Insulin-like Growth Factor-binding Protein-5 Activates Plasminogen by Interaction with Tissue Plasminogen Activator, Independently of Its Ability to Bind to Plasminogen Activator Inhibitor-1, Insulin-like Growth Factor-1, or Heparin

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Transgenic mice expressing IGFBP-5 in the mammary gland exhibit increased cell death and plasmin generation. Because IGFBP-5 has been reported to bind to plasminogen activator inhibitor-1 (PAI-1), we determined the effects of this interaction in HC11 cells. PAI-1 prevented plasmin generation from plasminogen and inhibited cleavage of focal adhesions, expression of caspase 3, and cell death. IGFBP-5 could in turn prevent the effects of PAI-1. IGFBP-5 mutants with reduced affinity for IGF-I (N-term) or deficient in heparin binding (HEP- and C-term E and F) were also effective. This was surprising because IGFBP-5 reportedly interacts with PAI-1 via its heparin-binding domain. Biosensor analysis confirmed that, although wild-type IGFBP-5 and N-term both bound to PAI-1, the C-term E had greatly decreased interaction with PAI-1. This suggests that IGFBP-5 does not antagonize the actions of PAI-1 by a direct molecular interaction. In a cell-free system, using tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) to activate plasminogen, PAI-1 inhibited plasmin generation induced by both activators, whereas IGFBP-5 prevented the effects of PAI-1 on tPA but not uPA. Furthermore, we noted that IGFBP-5 activated plasminogen to a greater extent than could be explained solely by inhibition of PAI-1, suggesting that IGFBP-5 could directly activate tPA. Indeed, IGFBP-5 and the C-term E and F were all able to enhance the activity of tPA but not uPA. These data demonstrate that IGFBP-5 can enhance the activity of tPA and that this can result in cell death induced by cleavage of focal adhesions. Thus IGFBP-5 can induce cell death by both sequestering IGF-I and enhancing plasmin generation.

We have shown previously a large increase in the concentration of IGFBP-5 protein in the milk of rats after 48 h of mammary involution induced by removal of the suckling young, and we propose that this acts to inhibit IGF-I interaction with its receptor on the epithelial cells, resulting in cell death (1). We subsequently showed this to be the case in three independent studies, involving transgenic animals expressing IGFBP-5 in the mammary gland (2) as well as exogenous treatment with IGFBP-5 both in vivo (3) and in vitro (4). More recently we have shown that IGFBP-5 mRNA levels are also significantly increased during involution in the mouse mammary gland (5). Changes consistent with a role for IGFBP-5 as an inhibitor of IGF-I-mediated cell survival were evident, because phosphorylation of the type I IGF receptor and a downstream signaling molecule Akt were both decreased in IGFBP-5 transgenic mice. Furthermore, these mice exhibited decreased concentrations of two pro-survival molecules, Bcl-2 and Bcl-xL, and increased activity of caspase-3. Most intriguingly, these mice also exhibited increased concentrations of plasmin in their mammary glands.

The remodeling of the mammary gland, which occurs at the end of lactation, involves an initial phase in which there are dramatic increases in the rates of apoptosis and a second phase involving extracellular proteases, including the plasminogen system and matrix metalloproteases, which are involved in degrading the extracellular matrix in the later stages of tissue remodeling (6). IGFBP-5 has been reported to bind to PAI-1 and therefore to influence IGF-mediated cell responses (7). Recent studies have suggested that the effects of IGFBP-3 and -5 can be both IGF-dependent and IGF-independent and that interactions with particular extracellular matrix components might influence these responses (8, 9). These observations led us to consider whether the extremely high concentrations of IGFBP-5 in milk from involuting mammary glands (~50–100 μg/ml) were present solely to inhibit IGF actions or whether they were also involved in additional and perhaps IGF-independent actions (10). Thus we were interested in examining whether IGFBP-5 is also involved in the regulation of tissue remodeling via a direct influence upon the plasminogen system. We have identified a specific interaction of IGFBP-5 with α2-casein (11). This milk protein has also been shown to bind plasminogen and its activator, tPA (12), and the physical apposition of all of these molecules adds further weight to the hypothesis that IGFBP-5 may directly influence aspects of plasminogen activation.

The aims of this study were therefore to determine whether IGFBP-5 and IGFBP-5 mutants that are unable to bind to IGF-1 or heparin are able to interact with PAI-1 and influence plasminogen activation. If so, this could provide a mechanism by which IGFBP-5 could coordinate the induction of cell death (by sequestering IGF-I) with the activation of extracellular matrix degradation by generation of the extracellular protease cascades initiated by plasminogen activators.

3 The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; WT, wild type; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1; EACA, ε-amino caproic acid; DTT, dithiothreitol; pNA, p-nitroanilide; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; RP-HPLC, reverse phase-high pressure liquid chromatography; VLL-pNA, H-Val-Leu-Lys-p-nitroanilide; GST, glutathione S-transferase.
IGFBP-5 and Plasminogen Activation

MATERIALS AND METHODS

Cell Culture—The mouse mammary epithelial cell line HC11 (a subclone of COMMA-1D cells derived from mid-pregnant mice) (13) was a kind gift from Dr. Bruce Whitelaw, Roslin Institute, Edinburgh, Scotland, UK, and was cultured as described previously (14). Bovine insulin (I-6634), epidermal growth factor (E-4127), and uPA were from Sigma. tPA, plasminogen activator inhibitor-1 (PAI-1), and plasminogen were from Calbiochem. PAI-1 was a mutant form (K154T, Q319L, M354L, and N150H), which is virtually unable to be converted to the latent form (half-life = 145 h at 37 °C, pH 7.4) (15). DMEM (phenol red free 31053-028), penicillin/streptomycin (15140-122), L-glutamine (25030-024), and fetal calf serum were from Invitrogen. When cells reached confluence, cultures were washed with serum-free medium and treated with various combinations of plasminogen, PAI-1, and wtIG-FBP-5 or mutant IGFBP-5 proteins in serum-free DMEM containing 0.1% BSA. Cells were cultured for a further 24 h, at which time conditioned medium was removed for analysis of plasmin activity. At the end of the culture period, cell monolayers were washed with PBS, fixed with 4% paraformaldehyde, and stained with crystal violet for histological analysis. After photographing the cells, the crystal violet stain was eluted in PBS containing 1% Triton X-100, and absorbance was read at 590 nm. Treatments were performed in duplicate or triplicate.

Focal Adhesions—After fixation, cells were incubated with phalloidin and antibodies to vinculin to identify focal adhesions and actin stress fibers, exactly as described previously (16).

Cell Death—Caspase-3 activity was measured using a colorimetric assay kit (Sigma, CASP-3-C) according to the manufacturer’s instructions. After 24 h of treatment, cells were lysed on ice for 10 min in cell lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM EDTA). Lysates were then incubated with 0.2 mM Ac-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNA) in reaction buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM EDTA) at 37 °C, and the absorbance at 405 nm was read at intervals to measure released pNA.

Assay of Plasmin Activity—Plasmin activity was measured by incubating conditioned media, from cells cultured in serum-free conditions, in 50 mM Tris-HCl, 0.1% Tween 20, pH 7.4, with 25 µl of the artificial plasmin substrate 6 mM H-Val-Leu-Lys-p-nitroanilide (VLL-pNA) from Bachem (0554101). The absorbance at 405 nm was measured at 15-min intervals for 4 h, and plasmin activity was determined by the rate of change in absorbance.

Plasminogen Activator Assays—tPA and uPA activities were measured indirectly by their ability to convert plasminogen to plasmin followed by cleavage of the plasmin substrate VLL-pNA. tPA or uPA was incubated in the presence or absence of PAI-1 and IGFBP-5 in 96-well plates containing 2 µM plasminogen, 10 µl of 6 mM VLL-pNA, and buffer (50 mM Tris-HCl plus 0.1% Tween 20, pH 7.4). Absorbance at 405 nm was measured at 15-min intervals for 3 h. tPA standards (5–200 ng/ml) were used to determine a dose-response curve. After an initial lag phase, the generation of plasmin increased over the first 15–75 min (depending upon the quantity of tPA), and this period was used to quantitatively measure the effects of IGFBP-5 and PAI-1 on the activity of tPA, using the tPA standards as a reference. tPA activity was also measured directly using the Chromozym-tPA assay (Roche Diagnostics) according to the manufacturer’s instructions with the tPA substrate N-methylsulfonyl-d-Phe-Gly-Arg-4-nitroanilide acetate. tPA (20 ng/ml) was incubated either alone or in the presence of varying concentrations of IGFBP-5. Absorbance readings were monitored at 405 nm for 4 h.

Comparison of tPA Activating Activities of IGFBP-5 with Fibrin—Soluble fibrin fragments were prepared from a suspension of fibrin from human plasma (Sigma). 100 mg/ml fibrin suspension was centrifuged at 14,000 × g for 20 min, and the soluble fraction in the supernatant was used. The protein concentration of soluble fibrin was determined by using the Bradford assay. Such soluble fragments of fibrin have been shown to mimic the effect of polymeric fibrin (17, 18). tPA (5 ng/ml) was incubated in the absence or presence of soluble fibrin (1–100 mg/ml) or IGFBP-5 and mutants thereof (1–100 mg/ml) in 96-well plates containing 2 µM plasminogen, 10 µl of 6 mM VLL-pNA and buffer (50 mM Tris-HCl plus 0.1% Tween 20, pH 7.4). Absorbance at 405 nm was measured at 15-min intervals for 3 h.

The ability of fibrin and IGFBP-5 to enhance the activity of tPA was also investigated in the presence of two inhibitors of fibrin-mediated tPA enhancement. Fibrin activation of tPA involves fibrin fragments containing C-terminal lysine residues, and we thus used a competitive inhibitor EACA, a lysine analogue, and carboxypeptidase-B that enzymatically removes C-terminal lysine residues. tPA (5 ng/ml) was incubated with fibrin or IGFBP-5 (both at 100 mg/ml) in the absence or presence of 50 mM EACA (Sigma) or 10 mg/ml pancreatic carboxypeptidase-B (Sigma) in 96-well plates containing 10 µl of 2 µM plasminogen, 10 µl of 6 mM VLL-pNA and buffer (50 mM Tris-HCl plus 0.1% Tween 20, pH 7.4). Absorbance at 405 nm was measured at 15-min intervals for 4 h.

Production of Recombinant Mouse IGFBP-5—Expression of recombinant IGFBP-5 proteins was carried out using conditions identical to those described by Allan et al. (3). cDNAs for mouse wtIG-FBP-5 and various IGFBP-5 mutants minus the signal peptide-encoding sequence were cloned into the pGEX 6P-1 vector (Amersham Biosciences) between BamHI and EcoRI in the multiple cloning site, so that the proteins would have an N-terminal glutathione S-transferase (GST) tag. The mutants used in this study included N-term, a mutant with five mutations in the N-terminal domain (K68N/P69Q/L70Q/K73Q/L74Q) with greatly reduced affinity for IGFs (19, 20), and three C-terminal mutants. The C-terminal mutants consisted of HEP−, in which four basic residues in the C terminus were mutated (R201L/K202E/K206Q/R214A (21)) and C-term E (R201A/K202E/K206A/R207A/K208A/K211A/R214A/R216A/K217A/R218A (22)), and a mutant, C-term F, based upon C-term E but with additional mutations in the central domain of IGFBP-5 (R136A/R137A), which are involved in a second putative heparin-binding domain (23).

Purification of IGFBPs—IGFBPs were purified from glutathione affinity columns after release from GST using the PreScission Protease system (3).

IGFBP-5 proteins were further purified in two ways, either on IGF affinity columns and quantified as described previously (23) or by RP-HPLC. This involved using a polymeric column (PLRP-S, 300 Å; 8-µm beads; 4.6 mm diameter × 150 mm; Polymer Laboratories Ltd., Church Stretton, UK) equilibrated with a mobile phase of 25% acetonitrile in 0.1% trifluoroacetic acid. 1-ml aliquots of GST-purified protein (2–4 mg of protein) in cleavage buffer were injected onto the column, which was run at 1 ml/min. Isocratic elution at 25% acetonitrile for 5 min was followed by a gradient of 25–55% acetonitrile over the next 60 min. Absorbance of column eluate was monitored at 220 nm, and fractions were assessed for protein purity by SDS-PAGE and by Western immunoblotting with anti-IGFBP-5, as described previously (24), with quantitation of protein by the Bradford assay. Fractions were lyophilized and stored at −70°C. Proteins were redissolved in Tris-HCl/Tween 20 buffer, pH 7.4, for use.

Biosensor Analysis—Biosensor studies were performed on a fully automated BIAcore 3000 instrument under conditions as described previously (19, 25). The human PAI-1 mutant was dissolved at 10 µg/ml in sodium acetate, pH 5.0, and immobilized to the surface of a carboxymethyl (CM-5) biosensor chip using standard amine coupling
**IGFBP-5 and Plasminogen Activation**

Although the IGFBP-5 proteins purified by glutathione-Sepharose were relatively pure, we did note, in some preparations, the presence of impurities, particularly IGFBP-5 fragments (see Fig. 1). The majority of these fragments was derived from IGFBP-5 as demonstrated by Western immunoblotting using an antiseraum to IGFBP-5 (results not shown). We further purified IGFBP-5, either on an IGF affinity column or by RP-HPLC (Fig. 1). These subsequent purifications produced IGFBP-5, and mutants thereof, devoid of fragments. Wild-type, N-term, and C-term E are shown as examples (Fig. 1). Furthermore, the effects of IGFBP-5 described in our studies were not because of artifactual generation of IGFBP-5 polymers during the purification process, because our preparations ran as monomeric proteins under native conditions, which are routinely used for Western ligand blotting (results not shown).

HC11 cells attached to the substratum via numerous focal adhesions and exhibited actin stress fibers when cultured in serum-free medium in the absence of plasminogen (Fig. 2a). In contrast, in the presence of plasminogen, there was evidence of cleavage of focal adhesions, some cell migration, and increased cell-cell contact before cells died via anoikis. Without plasminogen, showed no intrinsic ability to modify cell attachment or survival (Fig. 2b and c). This was accompanied by a 4-fold increase in the activity of caspase 3 in the cells 24 h post-treatment, an effect that was inhibited in a dose-dependent fashion by PAI-1 (results not shown).

Fig. 3a shows a confluent monolayer of HC11 cells cultured in serum-free conditions, stained with crystal violet. IGFBP-5, in the absence of plasminogen, showed no intrinsic ability to modify cell attachment or survival (Fig. 3b). Addition of plasminogen, in contrast, resulted in cell migration and ultimately cell death, indicating the conversion of plasminogen to plasmin by plasminogen activator(s) produced by the cells (Fig. 3c). These plasminogen-induced effects were completely pre-

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**RESULTS**

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**FIGURE 1. Purification of IGFBP-5 expressed as a GST fusion protein in Escherichia coli.** The soluble extract from *E. coli* (lane 1) was subjected to glutathione-Sepharose affinity chromatography. Unbound material (lane 2) was discarded and IGFBP-5 eluted using the PreScision Protease system. The eluted material (lane 3) contained a major band at the appropriate molecular WT for intact IGFBP-5 (indicated by arrowhead) along with smaller molecular WT fragments and some high molecular WT contaminants. Subsequent purification of this material on an IGF-I affinity column produced an unbound fraction (lane 4) containing contaminating proteins and a bound fraction that was eluted (lane 5) and contained essentially pure intact IGFBP-5. Alternatively, material from lane 3 was purified by RP-HPLC. Fractions 35–40 of the elution are shown in lanes 6–11, respectively, and demonstrate the ability to almost completely separate intact IGFBP-5 (lanes 9 and 10) from smaller molecular WT contaminants (lanes 7 and 8). Mutant-binding proteins were also successfully purified via HPLC and are shown in lanes 14 and 15. Note the size differences between the various proteins analyzed as follows: the mature (minus signal peptide) WT mouse IGFBP-5 protein (28,461 Da), plus N-terminal pentapeptide extension (GPLGS) from pGEX-6P1 is 28,872 Da (Fig. 1). These subsequent purifications produced IGFBP-5, and mutants thereof, devoid of fragments. Wild-type, N-term, and C-term E are shown as examples (Fig. 1). Furthermore, the effects of IGFBP-5 described in our studies were not because of artifactual generation of IGFBP-5 polymers during the purification process, because our preparations ran as monomeric proteins under native conditions, which are routinely used for Western ligand blotting (results not shown).

HC11 cells attached to the substratum via numerous focal adhesions and exhibited actin stress fibers when cultured in serum-free conditions in the presence of 2 mM plasminogen. This resulted in cell migration and ultimately cell death, indicating the conversion of plasminogen to plasmin by plasminogen activator(s) produced by the cells (Fig. 3c). These plasminogen-induced effects were completely pre-

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**FIGURE 2. Plasmin disrupts focal adhesions in HC11 cells resulting in cell death.** Control cells (a) cultured for 24 h in serum-free conditions show numerous focal adhesions (green) and actin stress fibers (red) indicative of strong cell-substratum attachment. Cultures for 6 h (b) or 24 h (c) in serum-free conditions in the presence of 2 μg plasminogen resulted in reduced numbers of focal adhesions, reduced cell spreading, increased cell migration, and ultimately loss of cell adhesion to the substratum and death by anoikis.

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**FIGURE 3. Effects of plasminogen, PAI-1, and wtIGFBP-5 on HC11 cells.** HC11 cells were seeded into 96-well plates and were grown to confluence in DMEM. Cells were washed with PBS and treated with various combinations of plasminogen, PAI-1 (500 ng/ml), and IGFBP-5 (25 μg/ml) under serum-free conditions. Cells were incubated at 37 °C for 24 h, after which the culture medium was removed for determination of plasmin activity. The cells were then fixed with 4% paraformaldehyde, stained with crystal violet, rinsed, and photographed. 

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IGFBP-5 and Plasminogen Activation

vented by PAI-1 (Fig. 3d). However, the addition of IGFBP-5 to cultures containing PAI-1 and plasminogen antagonized the effects of PAI-1 and induced cell detachment (Fig. 3e). In contrast, the pGEX-6P1 empty vector control (potentially containing contaminating proteases from the bacterial expression system) failed to influence the actions of PAI-1 (Fig. 3f). Cell death was also assessed quantitatively after solubilization of crystal violet staining (Fig. 3g) and confirmed the visual changes induced in the cells by the various treatments. These effects were because of plasmin generation as confirmed by measuring plasmin activity in the medium (Fig. 4). After 24 h of culture in serum-free medium, in the absence of exogenous plasminogen, there was no evidence of plasmin activity, either in the presence or absence of IGFBP-5. However, if plasminogen was added to the HCl1 cultures, plasmin activity was readily detectable in the medium removed at the end of the 24-h culture period. The addition of PAI-1, together with plasminogen, prevented plasmin formation. When IGFB-5 was added together with PAI-1 and plasminogen, it was able to prevent the effects of PAI-1, resulting in increased plasmin generation. The pGEX-6P1 plasmid (empty vector control) failed to influence plasmin generation, indicating that any trace contamination of bacterial proteins/proteases following glutathione purification had no effect in these assays.

A mutant IGFBP-5 that had greatly reduced affinity for IGF-I (N-term) was as effective as wtIGFBP-5 protein in terms of cell remodeling (Fig. 5e). A mutant HEP−, which had a greatly reduced affinity for heparin, showed only a small decrease in potency when compared with wtIGFBP-5 (Fig. 5f). Details of the properties of these mutants have been reported previously (22, 23). Similar observations were made for the C-term E and C-term F, two additional non-heparin-binding mutants (results not shown). These findings were supported by determination of the plasmin concentration in the medium 24 h after addition of the treatments in serum-free medium that paralleled the phenotypic changes (results not shown). These observations indicated that the effect of IGFBP-5 on PAI-1 action was almost entirely unrelated to its ability to bind to IGFs or heparin. Biosensor analysis demonstrated that wtIGFBP-5 bound to PAI-1 with a Kd of ~100 nM (Fig. 6a). However, the non-heparin-binding mutant C-term E had a greatly reduced affinity for PAI-1 (Fig. 6b), whereas the N-term mutant had a similar affinity to wtIGFBP-5 (Fig. 6c). Given that the C-term E had little affinity for PAI-1, it seemed unlikely that it could antagonize the effects of PAI-1 by a direct molecular interaction. We therefore examined these effects of IGFBP-5 on tPA, uPA, and PAI-1 activities in greater detail by using cell-free studies. As shown in Fig. 7, tPA and uPA generated plasmin from exogenous plasminogen, and PAI-1 could inhibit this in a dose-dependent manner. When IGFBP-5 was added into the tPA/PAI-1 mixture, as anticipated, it markedly inhibited the effect of PAI-1 (Fig. 7a). However, we also noted that, at a lower dose of PAI-1, IGFBP-5 not only inhibited the effect of PAI-1 but actually increased plasmin production to levels even greater than with tPA alone (Fig. 7b). Furthermore, when IGFBP-5 was incubated with uPA plus PAI-1, IGFBP-5 failed to inhibit the action of PAI-1 (Fig. 7c). These results suggested that IGFBP-5 was acting not by inhibiting PAI-1 but by specifically enhancing the activity of tPA but not uPA.

We therefore tested the effect of IGFBP-5 on uPA or tPA in the absence of PAI-1. This confirmed our hypothesis, because IGFBP-5 enhanced the effect of tPA on plasminogen in a dose-dependent fashion (Fig. 8a). BSA was used as a control and showed no ability to enhance tPA (Fig. 8b) or uPA activity. In complete contrast, plasmin generation by uPA was not enhanced by IGFBP-5 (Fig. 8c). More importantly, we demonstrated that, in the absence of tPA or uPA, IGFBP-5 possessed no enzymatic activity capable of cleaving plasminogen to plasmin (Fig. 8c). The ability of IGFBP-5 to enhance tPA activity was similar in preparations that were derived from the glutathione column (containing some fragments) as well as the highly purified preparations from HPLC or IGF affinity purifications that were devoid of IGFBP-5 fragments, suggesting that proteolytic fragments of IGFBP-5 present in the bacterially expressed proteins, potentially containing C-terminal lysines, were not major contributors to this effect of IGFBP-5 (results not shown). To examine the possibility that such C-terminal lysines were produced during the incubation with plasminogen, we used EACA, a lysine analogue, and carboxypeptidase-B to inhibit interactions of tPA and plasminogen with the C-terminal lysine residues, potentially present in breakdown products of IGFBP-5. Fibrin was used.
as a positive control and was able to enhance the activity of tPA in a dose-response fashion, resulting in increases of tPA activity greater than 40-fold (Fig. 9A). IGFBP-5 (Fig. 9C) and C-term E (results not shown) were as potent as soluble fibrin in enhancing tPA activity. EACA inhibited the effects of fibrin in a dose-dependent manner, with 100 mM EACA being completely inhibitory (Fig. 9A). Carboxypeptidase-B also markedly attenuated fibrin-mediated enhancement of tPA (Fig. 9B), and similar effects were evident when IGFBP-5 replaced fibrin (Fig. 9C). We finally determined whether the enhancement of tPA-mediated plasminogen activation by IGFBP-5 was exerted through an induced conformational change in tPA, which enhanced its direct catalytic activity. To examine this, the effect of IGFBP-5 on tPA activity was investigated in a direct amidolytic assay for tPA. IGFBP-5 showed a dose-dependent ability to increase the activity of tPA, although this was more modest (2-fold) than the effect achieved in the indirect assay (results not shown).

**DISCUSSION**

By utilizing the epithelial cell line HC11, we were able to show that plasmin generation induced proteolytic cleavage of focal adhesions and disruption of actin stress fibers, which ultimately led to cell death as indicated by activation of caspase-3 and eventual cell detachment. These effects were inhibited by PAI-1, and we now show for the first time that IGFBP-5 can prevent these effects of PAI-1. This clearly suggests that the physical interaction of IGFBP-5 with PAI-1, as described previously (7), serves to inhibit the actions of PAI-1. Subsequently, we showed that IGFBP-5 mutants that were unable to bind either to IGF-1 or heparin were also able to antagonize the effects of PAI-1. This was somewhat surprising given that PAI-1 has been described to interact with IGFBP-5 via its heparin-binding domain (amino acids 201–218). We thus examined IGFBP-5 interactions with PAI-1 using biosensor analysis and showed that IGFBP-5 did indeed bind to PAI-1, whereas a heparin-binding mutant (C-term E) used in this study showed greatly compromised binding to PAI-1, supporting the earlier findings of Clemmons and co-workers (7). It was thus puzzling that a mutant with limited ability to bind to PAI-1 could nevertheless prevent the effects of PAI-1 on plasmin generation. However, we were able to show that IGFBP-5 activated plasminogen independently of PAI-1 by specifically enhancing the activity of tPA directly, although it was unable to influence the effects of uPA and had no intrinsic plasminogen cleaving activity. This finding implies that the interaction of IGFBP-5 with tPA lies out with the basic residues of the heparin-binding domain of IGFBP-5 and presumably out with the major IGF-binding site present in the N terminus of IGFBP-5, because C-term E and N-term were able to enhance tPA activity to a similar extent as wild-type IGFBP-5. The manner in which IGFBP-5 enhanced tPA activity is similar to that of fibrin. The intrinsic activity of tPA against plasminogen is very low but is considerably enhanced by fibrin (26). This involves two mechanisms. In the first, fibrin creates a ternary complex with tPA and plasminogen, involving interactions of tPA and plasminogen with C-terminal lysine residues of fibrin, thereby retaining the molecules in close apposition. This mechanism was also evident for IGFBP-5 because the lysine analogue EACA and carboxypeptidase-B inhibited the actions of both IGFBP-5 and fibrin. Carboxypeptidase-B or thrombin-activable fibrinolytic inhibitor inhibits fibrinolysis by removing the C-terminal lysine residues from fibrin that are required for efficient plasmin formation (27, 28). The second mechanism involves binding of fibrin, or fragments thereof, to tPA, which induces favorable conformational changes in both tPA and plasminogen (29). IGFBP-5 was also able to act in this way, and this role of IGFBP-5 thus contrasts with that of a-s2 casein, which...
enhances the activation of tPA exclusively by ternary complex formation and exhibits no ability to enhance tPA activity in the direct amido-lytic assay (12). Most intriguingly, we have shown that IGFBP-5 binds to casein micelles and interacts exclusively with α-s2 casein on ligand blots (11). It has been proposed that casein micelles thus provide a matrix for plasmin generation in milk and that this serves to prevent formation of casein (milk) clotting. The presence of IGFBP-5 at high concentrations in these matrices, coupled with its ability to bind to PAI-1, provides additional support for a physiological role of IGFBP-5 in the process of plasminogen activation in vivo, at least in the mammary gland. The processes of clotting of milk and blood share numerous features on a molecular and hydrodynamic level (30). The fact that IGFBP-5 can serve as an activator of tPA (an important fibrinolytic agent) and the fact that IGFBP-5 is also expressed in situations where atherosclerotic plaques are evident (31, 32) and that it binds to thrombospondin and osteopontin (33), which are involved in this process, suggest that IGFBP-5 may, in addition, play an important role in processes such as thrombolysis and wound healing. The possibility that it plays a role in localized inhibition of blood clotting is worthy of investigation.

Although IGFBP-5 may play a role in maintaining the patency of mammary milk ducts, the plasminogen activator content of the rodent mammary gland is also correlated with involutionary processes, and thus plasminogen activation is also considered to be involved in the later stages of tissue remodeling (34). For example, in rodents, an increase in plasminogen activator production and a decrease in PAI-1 activity are correlated with the destruction of the basement membrane and loss of the secretory cells during mammary gland involution (34–36). Thus IGFBP-5 potentially plays a dual role in mammary gland remodeling during the involutionary process. First, it sequesters IGF-I and induces cell death; and second, it activates tPA and thereby induces the proteolytic cascades, including activation of matrix metalloproteinases, which are involved in degradation of the extracellular matrix in the mammary gland. It is thus likely that IGFBP-5 serves to coordinate these events.

**FIGURE 8.** Effects of IGFBP-5 upon tPA- or uPA-mediated plasminogen activation. A, 5 ng/ml tPA was incubated alone (solid diamonds) or with various concentrations of IGFBP-5 in the presence of 10 µl of 6 mM VLL-pNA and 2 µM plasminogen in Tris-HCl buffer, pH 7.4, containing 0.1% Tween. IGFBP-5 concentrations (µg/ml) were 3 (open triangles), 6 (solid triangles), 12.5 (open circles), 25 (solid circles), or 50 (solid squares). B, same as for A but with BSA used as a control in place of IGFBP-5. C, same as for A except that uPA (4 ng/ml) replaced the tPA. In addition, 50 µg/ml IGFBP-5 was incubated with 10 µl of 6 mM VLL-pNA and 2 µM plasminogen in Tris-HCl buffer, pH 7.4, containing 0.1% Tween in the absence of tPA or uPA (open diamonds), demonstrating the absence of any intrinsic plasminogen-activating properties in IGFBP-5. Absorbance at 405 nm was measured at 15-min intervals for 3 h. Results are means of triplicate wells representative of studies that were performed on at least four occasions.

**FIGURE 9.** Influence of EACA and carboxypeptidase-B on tPA-catalyzed plasminogen cleavage. A, 5 ng/ml tPA was incubated alone (diamonds), with 100 µg/ml fibrin (squares), or with fibrin plus EACA at concentrations of 1 (circles), 10 (triangles), or 100 mM (crosses). B, 5 ng/ml tPA was incubated alone (diamonds), with 100 µg/ml fibrin (squares), or with fibrin plus 50 mM EACA (triangles) or with fibrin plus 10 mg/ml carboxypeptidase B (circles). C, same as for B except that 100 µg/ml IGFBP-5 replaced fibrin. All wells contained 10 µl of 6 mM VLL-pNA and 2 µM plasminogen in Tris-HCl buffer, pH 7.4, containing 0.1% Tween. Absorbance at 405 nm was measured at 15-min intervals for 3 h.
In summary, despite the fact that we were able to confirm by biosensor analysis that IGFBP-5 binds to PAI-1, we were unable to show any direct effect of IGFBP-5 on PAI-1 activity. Rather, the ability of IGFBP-5 to counteract the effects of PAI-1 appeared to be indirect, due to activation of tPA but not uPA. Of course, this does not rule out the possibility that IGFBP-5 can influence other actions of PAI-1, such as its ability to modulate cell migration, involving a plasmin-independent process (20, 37).

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