HBD), which is highly conserved in IGFBP-3 and the closely related protein IGFBP-5, heparin and certain other glycosaminoglycans apparently change the conformation of these IGFBPs, resulting in significantly lower affinity for IGF-I (9, 12). In addition, IGFBP-3 proteolysis has been described in many in vitro situations, resulting in IGFBP-3 fragments with low affinity for IGFs (1, 13–15). In these situations, IGF-I may be released from IGFBP-3 to type I receptors on the cell surface with subsequent induction of IGF-I effects (1, 2, 8, 13–15).

The IGF system plays an important role in wound healing (16, 17), and both IGF-I and IGFBP-3 are present in wound fluid in significant concentrations (16–19). Recent studies show that plasminogen (Glu-Pg) binds IGFBP-3 and the binary IGFBP-3-IGF-I complex with high affinity by interacting directly with the IGFBP-3 HBD (20). This suggests that Glu-Pg, which plays a crucial role in wound healing and binds to the fibrin clot with high affinity (21–23), may localize IGF-I to the wound site by binding directly to both IGFBP-3-IGF-I complexes and the fibrin clot. However, another possibility is that the IGFBP-3-IGF-I complex binds directly to fibrin; this would be reminiscent of the ability of fibrinogen/fibrin to bind with high affinity to basic fibroblast growth factor, another mitogen that plays a role in the wound healing process (24). This paper describes studies which show that IGFBP-3 and IGFBP-3-IGF-I complexes do indeed bind with high affinity to fibrinogen/fibrin and to fibrin clots, and suggest that the IGFBP-3 HBD participates in this binding process.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGF-I and LR²-IGF-I were purchased from GroPep, Ltd (Adelaide, Australia). Recombinant human IGFBP-5 was produced in a baculovirus expression system and purified by affinity chromatography and reverse phase-high performance liquid chromatography (25). Most studies used recombinant human IGFBP-3 expressed in Chinese hamster ovary cells and purified as described previously (20, 26). Some experiments used recombinant human IGFBP-3α² and IGFBP-3αββ² produced in a baculovirus expression system and purified as described previously (20, 26); IGFBP-3αββ² is identical to native IGFBP-3, except that the heparin binding domain has been replaced by the homologous but non-heparin binding sequence of IGFBP-1 (26). IGFBP-3 was purified from human amniotic fluid by affinity chromatography and reverse phase-high performance liquid chromatography (20); quantitation was by radiommunoassay using a kit from Diagnostic Systems Laboratories, Inc. ( Webster, TX). Fibrinogen was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN). Peptide IGFBP-3bd (KKG-
IGFBP-3 binds fibrinogen/fibrin

**Solid-phase Plate Binding Assay**—Binding of IGFBP-3 to fibrinogen and fibrin was characterized using an immobilized ligand-based assay system adapted from a similar system used to characterize IGFBP interactions with other proteins (20, 26, 27). Optimal binding conditions, fibrinogen coating concentration, plate type, and incubation parameters were determined and are presented in the following assay protocol. 96-well immunological plates (Polysorb, Nunc, Fisher Scientific, Pittsburgh, PA) were coated with 5 μg/ml fibrinogen, unless otherwise stated, in 0.1 M Na2CO3, pH 9.8, overnight at 4 °C. The plates were rinsed with 200 μl of 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.01% Tween 20, 0.2% bovine serum albumin (BSA), 0.2% NaN3. To convert fibrinogen to fibrin, 100 μl of 20 NIH units/ml human thrombin (Calbiochem, San Diego, CA) in 10 mM sodium phosphate, pH 7.4, 150 mM NaCl, 0.01% Tween 20, 0.2% BSA, 0.2% NaN3, 1 mM CaCl2 was added to washed wells and incubated for 1 h at 37 °C. Fibrinogen conversion to fibrin was terminated by rinsing the plates three times with 1 mM Na2CO3, 8 mM CaCl2, 0.01% Tween 20, 0.2% NaN3. An additional rinse was performed with 5 mM sodium phosphate, pH 6.8, 0.01% Tween 20, 0.2% NaN3. Both fibrinogen and fibrin plates were blocked in 10 mM sodium phosphate, pH 6.8, 150 mM NaCl, 0.2% BSA, 10 mM lysine, 0.2% NaN3, tightly sealed in plastic wrap; and stored for up to 1 month.

For an assay, plates were rinsed twice with 200 μl of 30 mM Tris-acetate, pH 7.4, 10 mM sodium phosphate, 0.1% Tween 20, 0.2% NaN3 (assay buffer). IGF-1, and IGFBP-3 expressed in Chinese hamster ovary cells, were each iodinated by the chloramine-T method to a specific activity of ~150 μCi/μg protein (13). 125I-IGFBP-3 (50,000 cpm) or 125I-IGF-I (50,000 cpm) were incubated with various concentrations of IGFBP-3, IGFBP-3BV, IGFBP-3hbdBP1BV, IGFBP-1, IGFBP-5, IGFBP-3hbd, IGF-I, fibrinogen, Pm, Glu-Pg, e-aminocaproic acid (eACA), arginine, or heparin in 100 μl of assay buffer; unless stated otherwise, incubations were for 1 h at 37 °C. Unbound radioactivity was removed by rinsing the wells twice with 200 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**—96-well immunological plates (MaxiSorb, Nunc) were coated with 5 μg/ml rabbit anti-human fibrinogen antibody (Enzyme Research Laboratories) overnight at 4 °C or for 3 h at 22 °C in 100 μl of 1 mM Na2CO3, pH 9.8. 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 fibrinogen, IGFBP-3BV, 125I-IGFBP-3, and/or human citrate plasma were incubated in a total volume of 200 μl of assay buffer for 1 h at 37 °C; this allows all reactants to associate in the fluid phase. At the end of the incubation period, 100 μl of reagents was transferred to the block and rinsed 96-well plates that were coated with the anti-fibrinogen antibody. After 30 min at 23 °C, unbound radioactivity was removed by rinsing wells twice with assay buffer. Bound radioactivity, representing IGFBP-3/fibrinogen 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 fibrinogen served as an estimate of nonspecific binding.

IGFBP-3 or was estimated by determining the binding of IGFBP-3 to wells in which buffer without fibrinogen/fibrin was absorbed; this value was subtracted from total binding to give specific binding. Each data point is the mean of triplicate determinations. A, 125I-IGFBP-3 binds to fibrinogen/fibrin. ~100,000 cpm of 125I-IGFBP-3 was equilibrated with either solid-phase fibrinogen or fibrin for 1 h at 37 °C. B, competition curve for ~50,000 cpm of 125I-IGFBP-3 was equilibrated with solid-phase fibrinogen for 1 h at 37 °C either without unlabeled IGFBPs or in the presence of increasing concentrations of IGFBP-1, IGFBP-3, or IGFBP-5. C, 125I-IGFBP-3 binding to fibrinogen/fibrin is stable but reversible. ~50,000 cpm of 125I-IGFBP-3 was equilibrated with either solid-phase fibrinogen or fibrin for 1 h at 37 °C. After unbound 125I-IGFBP-3 was removed by rinsing, either buffer alone or buffer containing 0.5 mM arginine, pH 7.4, was added and the incubations continued at 37 °C. At the indicated time points, specific wells were rinsed and bound radioactivity was measured.

**Fig. 1.** 125I-IGFBP-3 binding to fibrinogen/fibrin immobilized on immunocapture plates. Except where noted, plates were coated overnight at 4 °C with 5 μg/ml fibrinogen. In some experiments, fibrinogen was converted to fibrin as described under "Experimental Procedures." Nonspecific binding was determined by adding unlabeled FYKKKQCRPSKGRKQ, which encodes the heparin binding domain of IGFBP-3 (20), was synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). Glu-Pg was purified as described previously (20). Plasmin (Pm) was obtained from American Diagnostics (Greenwich, CT). Human citrate plasma was obtained from outdated blood bank supplies or from laboratory personnel.
**IGFBP-3 Binds Fibrinogen/Fibrin**

Fibrinogen, Glu-Pg, and IGFBP-1 (10 μg each) were separated by SDS-PAGE on a 7.5–15% gradient gel and then transferred to a nitrocellulose membrane. The membrane was incubated overnight at 4 °C with bn-IGFBP-3, washed, and then visualized as described under “Experimental Procedures.” The molecular mass, in Kₑ, of protein markers is shown on the left.

**Fibrin Clot Binding Assay**—Fibrin clots were formed in 1.5-ml Eppendorf tubes by mixing citrate plasma with assay buffer containing CaCl₂; total reaction volume was 100 μl, final CaCl₂ concentration was 5 mM and, unless stated otherwise, 25 μl of citrate plasma were added. After adding ~100,000 cpm of ¹²⁵I-IGF-I or ¹²⁵I-IGFBP-3, either with or without IGFBP-3, the mixture was incubated for 90 min at 37 °C. Nonspecific incorporation of trace into fibrin clots was determined in mixtures not containing CaCl₂. To terminate incubations, fibrin clots were carefully suspended in 500 μl of ice-cold assay buffer and then pelleted by centrifuging at 14,000 × g for 2 min. After this wash step was repeated, the vial tips were cut off and then counted for radioactivity.

**Biotinylation of IGFBP-3**—IGFBP-3 BV (13 g) was diluted in 0.25 M NaHCO₃, pH 9.5, to a total volume of 45 μl. After 1.4 mg of NHS-LS-biotin (Pierce) was dissolved in 40 μl of H₂O, 5 μl of this solution were mixed with the IGFBP-3 BV solution. The mixture was placed on an orbital shaker for 1 h at 22 °C, after which the reaction was terminated by adding 200 μl of 1 M Tris-HCl, pH 7.4. Unincorporated biotin was separated from biotinylated IGFBP-3 BV (bn-IGFBP-3) using a Quick-spin column as instructed by the manufacturer (Roche Molecular Biochemicals). By SDS-PAGE, IGFBP-3 BV in bn-IGFBP-3 was the size expected for the intact native protein. Preparations of bn-IGFBP-3 were stored at 4 °C.

**Western Ligand Blotting Using bn-IGFBP-3**—bn-IGFBP-3 BV (13 g) was diluted in 0.25 M NaHCO₃, pH 9.5, to a total volume of 45 μl. After 1.4 mg of NHS-LS-biotin (Pierce) was dissolved in 40 μl of H₂O, 5 μl of this solution were mixed with the IGFBP-3 BV solution. The mixture was placed on an orbital shaker for 1 h at 22 °C, after which the reaction was terminated by adding 200 μl of 1 M Tris-HCl, pH 7.4. Unincorporated biotin was separated from biotinylated IGFBP-3 BV (bn-IGFBP-3) using a Quick-spin column as instructed by the manufacturer (Roche Molecular Biochemicals). By SDS-PAGE, IGFBP-3 BV in bn-IGFBP-3 was the size expected for the intact native protein. Preparations of bn-IGFBP-3 were stored at 4 °C.

**RESULTS**

125I-IGFBP-3 Binds Immobilized Fibrinogen/Fibrin—125I-IGFBP-3 bound specifically to fibrinogen or fibrin immobilized on immunocapture plates. As shown in Fig. 1A, increasing the amount of fibrinogen/fibrin immobilized on the plates resulted in increased binding of 125I-IGFBP-3. IGFBP-5 and IGFBP-3, but not IGFBP-1, efficiently competed with 125I-IGFBP-3. Each data point is the mean ± S.E. of triplicate determinations.

**Scatchard analysis revealed that:**

1) IGFBP-3 bound comparatively to fibrinogen (Kₛ = 0.67 ± 0.2 nM; mean ± S.E. of three independent experiments) and fibrin (Kₛ = 0.7 ± 0.2 nM; mean ± S.E. of three independent experiments); 2) 1 mol of fibrinogen bound 0.15 mol of IGFBP-3; and 3) 1 mol of fibrin had 59 ± 15% more 125I-IGFBP-3 binding sites than did 1 mol of fibrinogen. As shown in Fig. 1C, IGFBP-3 binding to immobilized fibrinogen or fibrin was quite stable but was rapidly reversible in the presence of 500 mM arginine.

IGFBP-3 also bound fibrinogen by ligand blot. Fibrinogen, Glu-Pg, and IGFBP-1 were electrophoresed by SDS-PAGE,
transferred to a nitrocellulose membrane, and then incubated with bn-IGFBP-3. As shown in Fig. 2, bn-IGFBP-3 bound as expected to the ∼86-kDa Glu-Pg protein but not to IGFBP-1. In addition, bn-IGFBP-3 bound to at least two proteins of mass >200 kDa in the fibrinogen lane; the size of these proteins is consistent with the expected size of the common forms of plasma fibrinogen (24, 28).

Role of the IGFBP-3 HBD in Fibrinogen/Fibrin Binding—Since the IGFBP-3 HBD participates in binding to plasminogen, prekallikrein, and the IGF acid-labile subunit binary complex (4, 20, 26), it seemed likely that the IGFBP-3 HBD participates in fibrinogen binding. To test this hypothesis, heparin and a synthetic 18-amino acid peptide encoding the IGFBP-3 HBD were evaluated for their ability to compete with 125I-IGFBP-3 for binding to fibrinogen/fibrin. To determine the affinity of IGF for IGFBP-3 bound to immobilized fibrinogen/fibrin, increasing amounts of unlabeled IGF-I were coincubated with 125I-IGFBP-3 (100 ng/ml) in the presence of 125I-IGFBP-3 (70 fmol/well) or IGFBP-3-fibrin complexes (115 fmol/well). As shown in Fig. 4B, IGFBP-3 (100 ng/ml) was bound to solid-phase fibrinogen or fibrin for 1 h at 37 °C. Unbound IGFBP-3 was removed by rinsing. 125I-IGF-I and increasing concentrations of unlabeled IGF-I were equilibrated with bound IGFBP-3 for 1 h at 37 °C. Bound 125I-IGF-I was determined. Each data point is the mean ± S.E. of triplicate determinations. C, IGFBP-3 (100 ng/ml) was bound to solid-phase fibrin at least 1 h at 37 °C. Unbound IGFBP-3 was removed by rinsing. 125I-IGF-I and increasing concentrations of unlabeled IGF-I or LR3-IGF-I were equilibrated with bound IGFBP-3 for at least 1 h at 37 °C. Bound 125I-IGF-I was determined. Each data point is the mean ± S.E. of triplicate determinations. D, ~50,000 cpm of 125I-IGFBP-3 and increasing concentrations of IGF-I were equilibrated with either solid-phase fibrinogen or fibrin for at least 1 h at 37 °C. Specifically bound 125I-IGFBP-3 was determined. Each data point is the mean ± S.E. of two separate experiments performed in duplicate.

IGFBP-3 mediates the binding of 125I-IGF-I to fibrinogen/fibrin. In each study, incubations were performed in fibrinogen- or fibrin-coated plates using ∼50,000 cpm 125I-IGF-I. A, 125I-IGF-I was coincubated with increasing amounts of unlabeled IGFBP-3. Bound 125I-IGF-I was measured. Nonspecific binding was determined by measuring the binding of 125I-IGF-I in the absence of IGFBP-3. Each data point is the mean ± S.E. of triplicate determinations. B, IGFBP-3 (100 ng/ml) was bound to solid-phase fibrinogen or fibrin for 1 h at 37 °C. Unbound IGFBP-3 was removed by rinsing. 125I-IGF-I and increasing concentrations of unlabeled IGF-I were equilibrated with bound IGFBP-3 for 1 h at 37 °C. Bound 125I-IGF-I was determined and Scatchard plot calculated. Each data point is the mean of triplicate determinations. C, IGFBP-3 (100 ng/ml) was bound to solid-phase fibrin at least 1 h at 37 °C. Unbound IGFBP-3 was removed by rinsing. 125I-IGF-I and increasing concentrations of unlabeled IGF-I or LR3-IGF-I were equilibrated with bound IGFBP-3 for at least 1 h at 37 °C. Bound 125I-IGF-I was determined. Each data point is the mean ± S.E. of triplicate determinations. D, ~50,000 cpm of 125I-IGFBP-3 and increasing concentrations of IGF-I were equilibrated with either solid-phase fibrinogen or fibrin for at least 1 h at 37 °C. Specifically bound 125I-IGFBP-3 was determined. Each data point is the mean ± S.E. of two separate experiments performed in duplicate.
IGFBP-3 Binds Fibrinogen/Fibrin

only minimally (−30%) for 125I-IGFBP-3 binding to immobilized fibrinogen, suggesting that IGFBP-3 binding to a fibrin clot can occur in the presence of physiologic concentrations (−3–4 mg/ml) of plasma fibrinogen (30).

IGFBP-3 Increases IGF-I Binding in Fibrin Clots—Native fibrin clots, induced using calcium saturation of citrate plasma, specifically bound 125I-IGFBP-3 (10,600 ± 130 cpm specifically bound/100,000 cpm 125I-IGFBP-3 added; mean ± S.E. of three experiments performed in triplicate). This suggests that IGFBP-3 can be bound during clot formation. Furthermore, the amount of 125I-IGF-I specifically bound within these fibrin clots is directly related to the amount of plasma used to generate the clot (data not shown). As IGFBP-3 is the primary circulating IGFBP, it is likely that IGFBP-3 is primarily responsible for immobilizing IGF-I in the fibrin clot. When forming fibrin clots using 25 μl of citrate plasma, 125I-IGF-I was specifically bound (6,200 ± 140 cpm specifically bound/100,000 cpm 125I-IGF-I added), and 125I-IGF-I specific binding increased by 62 ± 11% upon the further addition of 100 ng of IGFBP-3 (mean ± S.E. of two experiments performed in quadruplicate). These data suggest that IGF-I is immobilized in fibrin clots by associating with fibrin-bound IGFBP-3.

Fibrin Clots Contain Both IGFBP-3 and Glu-Pg—Immunofluorescent staining was also used to determine whether IGFBP-3 was incorporated into native fibrin clots. As shown in Fig. 6, specific antibodies to IGFBP-3 and Glu-Pg did indeed recognize each protein in cross-sections of fibrin clots. This is consistent with the ability of Glu-Pg to bind fibrin with high affinity (22, 23). However, both fibrin and Glu-Pg bind with high affinity to IGFBP-3, and Fig. 6 does not discriminate which protein binds IGFBP-3 in fibrin clots.

IGFBP-3 Binds Fibrinogen Both Directly and via Glu-Pg—As shown in Fig. 7A, partial proteolysis of immobilized fibrinogen by 5 ng/ml Pm completely inhibits IGFBP-3 binding, presumably by destroying IGFBP-3 binding sites. However, coinoculation of IGFBP-3 with Glu-Pg allows IGFBP-3 binding to fibrinogen partially proteolyzed by 5 ng/ml Pm. This increase is likely due to IGFBP-3 binding to fibrin-bound Glu-Pg, since partial digestion of fibrinogen may actually increase Glu-Pg binding by uncovering previously hidden Glu-Pg binding sites (22).

Previous studies found that 10 mM eACA completely blocks Glu-Pg binding to fibrinogen (31). However, as shown in Fig. 7B, 25 mM eACA only weakly inhibits fibrinogen binding to IGFBP-3; as expected, arginine is a potent inhibitor of fibrinogen binding to IGFBP-3. This suggests that eACA can be used to discriminate between IGFBP-3 binding to fibrinogen and to fibrinogen-bound Glu-Pg. Indeed, as shown in Fig. 7C, when solid-phase fibrinogen is pre-equilibrated with Glu-Pg, IGFBP-3 binding is inhibited 35–40% in the presence of 10 or 25 mM eACA, suggesting that 35–40% of IGFBP-3 binds to fibrinogen-bound Glu-Pg.

DISCUSSION

IGF-I participates in wound healing by acting as a chemotactic agent supporting the early migration ofstromal cells into...
the fibrin clot at the wound site, and by stimulating proliferation of fibroblasts and endothelial cells (16, 17). Potential sources for IGF-I found at the wound site include plasma, platelets (32), monocytes (33), and macrophages (34). As with IGFs in other body fluids, IGF-I in wound fluid is complexed with IGF-binding proteins, primarily IGFBP-3, which is abundant in serum and in platelets (18, 19, 32). Studies presented in this report, which show that IGFBP-3-1GF-I binary complexes bind with high affinity to fibrinogen/fibrin, suggest that direct binding of IGFBP-3-IGF-I complexes to the fibrin clot is an important mechanism by which IGF-I levels may be maintained at the wound site.

Complex formation between IGFBP-3 and fibrinogen or fibrin was shown by solid- and fluid-phase plate binding assays and by Western ligand blotting using biotinylated IGFBP-3. Similar techniques have been used to identify other proteins which bind IGFBP-3 (20, 26). The binding affinity of IGFBP-3 for immobilized fibrinogen and fibrin was $K_{d} = 0.87$ and 0.7 nM, respectively. These values suggest that fibrinogen/fibrin affinity for IGFBP-3 is comparable to or stronger than fibrinogen/fibrin affinity for Glu-Pg, tissue plasminogen activator, plasminogen activator inhibitor, apolipoprotein(a), thrombin, factor Xa, factor XIII, fibronectin, thrombospondin and basic fibroblast growth factor (22–24, 35–41). The binding affinities of IGFBP-3 for fibrinogen/fibrin are roughly comparable to the affinity of the IGFBP-3-IGF-I binary complex for acid labile subunit (5), and to the affinity of IGFBP-3 for Glu-Pg and prekallikrein (20, 26).

Fibrinogen and fibrin appear to interact with a specific HBD of IGFBP-3, which is rich in lysine groups and which participates in IGFBP-3 binding to acid-labile subunit, Glu-Pg, and prekallikrein (4, 20, 26). The sequence and location of the IGFBP-3 HBD is conserved in IGFBP-5 (12). Studies presented here show that IGFBP-5 efficiently competes with IGFBP-3 binding to fibrinogen or fibrin, suggesting that IGFBP-5 also binds fibrinogen and fibrin with high affinity. In contrast, the IGFBP-3 HBD sequence is not conserved in IGFBP-1 (12), IGFBP-1 does not bind appreciably to fibrinogen, and replacing the IGFBP-3 HBD sequence with homologous sequence from IGFBP-1 results in a 10-fold loss of affinity for fibrin. The residual ability of IGFBP-3-hbdBP1BV to compete with native IGFBP-3 for binding to fibrin suggests that, in addition to the HBD, other IGFBP-3 regions may participate in binding to fibrinogen/fibrin.

Polymerization of fibrinogen into fibrin did not alter affinity for IGFBP-3, but it did increase the number of $^{125}$I-IGFBP-3 binding sites by 59% and the number of $^{125}$I-IGFBP-3 binding sites by 63%. These data suggest that cleavage of fibrinogen exposes new binding sites for IGFBP-3, just as it exposes new binding sites for Glu-Pg (22). These data also suggest that formation of binary complexes between IGFBP-3 and immobilized fibrin does not prevent the further binding of fibrinogen to fibrin.

**Fig. 6.** Both Glu-Pg and IGFBP-3 are detectable in fibrin clots. Normal human citrate plasma was clotted by addition of CaCl$_2$ to a final concentration of 5 mM. After 90 min, the resultant fibrin clot was rinsed and cryosectioned into 5-µm sections. Sequential sections were incubated with normal rabbit serum (NRS) or with rabbit antiserum to plasminogen or IGFBP-3. Immunofluorescent staining was recorded using fluorescein isothiocyanate-labeled donkey anti-rabbit IgG as the secondary antibody.

**Fig. 7.** IGFBP-3 binds to fibrinogen both directly and via Glu-Pg. A, fibrinogen immobilized on immunocapture plates was pretreated with increasing concentrations of Pm for 1 h at 37 °C. Pm was removed by rinsing wells extensively with 200 mM eACA followed by buffer alone. $\sim 50,000$ cpm $^{125}$I-IGFBP-3 was removed by rinsing. Bound radioactivity was determined by addition of $1 \times$ NaOH to well contents for 10 min at 23 °C. After nonspecific binding was subtracted out, specific binding was normalized to % control values (i.e. binding of IGFBP-3 to solid-phase fibrinogen not exposed to Pm). Each data point is the mean ± S.E. of quadruplicate determinations. B, $\sim 50,000$ cpm $^{125}$I-IGFBP-3 were equilibrated with solid-phase fibrinogen and with increasing concentrations of either arginine or eACA for at least 1 h at 37 °C. Unbound $^{125}$I-IGFBP-3 was removed by rinsing. Bound radioactivity was determined by addition of $1 \times$ NaOH to well contents for 10 min at 23 °C. Each data point is the mean ± S.E. of duplicate determinations. C, $\sim 50,000$ cpm $^{125}$I-IGFBP-3 were equilibrated with solid-phase fibrinogen and either with or without 10 µg of Glu-Pg along with 0, 10, or 25 mM eACA for at least 1 h at 37 °C. Unbound $^{125}$I-IGFBP-3 was removed by rinsing. Bound radioactivity was determined by addition of $1 \times$ NaOH to well contents for 10 min at 23 °C. After nonspecific binding was subtracted out, specific binding was normalized to percentage of control values (i.e. $^{125}$I-IGFBP-3 alone or $^{125}$I-IGFBP-3 + Glu-Pg). Each data point is the mean ± S.E. of quadruplicate determinations.
IGF-I to IGFBP-3, thus forming a ternary complex. Additional studies confirmed that IGF-I binds to IGFBP-3 complexed with immobilized fibrin (Kd = 2.3 nM), and also found that IGF-I binds to IGFBP-3 complexed with immobilized values of fibrin (Kd = 2.9 nM). These Kd values are higher than the range of values (0.03–5 nM) reported previously for IGF-I binding to IGFBP-3 (42), and are comparable to the affinity of IGFBP-3 for the type I IGF receptor (43). This suggests that type I IGF receptors on fibroblasts and other cells that are migrating into the fibrin clot can remove IGF-I from fibrin-bound IGFBP-3, resulting in proliferation of these cells at the wound site.

In addition to the direct binding of IGFBP-3 to fibrinogen, studies presented here suggest that IGFBP-3 also binds indirectly, to fibrinogen-bound Glu-Pg. It is well known that Glu-Pg binds with high affinity to fibrinogen/fibrin and that, through this interaction, Glu-Pg plays an important role in the wound healing process (21–23). It is also known that IGFBP-3 and IGF-I-IGFBP-3 complexes bind Glu-Pg with high affinity, and that IGFBP-3 binds to multiple sites on Glu-Pg (20). Thus, a complex of fibrin-Glu-Pg-IGFBP-3-I GF-I is likely to form; in one possible arrangement, the kringle 1–3 regions of Glu-Pg bind to lysines in fibrinogen/fibrin, while the kringle 5/catalytic region of Glu-Pg binds to the HBD of IGFBP-3. If formed, this complex would provide another mechanism for releasing IGF-I at the wound site, since tissue plasminogen activator can activate immobilized Glu-Pg that is complexed with IGFBP-3-I GF-I, resulting in IGFBP-3 proteolysis and IGF-I release (20). Indeed, in tissue culture, activation of Glu-Pg to Pm results in IGFBP-3 proteolysis and release of bound IGF-I to cultured cells where mitogenic and metabolic pathways are stimulated (44, 45); thus, activation of Glu-Pg that is complexed both with fibrinogen and with IGFBP-3-I GF-I should ultimately make IGF-I available to stromal cells present at the wound site. In support of this hypothesis, additional studies show that plasminogen activators can induce proteolysis of IGFBP-3 complexed with fibrin-bound Glu-Pg, resulting in release of IGF-I (manuscript in preparation).

The observations that 125I-IGFBP-3 and 125I-IGF-I localize to fibrin clots generated from plasma in vitro, and that addition of exogenous IGFBP-3 greatly increases the incorporation of 125I-IGF-I into these clots, suggest that IGF-I-IGFBP-3 complexes bind to fibrin clots in vivo at wound sites. This possibility is supported by immunoblot detection of IGFBP-3 in these fibrin clots. The further detection of Glu-Pg in these fibrin clots by immunoblotting suggests that IGFBP-3 may bind these clots directly through fibrin and indirectly through fibrin-bound Glu-Pg. Thus, data obtained from the study of these fibrin clots support the hypothesis that IGFBP-3-I GF-I complexes bind to fibrin clots formed at wound sites in vivo, and are consistent with the possibility that at least two mechanisms exist for the ready release of bound IGF-I to fibroblasts and endothelial cells migrating into the wound site.

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

REFERENCES
1. Jones, J., and Clemons, D. R. (1995) Endocrin. Rev. 16, 3–32
2. Durham, S. K., Mohan, S., Liu, F., Baker, B. K., Lee, P. D. K., Hintz, R. L.,
Conover, C. A., and Powell, D. R. (1997) Pediatr. Res. 42, 335–341
3. Cubbage, M. L., Suwanichkul, A., and Powell, D. R. (1999) J. Biol. Chem. 263, 12642–12649
4. Baxter, R. C., and Firth, S. M. (1995) Prog. Growth Factor Res. 6, 215–222
5. Leong, S. R., Baxter, R. C., Camerato, T., and Wood, W. I. (1992) Mol. Endocrinol. 6, 870–876
6. Jones, J., Doer, M. E., and Clemons, D. R. (1995) Prog. Growth Factor Res. 6, 319–327
7. Werner, H., and Leroith, D. (1997) Crit. Rev. Oncog. 8, 71–92
8. Conover, C. A., Ronk, M., Lombana, F., and Powell, D. R. (1996) Endocrinology 137, 2797–2803
9. Arai, T., Parker, A., Busby, W. H., Jr., and Clemons, D. R. (1994) J. Biol. Chem. 269, 20388–20393
10. Jones, J., Goshko, A., Busby, W. H., Jr., Camacho-Hubner C., and Clemons, D. R. (1993) J. Cell Biol. 121, 679–687
11. Campbell, P. G., and Andress, D. L. (1997) Am. J. Physiol. 273, E1005–E1013
12. Booth, B. A., Boes, M., Andress, D. L., Dake, B. L., Kiefc, M. C., Maack, C., Linhardt, R. J., and Barr, K. (1995) J. Biol. Chem. 270, 25–30
13. Kirstein, M., Aston, C., Hintz, R., and Vlassara, H. (1992) J. Clin. Invest. 90, 1028–1034
14. Conover, C. A., Ronk, M., Lombana, F., and Powell, D. R. (1996) Endocrinology 137, 2797–2803
15. Frost, V. J., Macaulay, V. M., Wess, J. A. H., and Holly, J. M. P. (1993) J. Endocrinol. 135, 259–264
16. Campbell, P. G., and Andress, D. L. (1997) J. Biol. Chem. 272, 2318–2326
17. Clark, R. E. (1996) Molecular and Cellular Biology of Wound Repair, pp. 1–50, Plenum Press, New York
18. Robertson, J. G., Pickering, K. J., and Belford, D. A. (1996) Endocrinology 137, 2774–2784
19. Vogt, P. M., Lehnhardt, M., Wagner, D., Jansen, V., Krieg, M., and Steinau, H. (1998) Plast. Reconstr. Surg. 102, 117–123
20. Campbell, P. G., Durham, S. K., Suwanichkul, A., Hayes, J. D., and Powell, D. R. (1998) Am. J. Physiol. 275, E321–E331
21. Romer, J., Bagge, T. H., Pyke, C., Lund, J. L., Flick, M. J., Degen, J. L., and Durano, K. (1996) Eur. J. Biochem. 239, 599–600
22. Tran-Thang, C., Kruithof, E. K. O., Atkinson, J., and Bachmann, F. (1986) Eur. J. Biochem. 160, 599–600
23. Rouy, D., Koschinsky, A., Fleury, V., Chapman, J., and Angles-Cano, E. (1992) Biochemistry 31, 6333–6339
24. Sahni, A., Odrdijen, T., and Francis, C. W. (1998) J. Biol. Chem. 273, 7554–7559
25. Andress, D. L., Loop, S. M., Zapf, J., and Kiefer, M. C. (1995) Biochem. Phosphoproteins. Res. Commun. 130, 559–565
26. Durham, S. K., Suwanichkul, A., Hayes, J. D., Herington, A., Powell, D. R., and Campbell, P. G. (1999) Horm. Metab. Res. 31, 16–25
27. Campbell, P. G., and Andress, D. L. (1997) J. Am. J. Physiol. 273, E996–E1004
28. Ballard, F. J., Walton, P. E., Bastian, S., Tomas, F. M., Wallace, J. C., and Francis, G. L. (1998) J. Clin. Invest. 101, 439–446
29. Rappolee, D. A., Mark, D., Banda, M. J., and Werb, Z. (1998) Science 281, 708–712
30. Keijer, J., Linders, M., van Zonneveld, A. J., Ehrlich, H. J., de Boer, J. P., and Pannekoek, H. (1991) Blood 78, 1011–1019
31. Ichinose, A., Takio, K., and Fujikawa, K. (1986) J. Clin. Invest. 78, 139–146
32. Greenberg, C. S., Doehson, J. V., and Miraglia, C. C. (1985) Blood 66, 1028–1034
33. Tuszynski, G. P., Srivastava, S., Viale, P., Auer, R. B., and Viale, G. (1996) J. Biol. Chem. 271, 7554–7559
34. Xia, Y., Viale, P., and Castegna, A. (2001) J. Biol. Chem. 276, 10222–10228
35. Xia, Y., Viale, P., and Castegna, A. (2001) J. Biol. Chem. 276, 10222–10228