Tissue factor pathway inhibitor (TFPI) is a plasma serine protease inhibitor that directly inhibits coagulation factor Xa and regulates blood coagulation via inhibition of factor VIIa-tissue factor enzymatic activity. We previously demonstrated that >90% of TFPI bound to a single population of low affinity binding sites on hepatoma cells (2 × 10^6 sites/cell, K_d = 30 nM), and, that following binding, the low density lipoprotein receptor-related protein (LRP) mediated TFPI uptake and degradation. We subsequently reported heparan sulfate proteoglycans (HSPGs) constitute a second receptor system involved in TFPI catabolism. In the present study, mouse embryonic fibroblasts heterozygous and homozygous-negative for disruption of the LRP gene were used to further examine the roles of LRP and HSPGs in TFPI endocytosis. We demonstrate that LRP is absolutely required for degrading 125I-TFPI. LRP heterozygous and homozygous-negative cells bind 125I-TFPI similarly, and the 39-kDa protein, an inhibitor of all known ligand interactions with LRP, does not alter 125I-TFPI binding to these cells. TFPI can be cross-linked to LRP on [35S]cysteine-labeled hepatoma and LRP-heterozygous cells but not LRP-negative cells. When HSPGs are blocked with protamine, 125I-TFPI binds in a 39-kDa protein-inhibitable manner to 41,000 high affinity sites/hepatoma cell (K_d = 2.3 nM). Blockade of HSPGs with protamine results in significantly more 125I-TFPI degradation by LRP-positive cells. TFPI can be cross-linked to LRP in the absence and presence of protamine. However, in the presence of protamine, relative to the total pool of cross-linked proteins, 5-fold more TFPI is cross-linked to LRP. Finally, we show TFPI inhibits 125I-α2-macroglobulin-methylamine binding to hepatoma cells and that carboxyl-terminal residues 115–319 of the 39-kDa protein inhibit both 125I-TFPI degradation and binding when binding conditions contain protamine. Together, our results suggest that while the majority of TFPI binds to cell surface HSPGs, LRP can function independently from HSPGs in the binding and uptake of TFPI.

* This work was supported in part by National Institutes of Health Grants HL52040, HL53280, and HL34462. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. § Supported in part by Cardiovascular Training Grant T32-HL07275. † Lucille P. Markey Scholar. Supported by Grant HL20948, the Perot Family Foundation, and the Syntex Scholar Program. ‡ To whom correspondence should be addressed: Dept. of Pediatrics, Children’s Hospital, Washington University School of Medicine, One Children’s Place, St. Louis, MO 63110. Tel.: 314-454-6005; Fax: 314-454-0537.

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The low density lipoprotein receptor-related protein (LRP) is a cell-surface glycoprotein composed of two subunits of ~515 kDa and 85 kDa. The ~515-kDa subunit binds ligands and is noncovalently associated with the 85-kDa subunit which contains a single membrane-spanning domain and two NPXY internalization sequences (1). LRP functions as an endocytosis receptor for a rapidly increasing number of diverse ligands that are involved in lipoprotein metabolism, protease/coagulation regulation, and toxin entry (1–13). A 39-kDa protein, also termed receptor-associated protein, copurifies with and binds with high affinity to LRP (1, 14). Numerous studies have found that exogenously added 39-kDa protein inhibits the binding and/or cellular uptake of all ligands by LRP (1). In 1994, an LRP-negative cell line was established from the embryos of LRP-knockout mice (15). LRP-negative cell lines, in addition to the 39-kDa protein and antibodies directed against LRP, are an extremely useful means of establishing whether LRP functions as a receptor for candidate ligands.

Using hepatoma cells, we previously reported (10) that LRP mediated the uptake and cellular degradation of tissue factor pathway inhibitor (TFPI), a 42-kDa plasma glycoprotein that inhibits both coagulation factor Xa and tissue factor-initiated blood coagulation (16). We also reported that LRP was not the major cell surface TFPI receptor since the 39-kDa protein did not inhibit 125I-TFPI binding (10). Because 10% of prebound TFPI was degraded via LRP, we speculated that ≤10% of TFPI bound directly to LRP which was then internalized and degraded. We also considered the possibility that after TFPI bound to another molecule, TFPI could be transferred to LRP for uptake and degradation since several previously characterized LRP ligands (7, 17) initially bind to other cell surface molecules prior to LRP-mediated uptake and degradation. Heparan sulfate proteoglycans (HSPGs) appear to play an important role in TFPI catabolism (18–21). Evidence for HSPG involvement has come from the following observations. 1) Plasma TFPI levels increase severalfold in vivo (18, 20) following intravenous administration of heparin, an effect attributed to the release of TFPI from endothelial or liver cell HSPG/glycosaminoglycan-binding sites. Further, neutralization of heparin with protamine reduces TFPI levels to preheparin values, presumably by re-exposing TFPI to HSPG/glycosaminoglycan-binding sites (21). 2) Protamine, which competes for HSPG-binding sites, inhibits TFPI binding to hepatoma cells

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(Received for publication, May 21, 1996, and in revised form, July 22, 1996)
and prolongs the half-life of 125I-TFPI in mice (19). 3) Under conditions where TFPI is unable to bind to HS PGs, the 39-kDa protein inhibits 125I-TFPI binding to hepatoma cells (19). 4) In mice overexpressing the 39-kDa protein, the rapid clearance of 125I-TFPI is virtually eliminated in the presence of protamine (19).

The purpose of the present study was to demonstrate a direct interaction between TFPI and LRP, to define this affinity, and to further examine the role of HS PGs in TFPI binding and degradation. Domains on the 39-kDa protein required for inhibiting TFPI degradation and cross-competition studies between TFPI and previously characterized LRP-ligands have been examined.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human full-length TFPI was produced and purified from Escherichia coli (22). Recombinant full-length glutathione S-transferase (GST)-39-kDa protein, GST-39-kDa protein constructs, and GST-free 39-kDa protein were produced and purified from E. coli (23). α-2-Macroglobulin was purified from human plasma and activated with methyamine (to yield α2M*) (23). Carrier-free sodium 125Iiodide, [35S]cysteine, Amplify, and Hyperfilm-MP were purchased from Amer sham. IODO-GEN and 3,3′dithiobis(sulfosuccinimidylpropionate) (DTSSP) were from Pierce Chemical Co. Protamine sulfate, phenylmethylsulfonyl fluoride, Triton X-100, sodium deoxycholate, and phosphate-buffered saline (PBS) were from Sigma. Bovine serum albumin (BSA) (fraction V) was from Calbiochem. Protein A-agarose beads were from Repligen. Tissue culture media were from Life Technologies, Inc.

Cell Culture—Hepatoma MH C1 cells (24) and mouse embryonic fibroblasts heterozygous (PEA10 cells) and homozygous-negative (PEA13 cells) for disruption of the LRP gene (15) were grown as described previously.

Protein Iodinations—TFPI (50–200 μg) and α2M* (20 μg) were labeled with 125I using the IODO-GEN method (24). Specific activities were generally 0.5–4 × 104 cpm/μg of protein.

Antibodies—Anti-39-kDa protein (25), anti-TFPI (25), and anti-LRP antibodies (26) were prepared as described previously.

Cell Binding and Degradation Assays—Assay buffers for 125I-TFPI and 125I-α2M* were Earle’s minimum essential medium (with glutamine) containing 3% BSA and Dulbecco’s modified Eagle’s medium containing 6 mg/ml BSA and 5 mM CaCl2, respectively. Cell binding assays were performed as described previously (10). Briefly, washed cells were preincubated with assay buffer containing the indicated concentrations of 125I-ligand in the absence or presence of unlabeled competitor proteins for 2 h at 4°C. The cells were then washed and lysed. Radioactivity of cell lysates was determined in a Packard γ counter. Total 125I-ligand binding was determined in the presence of high affinity unlabeled ligand alone. Nonspecific 125I-ligand binding was determined in the presence of excess unlabeled ligand as specified in the text. Specific 125I-ligand binding was determined by subtracting nonspecific from total 125I-ligand binding.

Cell degradation assays (10) were performed by adding assay buffer containing 125I-TFPI in the absence or presence of unlabeled competitor proteins to washed cell monolayers. After incubation at 37 °C for 4 h, the overlying media were removed and precipitated with trichloroacetic acid. Degradation of ligand was defined as the appearance of radioactive ligand fragments in the overlying media that were soluble in trichloroacetic acid. Degradation of 125I-TFPI in parallel dishes that did not contain cells was subtracted from each point.

Metabolic Labeling and Chemical Cross-linking—Cells were grown in 10-cm dishes to 80% confluency. After incubation in medium lacking cysteine, metabolic labeling was initiated by the addition of [35S]cysteine (166 μCi/ml) for 5 h at 37 °C, washed, and incubated for 2 h at 4 °C with 100 nM TFPI (A) or 10 nM 39-kDa protein (B). Cells were either lysed without cross-linking or were lysed following cross-linking with 1 mM DTSSP. Lysates were immunoprecipitated with 10 μg of total anti-TFPI IgG (α-TFPI), 1 μg of affinity purified anti-LRP IgG (α-LRP), 3 μg of affinity-purified anti-39-kDa protein IgG (α-39K), or 10 μl of normal rabbit serum (N.R.). The immunoprecipitates were analyzed by 6% SDS-PAGE under nonreducing and reducing conditions. Gels were exposed to film for 30 h prior to developing. The position of LRP is indicated by an arrowhead. Molecular mass markers in kDa are indicated on the left.

RESULTS

Chemical Cross-linking of TFPI and the 39-kDa Protein to LRP on Hepatoma Cells—To demonstrate a direct interaction between TFPI and LRP, chemical cross-linking was performed using DTSSP, a thio-cleavable, water-soluble, and membrane-impermeant reagent. In Fig. 1, [35S]cysteine-labeled hepatoma cells were subjected to cross-linking following binding with TFPI or the 39-kDa protein. Cell lysates, immunoprecipitated with anti-TFPI, anti-39-kDa protein, anti-LRP IgGs, or normal rabbit serum were analyzed by SDS-PAGE. When cells were cross-linked to TFPI or the 39-kDa protein, a complex with very high apparent molecular mass was seen under nonreducing conditions.

FIG. 1. Chemical cross-linking of TFPI and the 39-kDa protein to LRP on [35S]cysteine-labeled MH C1 cells. Cells were metabolically labeled with [35S]cysteine (166 μCi/ml) for 5 h at 37 °C, washed, and incubated for 2 h at 4 °C with 100 nM TFPI (A) or 10 nM 39-kDa protein (B). Cells were either lysed without cross-linking or were lysed following cross-linking with 1 mM DTSSP. Lysates were immunoprecipitated with 10 μg of total anti-TFPI IgG (α-TFPI), 1 μg of affinity purified anti-LRP IgG (α-LRP), 3 μg of affinity-purified anti-39-kDa protein IgG (α-39K), or 10 μl of normal rabbit serum (N.R.). The immunoprecipitates were analyzed by 6% SDS-PAGE under nonreducing and reducing conditions. Gels were exposed to film for 30 h prior to developing. The position of LRP is indicated by an arrowhead. Molecular mass markers in kDa are indicated on the left.
conditions following immunoprecipitation with anti-TFPI (Fig. 1A) or anti-39-kDa protein (Fig. 1B) IgG. These high molecular mass bands migrated to the identical position as that seen in cell lysates immunopurified with anti-LRP antibody (Fig. 1, A and B), implying TFPI and the 39-kDa protein are binding to LRP. Under reducing conditions and resultant cleavage of the cross-linker, the ~515-kDa subunit of LRP was observed using anti-TFPI (Fig. 1A), anti-39-kDa protein (Fig. 1B), and anti-LRP IgGs (Fig. 1, A and B). In the absence of cross-linking, no high molecular mass complex was evident with anti-TFPI (Fig. 1A) or anti-39-kDa protein IgG (Fig. 1B). In the absence of cross-linking, LRP was not co-immunoprecipitated with anti-39-kDa protein IgG since immunoprecipitation conditions contain 1% SDS. Immunoprecipitation of non-cross-linked lysates with anti-LRP IgG (Fig. 1, A and B) resulted in the appearance of the ~515-kDa subunit of LRP under both nonreducing and reducing conditions. No proteins are immunoprecipitated under any condition using normal rabbit serum.

Chemical Cross-linking of TFPI to LRP on Cells Heterozygous for Disruption of the LRP Gene—We next examined whether TFPI could be cross-linked to LRP-heterozygous PEA10 cells. The identical cross-linking experiments were performed on LRP-negative PEA13 cells. Following [35S]cysteine labeling, cells were incubated with unlabeled TFPI and thereafter cross-linked with DTSSP. Fig. 2 demonstrates that LRP is synthesized by PEA10 cells but not by PEA13 cells since the ~515-kDa subunit of LRP is only immunoprecipitated with anti-LRP IgG from PEA10 cell lysates. The ~515-kDa subunit of LRP is also immunoprecipitated with anti-TFPI IgG from PEA10 cell lysates but not from PEA13 cell lysates, demonstrating TFPI binding directly to LRP.

Effect of the 39-kDa Protein on [125I]-TFPI Degradation and Binding by LRP-negative Cells—To investigate whether LRP-negative cells were capable of mediating the uptake and degradation of [125I]-TFPI, PEA10 and PEA13 cells were incubated with [125I]-TFPI in the absence or presence of the 39-kDa protein at 37 °C, and degradation products were assessed. Fig. 3A shows PEA10 cells actively degrade [125I]-TFPI and that the 39-kDa protein inhibits this degradation in a dose-dependent manner with an IC_{50} value of ~15 nM. PEA13 cells degrade [125I]-TFPI minimally, and the 39-kDa protein has no effect on the amount of [125I]-TFPI degraded. The small amount of [125I]-TFPI degraded by PEA13 cells (~40 fmol/10^6 cells/4 h) may represent an LRP-independent process or more likely is the result of pinocytic internalization since mouse fibroblasts nonspecifically pinocytose ligand at a rate of 0.9 μm^3/cell/min (28). Fig. 3B shows that PEA10 and PEA13 cells bind [125I]-TFPI similarly and that the 39-kDa protein does not alter [125I]-TFPI binding to either cell line.

Saturation Binding of [125I]-TFPI to MH1C1 Cells in the Absence and Presence of Protamine—We previously reported that the majority of [125I]-TFPI binding was not to LRP (10), and that protamine, a competitor for HSPG-binding sites, inhibited [125I]-TFPI binding to MH1C1 cells (19). We also reported that when protamine was included in the binding medium, the 39-kDa protein inhibited [125I]-TFPI binding (19). Thus, under conditions where TFPI was unable to bind to HS PGs, more TFPI was available for binding to LRP. Therefore, to examine the affinity of TFPI binding to LRP, saturation binding experiments were performed with [125I]-TFPI in the absence or presence of protamine. As seen in Fig. 4A, in the absence of protamine, [125I]-TFPI bound specifically to MH1C1 cells over the concentration range of 0.6–12 nM. Nonspecific binding increased linearly and accounted for 10% of total [125I]-TFPI binding. Saturation of specific binding was not reached at a [125I]-TFPI concentration of 12 nM. Scatchard analysis (29) yielded ~5 × 10^6 sites/cell with an apparent K_d value of 20 nM (Fig. 4A, inset), similar to our previous study (10). In Fig. 4B, saturation binding of [125I]-TFPI was performed in the presence of 100 μg/ml protamine, a concentration previously shown (19) to inhibit 90% of [125I]-TFPI binding to MH1C1 cells. In the presence of protamine, [125I]-TFPI bound specifically to MH1C1 cells, and saturation of specific binding was observed at a [125I]-TFPI concentration of ~7 nM. Protamine reduced [125I]-TFPI binding by >90%. Nonspecific binding increased linearly and accounted for ~40% of total binding. Scatchard analysis (29) yielded 41,000 sites/cell with an apparent K_d value of 2.3 nM (Fig. 4B, inset). Fig. 4B also
Increasing concentrations of 125I-TFPI in the presence of 100 ng/ml protamine. The 39-kDa protein inhibited 125I-TFPI degradation in a dose-dependent manner in MH1C1 (Fig. 5B). Since 39-kDa protein-inhibitable 125I-TFPI binding was observed in the absence or presence of protamine, the 39-kDa protein preferentially bound TFPI in the absence of protamine. Total binding (solid circles) and presence (solid squares) of 500 nM 39-kDa protein was determined. Nonspecific binding (open circles) was derived as the difference between total and nonspecific binding (solid triangles). Specific binding (solid squares) was derived as the difference between total and nonspecific 125I-TFPI binding. Symbols represent means of duplicate determinations. Inset, Scatchard plots of specific binding. B/F, bound/free 125I-TFPI; B, bound 125I-TFPI (pmol/10^6 cells).

**Fig. 4.** Binding of 125I-TFPI to MH1C1 cells in the absence and presence of protamine. A, cells were incubated for 2 h at 4 °C with increasing concentrations of 125I-TFPI in the absence or presence of a >400-fold molar excess of unlabeled TFPI. Total binding (solid circles) and nonspecific binding (open circles) are indicated. Specific binding (solid triangles) was derived as the difference between total and nonspecific TFPI binding. B, cells were incubated for 2 h at 4 °C with increasing concentrations of 125I-TFPI in the presence of 100 μg/ml protamine. Total binding in the absence (solid circles) and presence (solid squares) of 500 nM 39-kDa protein was determined. Nonspecific binding (open circles) was determined in the presence of a >400-fold molar excess of unlabeled TFPI and 100 μg/ml protamine. Specific binding (solid squares) was derived as the difference between total and nonspecific TFPI binding. Symbols represent means of duplicate determinations. Inset, Scatchard plots of specific binding. B/F, bound/free 125I-TFPI; B, bound 125I-TFPI (pmol/10^6 cells).

Effect of Protamine on the Cellular Degradation of 125I-TFPI in the Absence and Presence of the 39-kDa Protein—Since 39-kDa protein-inhibitable 125I-TFPI binding was observed in the presence of protamine (Fig. 4B and Ref. 19), we hypothesized that by blocking TFPI from binding to cell surface HS PGs, more TFPI could bind to LRP. Thus, the effect of protamine on 125I-TFPI degradation was examined. In the absence of protamine, the 39-kDa protein inhibited 125I-TFPI degradation in a dose-dependent manner in MH1C1 (Fig. 5A) and PEA10 (Fig. 5B). Cells were incubated for 2 h at 4 °C with increasing concentrations of 125I-TFPI in the presence of 100 μg/ml protamine. Total binding (solid circles) and presence (solid squares) of 500 nM 39-kDa protein was determined. Nonspecific binding (open circles) was derived as the difference between total and nonspecific 125I-TFPI binding. Symbols represent means of duplicate determinations. Inset, Scatchard plots of specific binding. B/F, bound/free 125I-TFPI; B, bound 125I-TFPI (pmol/10^6 cells).

**Fig. 5.** Effect of protamine on the cellular degradation of 125I-TFPI in the absence and presence of the 39-kDa protein. MH1C1 (A), PEA10 (B, triangles), and PEA13 (B, squares) cells were incubated with 2 nM 125I-TFPI in the absence (solid symbols) or presence (open symbols) of 100 μg/ml protamine and in the absence or presence of increasing concentrations of the 39-kDa protein for 4 h at 37 °C. Thereafter, the overlying media were removed and subjected to trichloroacetic acid-soluble precipitation. Trichloroacetic acid-soluble radioactivity, representing degraded 125I-TFPI, was converted to femtomole equivalents/10^6 cells. Each symbol is the mean of duplicate determinations.

**Fig. 6.** Chemical cross-linking of TFPI to LRP on [35S]cysteine-labeled MH1C1 cells in the absence and presence of proteamine. Cells were metabolically labeled with [35S]cysteine (166 μCi/ml) for 5 h at 37 °C, washed, and incubated for 2 h at 4 °C with 100 μg/ml TFPI in the absence or presence of 100 μg/ml protamine. Cells were either lysed without cross-linking or were lysed following cross-linking with 1 mM DTSSP. Lysates were immunoprecipitated with 10 μl of total anti-TFPI IgG (α-TFPI), 10 μl of anti-LRP serum (α-LRP), or 10 μl of normal rabbit serum (N.R.). The immunoprecipitates were analyzed by 6% SDS-PAGE under nonreducing and reducing conditions. Gels were exposed to film for 15 h prior to developing. The position of LRP is indicated by an arrowhead. Molecular mass markers in kDa are indicated on the right.

Chemical Cross-linking of TFPI to LRP in the Absence and Presence of Proamine—The ability of protamine to alter the amount of 125I-TFPI cross-linked to LRP was next examined. In Fig. 6, [35S]cysteine-labeled MH1C1 cells were subjected to cross-linking following binding with TFPI in the absence or presence of protamine. Under nonreducing conditions, a very high apparent molecular mass complex remaining largely in the stacking gel was immunoprecipitated with anti-TFPI antibody when TFPI was cross-linked to cells following binding in the absence (lane 1) and presence (lane 7) of protamine. Denitometry scanning revealed that >4-fold more radioactivity was retained in the stacking gel in the absence of protamine (lane 1) than in the presence of protamine (lane 7). As expected, essentially equivalent amounts of LRP were immunoprecipitated with anti-LRP antibody in the absence (lane 2) and presence (lane 8) of protamine since incubation of cells with protamine following metabolic labeling should not alter the amount of LRP synthesized or immunoprecipitated. Under reducing conditions, the cross-linked complexes immunoprecipitated with anti-TFPI antibody (lanes 4 and 10) migrated to the identical position as that seen in cell lysates immunoprecipitated with anti-LRP antibody (lanes 5 and 11). In the presence of protamine, 15% more LRP was immunoprecipitated with anti-TFPI antibody, relative to the total amount of LRP immunoprecipitated with anti-LRP antibody (lane 10/lane 11 versus lane 4/lane 5). Finally, the amount of LRP immunoprecipitated with anti-TFPI antibody under reducing conditions relative to...
LRP Mediates TFPI Endocytosis

FIG. 7. Inhibition of 125I-TFPI degradation by 39-kDa protein constructs on MH1C1 cells. Cells were incubated for 4 h at 37°C with 2 nM 125I-TFPI in the absence or presence of increasing concentrations of various 39-kDa protein constructs, and degraded 125I-TFPI in the overlying buffer was determined. In A, the constructs used were: GST/1–319 (solid circles), GST/115–319 (solid triangles), GST/115–287 (solid squares), GST/115–255 (solid diamonds), and, as a negative control, GST (open circles). In B, the constructs used were: GST/1–319 (solid circles), GST/115–319 (solid triangles), GST/151–319 (open triangles), GST/187–319 (open squares), GST/200–319 (open diamonds), GST/225–319 (open inverse triangles), and, as a negative control, GST (open circles). 125I-TFPI degraded in the absence of any 39-kDa protein construct was defined as 100%. Each symbol is the average of duplicate determinations.

The total amount of cross-linked material immunoprecipitated with anti-TFPI antibody under nonreducing conditions was 5-fold greater in the presence of protamine than in the absence of protamine (lane 10; lane 7 versus lane 4; lane 1). Equivalent amounts of LRP were immunoprecipitated under reducing conditions with anti-LRP antibody in the absence and presence of protamine and in the absence and presence of cross-linking (lanes 5, 11, and 17). In the absence of cross-linking, LRP was immunoprecipitated with anti-LRP antibody (lanes 14 and 17) and not with anti-TFPI antibody (lanes 13 and 16). No proteins were immunoprecipitated using normal rabbit serum (lanes 3, 6, 9, 12, 15, and 18).

Inhibition of 125I-TFPI Degradation by 39-kDa Protein Constructs—To define regions of the 39-kDa protein required for the inhibition of 125I-TFPI interaction with LRP, the ability of a series of GST-fusion proteins encoding distinct regions of the 39-kDa protein to inhibit 125I-TFPI degradation by MH1C1 cells was examined. Fig. 7A shows the GST-fusion protein encoding carboxyl-terminal residues 115–319 of the 39-kDa protein (GST/115–319) inhibits 125I-TFPI degradation identically to the full-length GST-39-kDa protein (GST/1–319). Both GST/1–319 and GST/151–319 inhibited 80% of 125I-TFPI degradation at concentrations of 1 μM. GST/115–319 (500 nM) also inhibited 85% of specific 125I-TFPI binding at 4°C when protamine (100 μg/ml) was included in the binding medium but did not alter 125I-TFPI binding when protamine was omitted (data not shown). Fig. 7A also shows when amino acids 287–319 or 255–319 are removed from GST/115–319, the resulting constructs, GST/115–287 and GST/115–255, do not effectively inhibit 125I-TFPI degradation, suggesting residues 287–319 are important. Fig. 7B demonstrates inhibition of 125I-TFPI degradation by constructs in which amino acids are deleted from the amino-terminal portion of GST/115–319. The order of potency by these constructs is GST/151–319 > GST/187–319 > GST/200–319 > GST/225–319. The amino-terminal construct GST/1–114 (500 nM) inhibited 40–50% of 125I-TFPI degradation and binding when the binding medium contained protamine (data not shown).

Cross-competition Binding Experiments between TFPI and LRP-Ligands—Competition binding experiments on MH1C1 cells were performed to determine whether TFPI could alter the binding of previously characterized LRP-ligands. As seen in Table I, the 39-kDa protein and TFPI inhibit 94% and 85% of specific 125I-αM* binding, respectively. Each inhibits 125I-αM* binding with an IC50 value of 20 nM (data not shown). Each also inhibits >95% of 125I-αM* degradation (data not shown). Neither the 39-kDa protein nor αM* alters 125I-TFPI binding. Protamine inhibits >95% of 125I-TFPI binding. When the binding medium contains protamine, the 39-kDa protein and αM* reduce 125I-TFPI binding by 85% and 50%, respectively. Tissue-type plasminogen activator does not alter 125I-TFPI or 125I-αM* binding (data not shown).

DISCUSSION

Using hepatoma cells, we previously demonstrated that LRP mediates the cellular degradation of 125I-TFPI since degradation was inhibited by antibodies directed against LRP and by the 39-kDa protein (10). In the present study, we demonstrate that LRP is required for the degradation of 125I-TFPI since LRP-heterozygous cells, in contrast to LRP-negative cells, actively degrade 125I-TFPI in a 39-kDa protein-inhibitable manner. Fragments of the 39-kDa protein, which differentially regulate αM* and tissue-type plasminogen activator binding to LRP (23), also differentially inhibit 125I-TFPI degradation and HSPG-independent 125I-TFPI binding. The observation that protamine enhances 125I-TFPI degradation by LRP-positive MH1C1 and PEA10 cells is potentially important. While prevention of TFPI from binding to HSPGs may provide additional TFPI for uptake and degradation by LRP, this is unlikely since the absolute concentration of free 125I-TFPI is essentially unchanged in the absence and presence of protamine. An alternative explanation may be that the “effective” 125I-TFPI concentration is increased by protamine; protamine may increase the affinity and/or efficiency of TFPI endocytosis by LRP. The finding that 1 μM 39-kDa protein inhibits >95% of 125I-TFPI degradation by LRP-positive cells in the presence of protamine whereas 70–80% is inhibited in the absence of protamine supports this possibility. Further studies will be necessary to specifically define this mechanism. Nonetheless, the use of protamine provides a strategy to dissect the role of LRP in TFPI endocytosis, and together our results suggest that the direct binding of TFPI to LRP, rather than the transfer of TFPI from HSPGs to LRP, is the predominant mechanism by which TFPI endocytosis occurs, i.e., if transfer was an efficient mechanism, protamine should not enhance, and should, in fact, inhibit 125I-TFPI degradation since protamine would prevent 125I-TFPI binding to HSPGs and subsequent transfer to LRP.

### Table I

| Unlabeled competitor | Specific femtomoles 125I-ligand bound/10⁶ cells |
|-----------------------|-----------------------------------------------|
| αM*                  | None                                          |
| 39-kDa protein        | 2.2                                           |
| TFPI                  | 0.13                                          |
| αM*                  | 0.33                                          |
| Protamine             | 95                                            |
| Protamine + 39-kDa protein | 14                                      |
| Protamine + αM*       | 48                                            |

Cross-competition binding experiments between TFPI and αM*.

MH1C1 cells were incubated with 6 nM 125I-TFPI or 50 pm 125I-αM* for 2 h at 4°C in the absence or presence of unlabeled 39-kDa protein (1 μM), αM* (200 nM), TFPI (1 μM), and protamine (100 μg/ml). Nonspecific binding, determined in the presence of 500 nM TFPI or 50 nM αM*, accounted for 25% and 10% of total binding, respectively, and has been subtracted from each point. Each value is the average of duplicate determinations.
In addition, if transfer occurred, the 39-kDa protein should inhibit 125I-TFPI degradation to a similar extent in the absence and presence of protamine, unless transfer was a very slow process.

Additional data supporting a direct interaction between TFPI and LRP and this mechanism as responsible for TFPI uptake and degradation are seen in our cross-linking experiments. After cross-linking unlabeled TFPI to [125I]lysylne-labeled MH, C4 and LRP-heterozygous cells, LRP is immunoprecipitated using antibodies directed against TFPI. No specific cross-linking of TFPI to any species is observed on LRP-nega-
tive cells. Consistent with the hypothesis that more TFPI binds to LRP when HSPGs are blocked with protamine is the obser-
vation that relative to the total cohort of cross-linked proteins, 5-fold more TFPI is cross-linked to LRP in the presence of protamine. In other words, in the presence of protamine, sub-
stantially less TFPI binds to the cell surface so fewer cell surface proteins would be cross-linked to TFPI. Indeed, under nonreducing conditions and in the presence of protamine, 4–fold less total radioactivity is immunoprecipitated with anti-
TFPI antibody. Under reducing conditions, protamine does not alter the amount of LRP immunoprecipitated with anti-TFPI antibody since saturating concentrations of TFPI (~5 times the Kd value) would saturate LRP in the presence of protamine and would saturate both LRP and the major TFPI binding species in the absence of protamine.

Following reduction and SDS-PAGE, the fate of the remain-
ning radioactivity initially retained in the stacking gel under nonreducing conditions in the absence of protamine could be attributed to the fact that when unlabeled TFPI is cross-linked to cell surface proteins, it is cross-linked not only to LRP but also to numerous other species of various molecular masses. Under nonreducing conditions, these cross-linked complexes are retained in the stacking gel, whereas under reducing con-
ditions, the radioactivity from these complexes is widely dis-
tributed throughout the gel lane.

As stated, 4-fold more radioactivity was retained in the stacking gel under nonreducing conditions in the absence of protamine than in the presence of protamine. Yet, under re-
ducing conditions, only LRP was evident in the absence (and presence) of protamine. In the absence of protamine, it is not clear why the major TFPI binding species was not also appar-
ent following reduction of the cross-linker. One possibility is that the majority of TFPI binding is to a cell surface carbohydrate moiety and will not be cross-linked to TFPI using DTSSP. Another possibility is that free amine groups on the major TFPI binding species are not accessible to cross-linking with DTSSP. It is also conceivable that there are no cysteines available for metabolic labeling within this TFPI binding species. Finally, this molecule may not be solubilized with 1% Triton X-100. Support for this latter explanation comes from a recent study (30) which reported that endogenous TFPI is bound to the endothelial cell surface by a glycosylphosphatidylinositol-
linked receptor that is insoluble in Triton X-100.

Another line of evidence supporting a direct interaction between TFPI and LRP comes from our saturation binding experi-
ments on hepatoma cells performed in the absence and presence of protamine. In the absence of protamine, TFPI binds to 2.5 × 105 low affinity sites/cell (Kd = 20–30 nM) (10) whereas in the presence of protamine, TFPI binds to 40,000 high affinity sites/cell (Kd = 2.3 nM). Previous reports on ligand binding affinities and numbers of binding sites/cell of LRP-
ligands are similar to that of TFPI binding in the presence of protamine. For example, the Kd value for tissue-type plasminogen activator binding sites on hepatoma cells is 60,000–100,000 (24, 31). Thus, the similarity in affinity and number of binding sites/cell between TFPI in the presence of protamine and pre-
viously characterized LRP-ligands is consistent with direct in-
teraction between TFPI and LRP.

Finally, the findings that TFPI inhibits 125I-αM* binding and that αM* partially inhibits 125I-TFPI binding when HSPGs are blocked with protamine also suggest that at least a fraction of cell surface TFPI binding is directly to LRP since αM* is known to bind only to LRP. The inability of unlabeled αM* to alter 125I-TFPI binding supports our hypothesis that only a small fraction of cell surface TFPI binding is to LRP. Our results further suggest TFPI and αM* may bind to the same site on LRP. Differences in on/off rates for TFPI and αM* could account for the varying extents of inhibition of binding ob-
erved. Alternatively, TFPI and αM* may function as noncom-
petitive inhibitors for binding and therefore bind to different sites on LRP. Future competition binding experiments between TFPI and αM* are required to resolve these issues.

In summary, we have shown that TFPI binds directly to LRP, that LRP is required for mediating the cellular degrada-
tion of TFPI, and that under conditions where TFPI is unable to bind to HSPGs, TFPI binds in a 39-kDa protein-inhibitable manner specifically and with high affinity to the hepatoma cell surface. Thus, binding, uptake, and degradation of TFPI can occur independent of HSPGs. The two receptor systems in-
volved in TFPI catabolism appear to have different functions: LRP serves to remove TFPI from the circulation, whereas HSPGs may anchor TFPI to the cell surface so that TFPI remains in contact with plasma proteins to function as an anticoagulant.

Acknowledgments—We thank the Monsanto Co. for TFPI, Guojun Bu for helpful comments, Andy Stephens for his enthusiastic help on densitometric scanning, and Susan Starbuck for help in preparing this manuscript.

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J. Biol. Chem. 1996, 271:25873-25879.
doi: 10.1074/jbc.271.42.25873

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