The Endothelial Cell Tissue Plasminogen Activator Receptor

SPECIFIC INTERACTION WITH PLASMINOGEN*

(Received for publication, January 17, 1991)

Katherine A. Hajjar‡

From the National Institutes of Health Specialized Center of Research in Thrombosis, Divisions of Hematology-Oncology, Departments of Pediatrics and Medicine, Cornell University Medical College, New York, New York 10021

Human endothelial cells (EC) assemble plasmin-generating proteins on their surface. We have previously identified an EC membrane protein (Mr ~40,000) which specifically binds tissue plasminogen activator (t-PA) but not urokinase (Hajjar, K. A., and Hamel, N. M. (1990) J. Biol. Chem. 265, 2905–2916). In the present study, t-PA receptor protein (t-PA-R) was purified to apparent homogeneity from a detergent extract of human placental tissue by diisopropyl fluorophosphate (t-PA) affinity chromatography and preparative gel electrophoresis. In a solid phase binding assay wells coated with t-PA-R bound both 125I-t-PA and 125I-Lys-plasminogen (PLG), but not 125I-urokinase in a specific, reversible, and noncompetitive fashion. Binding of 125I-Lys-PLG, but not 125I-t-PA, to t-PA-R was 80% inhibited by a 20–100-fold molar excess of the PLG-like lipoprotein(a), or by the lysine analog, ε-aminocaproic acid (50 μM). A polyclonal anti-t-PA-R antibody inhibited 66% and 79% of the specific 125I-t-PA and 125I-Lys-PLG binding, respectively, to EC monolayers. Biosynthetically labeled 40-kDa protein coprecipitated with t-PA- or Lys-PLG-Sepharose beads, but not with unconjugated Sepharose. In a functional assay, t-PA associated with immobilized t-PA-R generated 6.4 times more plasmin than an equivalent amount of t-PA in the fluid phase. These results suggest that t-PA-R can bind both t-PA and Lys-PLG in a manner that mimics the EC surface. This protein may play a role in modulating plasmin generation on cell surfaces.

Endothelial cells support the assembly of a membrane-oriented plasmin-generating system (1). Both plasminogen and its primary circulating activator (tissue plasminogen activator, t-PA), which is synthesized and secreted by endothelial cells, bind specifically to the endothelial cell surface (2–5). In the course of this interaction, the circulating form of plasminogen, N-terminal glutamic acid plasminogen (Glu-PLG) is converted to a plasmin-modified, truncated form (N-terminal lysine plasminogen (Lys-PLG) (6), which is not found in plasma under normal circumstances (7). Lys-PLG is activated by t-PA approximately 10–20 times more efficiently than Glu-PLG (8). Lys-PLG may represent a specialized cell surface form of plasminogen since it binds EC with greater affinity than Glu-PLG and since it is localized on the surface of EC in tissues (6). Plasminogen binding to endothelial cells is inhibited by lipoprotein(a), a low density lipoprotein-like particle, which appears to compete with plasminogen for its cellular binding site probably through molecular mimicry related to its plasminogen-like apolipoprotein(a) component (9–11).

We have previously identified a specific endothelial cell surface binding site for t-PA (12). This Mr, 40,000 protein (t-PA-R) is distinct from plasminogen activator inhibitor, type 1 (PAI-1), preserves the catalytic activity of t-PA, and differs from a separate urokinase binding site also identified in plasma membrane-enriched subcellular fractions (12). Since this receptor was originally identified in cultured human umbilical vein endothelial cells, we chose human placenta as a tissue source for further isolation (13).

The current paper describes the isolation and characterization of a t-PA binding protein from detergent extracts of human placenta. This protein is identical to the endothelial cell surface t-PA-R in terms of its functional properties, structural characteristics, and immunologic cross-reactivity. In spite of a small (2 kDa) difference in apparent molecular mass, both proteins specifically bind plasminogen as well as tissue plasminogen activator, but not urokinase. Thus, we postulate that t-PA-R represents a unique dual-ligand receptor that functions to support efficient generation of plasmin on the endothelial cell surface.

EXPERIMENTAL PROCEDURES

Materials—Nunc T-75 tissue culture flasks, 24-well cluster dishes, and Maxi-sorp microtiter plates (Laboratory Disposable Products) were employed. Diisopropyl fluorophosphate (DFP), phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, bovine serum albumin (essentially fatty acid- and globulin-free), and CHAPS were from Sigma. Polyvinylidene difluoride (Immobilon) transfer membranes were purchased from Millipore. p-Nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyphosphate p-toluidine, and dithiothreitol were purchased from Millipore.

* This work was supported by National Institutes of Health Grants HL-42493, HL-46403, and HL-18828 as well as by the American Heart Association, New York Affiliate, and the Council for Tobacco Research-U.S.A., Inc. 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 1754 solely to indicate this fact.

‡ Established Investigator of the American Heart Association and a Syntax Scholar. To whom correspondence and reprint requests should be addressed: Cornell University Medical College, 1300 York Ave., Room C-606, New York, NY 10021. Tel.: 212-746-2070; Fax: 212-746-8866.

1 The abbreviations used are: PLG, plasminogen; apo(a), apolipoprotein(a); CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; EACA, ε-aminocaproic acid; EC, human umbilical vein endothelial cells; Lp(a), lipoprotein(a); Lys-PLG, N-terminal lysine plasminogen; PBS, phosphate-buffered saline; t-PA, tissue plasminogen activator; t-PA-R, tissue plasminogen activator receptor, u-PA, high molecular weight urokinase; PAI-1, plasminogen activator inhibitor, type 1; ELISA, enzyme-linked immunoabsorbent assay; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfonate; PAGE, polyacrylamide gel electrophoresis.
were obtained from Bio-Rad. Cyagenen bromide-activated Sepharose and protein G-Sepharose were purchased from Pharmacia LKB Biotechnology Inc.

**Purified Proteins**—Human recombinant t-PA, 80-90% single-chain as judged by SDS-PAGE, was generously provided by Genentech, and human Lys-plasminogen, by Immuno, Vienna, Austria. High molecular weight urokinase was purchased from American Diagnostica. Human lipoprotein(a) was a kind gift from Dr. Peter Harpel, Mount Sinai Medical Center, New York, NY. Recombinant apolipoprotein(a) was generously provided by Dr. Daniel Eaton, Genentech Corp., South San Francisco, CA.

Cell Culture—Passage human umbilical vein endothelial cells were cultured and quantified as previously described (2).

**Isolation of t-PA**—Human placental tissue was obtained fresh at the time of delivery. For each preparative run, 50-100 g (average 65.3 g) of fresh or frozen (−70 °C) tissue was washed extensively in ice-cold column buffer (CB, 25 mM Tris, 50 mM NaCl, 0.1 mM EDTA, 0.02% NaN3, pH 7.6) and diced into 1-cm³ portions. The tissue was homogenized at 4 °C in an equal (ml/g) volume of column buffer containing 1 mM phenylmethylsulfonyl fluoride, 21 µM leupeptin, and 15 µM pepstatin A in a Cuisenart mini-mate grinder (6-s burst x 5, high setting, sharp blade), and then subjected to low speed centrifugation (500 g, 20 min, 4 °C). The post nuclear supernatant (8–10 mg protein/ml) was collected and extracted with an equal volume of column buffer containing 15 mM CHAPS (CB/CHAPS, 4 °C, 18 h, gentle stirring). Since, in preliminary studies, the yield of purified protein from the unfractonated postnuclear supernatant did not exceed 0.1%, the Pelleted membranes, whole postnuclear supernatant was used in subsequent extractions. The extract was centrifuged (30,000 g, 20 min), and the supernatant subjected to affinity chromatography.

DFP-t-PA-Sepharose columns were prepared by twice treating t-PA (10 µg) with diisopropyl fluorophosphate (25 mM, 24 h, 21 °C), after which plasmin-generating activity was quantified in a fluorometric assay (2). DFP-t-PA-Sepharose (98.3 ± 0.4% inactivated, S.E., n = 4) was coupled to CNBr-activated Sepharose (0.5 g dry weight, 18 h, 21 °C, coupling efficiency 94.0 ± 2.4%; S.E., n = 4), and unoccupied sites blocked with 0.1 M Tris (2 h, 21 °C). DFP-t-PA columns, connected in a series with unmodified Tris-quenched Sepharose precolumns were loaded with placental extract at 4 ml/h, 4 °C, washed with 10 column volumes of CB/CHAPS, and eluted with CB/CHAPS containing 1 M NaCl. Peak A280 fractions were pooled and concentrated (Centricon-10), and component proteins resolved by preparative SDS-PAGE using 9% Laemmli gels. The presence and position of the major DFP-t-PA binding protein (M, 42,000) was determined by ligand blotting of companion gel lanes using endothelial cell plasma membrane extract as a positive control (12). Corresponding bands were excised from preparative gels, and protein collected by electroelution, and characterized in further studies. DFP-t-PA columns were washed extensively, and stored at 4 °C in column buffer containing 5% glycerol.

The M, 42,000 protein was further purified by electroelution (200 V, 4 h, E-C Geluter II) from individual SDS-PAGE bands in precooled 25 mM Tris, 190 mM glycine buffer. Preactivated marker proteins placed in adjacent lanes were used as a guide. The eluted protein (30–150 µg/run) was dialyzed against a 1000-fold excess of CB/CHAPS (4 °C, 24–48 h). Protein concentration was determined by the bicinchoninic acid assay (Pierce Chemical Co.) using bovine serum albumin as a standard.

**Endothelial Cell Membrane Preparation**—A plasma membrane-enriched fraction from cultured human umbilical vein endothelial cells was prepared as previously described (12).

**Immunoligand Blotting**—Ligand blotting was carried out exactly as previously described (12).

**Radioisotope Labeling**—Human recombinant t-PA, Lys-plasminogen, and urokinase were labeled by the lactoperoxidase method as previously described (5, 6, 12).

**Radioligand Binding Studies**—Binding studies were carried out in a fluorogenic assay. Microtiter plates were coated with purified t-PA-R (8–10 µg/ml carbonate buffer, pH 9.6), bovine serum albumin (10 µg/ml carbonate buffer), or buffer alone (1 h, 4 °C). The plates were washed three times (200 µl PBS/well), and then exposed to t-PA (200 ng in PBS, 37 °C, 30 min). Following an additional three washes, substrate mixture consisting of PBS containing Lys-plasminogen (34 mM), and the fluorogenic plasmin substrate p-Val-Leu-Lys-chloromethylketone (100 µM; Enzyme Systems Products) was added to each well (400 µl/well). Substrate hydrolysis in quadruplicate wells was measured at 15-min intervals at excitation 400 nm, emission 505 nm, and slit widths 2 nm using a model 6500-00 fluorescence spectrophotometer. The fluorescent product formed by plasmin generation was measured using linear regression analysis of plots of relative fluorescence units versus t² as previously described (2, 5, 12).
RESULTS

Isolation of t-PA-R from Human Placenta—Preliminary SDS-PAGE and ligand blotting analyses of various placental fractions confirmed the feasibility of affinity chromatography for purification of t-PA-R (Fig. 1). Initial evaluation of a CHAPS extract of the placental postnuclear supernatant fraction revealed that, of a total of 30–40 proteins (Fig. 1A, lane 1), four (apparent M, ~100,000, 50,000, 44,000, and 42,000) (Fig. 1B, lane 3) interacted to some degree with t-PA or its alkaline phosphatase-conjugated detecting antibody (rabbit F(ab')2 anti-human t-PA). Under the same conditions, t-PA reacted with a single polypeptide (apparent M, 40,000) extracted from human endothelial cell plasma membranes as previously reported (Fig. 1B, lane 2) (12). When the starting placental extract was further fractionated by affinity chromatography on DFP-t-PA-Sepharose, a single t-PA-reactive polypeptide (apparent M, 42,000) was retained on the column and could be eluted along with several minor contaminants in a 1 M NaCl-containing buffer (Fig. 1A, lane 3 and Fig. 1B, lane 1). Essentially all other proteins were excluded by the column (Fig. 1A, lane 2).

When evaluated by ligand blotting, the partially purified M, 42,000 protein reacted strongly with t-PA (Fig. 1B, lane 1). In some experiments, a secondary band of apparent M, 40,000 was also detected by this procedure (Fig. 1B, lane 1). In a final step, the M, 42,000 protein was electroeluted from preparative SDS gels. The resulting protein migrated as a single band under reducing conditions (Fig. 1A, lane 4) and represented a ~2000-fold purification and 24% yield with respect to t-PA-binding activity in the initial detergent extract (Table I). Interestingly, Lys-PLG-binding activity copurified (~1800-fold, 21% yield) with t-PA-binding activity. The electrophoretic mobility of the t-PA-reactive 42-kDa protein was not altered by pretreatment with N-glycanase (Genzyme, Boston, MA) for 18 h (250 units/ml, 37 °C, pH 8.6), suggesting that it did not possess significant N-linked oligosaccharide.

Characterization of Placental t-PA-R—To determine the binding properties of t-PA-R with respect to t-PA and other potential ligands, ligand blotting as well as direct binding studies were carried out (Figs. 1C and 2). The 1 M NaCl column eluate was first tested for the ability to interact with t-PA, Lys-PLG, or u-PA by ligand blotting (Fig. 1C). Like t-PA (lane 1), Lys-PLG (lane 2) interacted with a single M, 42,000 band which comigrated exactly with the t-PA-reactive band (lane 1). u-PA, on the other hand, did not interact at all with the M, 42,000 protein (lane 3). This result suggested that both Lys-PLG and t-PA might interact with a common binding protein to the exclusion of u-PA. The alkaline phosphatase-conjugated anti-u-PA antibody did interact with a M, 100,000 band which probably represented u-PA in complex

| Table I
| Copurification of t-PA- and PLG-binding activity |
|-----------------|-----------------|-----------------|-----------------|
| Fraction | Total protein | 75% Inhibitory dose | Purification | Yield |
| mg | µg/ml | -fold | % |
| t-PA | PLG | t-PA | PLG | t-PA PLG |
| CHAPS extract | 945 | 162 | 122 | 1 | 1 | 100 | 100 |
| DFP-t-PA peak | 3.5 | 0.88 | 0.81 | 184 | 151 | 68 | 55 |
| 42-kDa protein | 0.070 | 0.080 | 0.068 | 2025 | 1794 | 24 | 21 |

* Polystyrene wells were coated with 42-kDa protein (4.4 µg/ml) and exposed to 125I-t-PA (10 nm) or 125I-Lys-PLG (10 nm) in the presence of graded doses of various test fractions (0–200 µg/ml). Ligand binding was quantified as described under "Experimental Procedures," and binding activity expressed as that concentration of test sample which inhibited 75% of total 125I-t-PA or 125I-Lys-PLG binding.

* Calculated from the 75% inhibitory dose.

![Fig. 1. Isolation of t-PA-R from human placenta. A, SDS-PAGE. The postnuclear supernatant (47 ml) from homogenate of human placental tissue (63 g) was extracted with CB/CHAPS (4 °C, 18 h). The 30,000 × g supernatant (7.8 mg/ml, lane 1, 100 µg) was applied to a 1.5-ml DFP-t-PA affinity column at 4 ml/h, and the flow through (7.7 mg/ml, lane 2, 100 µg) collected in a single fraction. The column was washed until the effluent Aprot approached 0, and then eluted in 1 ml fractions with 8 ml of CB/CHAPS/NaCl. Fractions 3–5 were pooled, concentrated to 5.1 mg/ml (lane 3, 51 µg), and subjected to preparative SDS-PAGE. The electroeluted polypeptide (lane 4, 16 µg, reduced gel) contained 180 µg/ml (total 90 µg). The small and large arrows show t-PA-R enriched in the high salt eluate and in final isolated form, respectively. B, t-PA ligand blot. 1 M NaCl column eluate (lane 1, 32 µg), endothelial cell membrane extract (lane 2, 35 µg), and unfractionated placental extract (lane 3, 35 µg) were run on 9% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The membranes were ligand blotted with t-PA (10 µg/ml) followed by alkaline phosphatase-conjugated rabbit anti-t-PA (1:1500), and developed as described previously (12). C, ligand blot of column eluate. The 1 M NaCl DFP-t-PA column eluate (112 µg/lane) was applied to a 1.5-ml DFP-t-PA affinity column at 4 ml/h, and the flow through (7.7 mg/ml, lane 1, 100 µg) followed by alkaline phosphatase-conjugated rabbit anti-t-PA (1:1500), anti-PLG (1:2000), or anti-u-PA (1:1000), and developed as described elsewhere (12). |

![Fig. 2. Radioligand binding isotherms. Polystyrene microtiter wells were coated with t-PA-R (6 µg/ml) to give 740 fmol of protein bound per well, as estimated by counting wells coated with 125I-t-PA-R labeled by the lactoperoxidase reaction. Washed wells were probed for ligand binding capacity with 125I-t-PA (5–250 nM, 126,100 cpm/pmnl), 125I-Lys-PLG (10–800 nM; 585,500 cpm/pmnl), or 125I-u-PA (5–250 nM; 740,190 cpm/pmnl) for 1 h at 37 °C. Bound and free radioactivity were sampled as described under "Experimental Procedures." Inset, Scatchard plot. Binding data for the isotherms for 125I-t-PA (△) and 125I-Lys-PLG (■) were analyzed using the Ligand program (14) to estimate binding affinity and capacity. Estimated values for Kd and Bmax for t-PA and Lys-PLG were subject to errors of 13 and 15%, while Bmax values varied by 54 and 63%, respectively. |
with plasminogen-activator inhibitor type 1 or 2.

In direct solid phase binding studies, \(^{125}\text{I}\)-t-PA interacted with t-PA-R (\(~740\) fmol/well) immobilized on non-tissue culture plastic in a dose-dependent fashion which approached saturation (Fig. 2). Binding was half-maximal at an input concentration of \(~50\) nM, and reached a plateau at doses of \(\geq 175\) nM or greater. Scatchard analysis, carried out both manually and using the Ligand program (14), yielded a \(K_d\) of 30 nM, and \(B_{max}\) of 650 fmol/well \((inset)\). When \(^{125}\text{I}\)-t-PA was added in the presence of a 50-fold molar excess of unlabelled ligand, approximately 86.5% of total binding was inhibited, indicating a specific interaction. When \(^{125}\text{I}\)-t-PA (200 nM) was added to wells coated only with buffer, binding represented 18.2% of that observed in wells coated with t-PA-R.

When evaluated in the same system, \(^{125}\text{I}\)-Lys-PLG bound specifically, saturably, and with high affinity to t-PA-R \((Fig. 2)\). Binding was half-maximal at an input dose of \(~165\) nM and plateaued at input doses greater than 500 nM. Scatchard analysis \((inset)\) revealed a \(K_d\) of 114 nM and \(B_{max}\) of 495 fmol/well. A 50-fold excess of unlabelled Lys-PLG blocked 85% of binding of the labeled ligand, indicating a highly specific interaction. Binding of \(^{125}\text{I}\)-Lys-PLG (500 nM) to uncoated wells was 11.9% of that observed for wells coated with t-PA-R. \(^{125}\text{I}\)-u-PA, on the other hand, displayed minimal specific binding \((Fig. 2)\). At input doses of 250 nM, t-PA-R specifically bound only 25 fmol of labeled u-PA versus 245 fmol of Lys-PLG or 555 fmol of t-PA. Thus, t-PA-R specifically bound both t-PA and Lys-PLG, but not u-PA.

To determine whether the interactions between \(^{125}\text{I}\)-t-PA and \(^{125}\text{I}\)-Lys-PLG with t-PA-R were the result of equilibrium binding, time course and reversibility experiments were carried out \((Fig. 3)\). When \(^{125}\text{I}\)-t-PA and \(^{125}\text{I}\)-Lys-PLG were incubated with immobilized t-PA-R for up to 60 min, binding approached a steady state at approximately 20 and 10 min, respectively. Upon "infinite dilution," 50% of total \(^{125}\text{I}\)-t-PA and 68% of \(^{125}\text{I}\)-Lys-PLG bindings were reversible. A similar proportion of t-PA and Lys-PLG binding \((67\) and \(71\), respectively) was blocked by excess unlabelled ligand in the same experiment. Thus, nearly all of the specific binding was reversible for both ligands, indicating an equilibrium type of interaction.

We next investigated whether t-PA and Lys-PLG interacted with the same or different binding domains of t-PA-R. A series of co-competition experiments were carried out with PLG and t-PA pretreated with DFP to avoid generation of plasmin \((Fig. 4)\). When \(^{125}\text{I}\)-DFP-t-PA was added to immobilized t-PA-R in the presence of increasing excess amounts of unlabelled DFP-t-PA, specific binding was 50% inhibited by a 3.0-fold molar excess \((15\) nM) of unlabelled ligand, and completely inhibited by a 50-fold molar excess \((250\) nM). Similarly, a 9.0-fold molar excess \((90\) nM) of unlabelled Lys-PLG efficiently blocked 50% of specific \(^{125}\text{I}\)-Lys-PLG binding to t-PA-R and a 100-fold molar excess \((1000\) nM) eliminated all of the specific binding. In contrast, however, neither Lys-PLG nor DFP-t-PA competed at all for binding of \(^{125}\text{I}\)-DFP-t-PA or \(^{125}\text{I}\)-Lys-PLG to t-PA-R, respectively, over a similar concentration range. This lack of cross-competition suggested that the two ligands could interact simultaneously at separate domains on t-PA-R in a mutually permissive fashion.

Binding of both \(^{125}\text{I}\)-t-PA and \(^{125}\text{I}\)-Lys-PLG to t-PA-R, furthermore, depended on the t-PA-R coating concentration \((Fig. 5)\). \(^{125}\text{I}\)-t-PA binding was linear over a range of coating concentrations \((0\) to \(100\) ng/ml). \(^{125}\text{I}\)-Lys-PLG binding, similarly, was linear at coating concentrations between 0 and 250 ng/ml. For both ligands, binding was maximal at coating concentrations in excess of 250 ng/ml.

Fluid-phase t-PA-R, moreover, specifically blocked binding of labeled t-PA or Lys-PLG to immobilized t-PA-R \((Fig. 6)\). When a fixed concentration of either \(^{125}\text{I}\)-t-PA \((50\) nM) or \(^{125}\text{I}\)-Lys-PLG \((100\) nM) was added to t-PA-R-coated wells in the presence of increasing molar quantities of fluid-phase t-PA-R, binding to the immobilized protein was reduced in a dose-dependent fashion. Half-maximal inhibition of \(^{125}\text{I}\)-t-PA and

---

**Fig. 3.** Time course and reversibility of ligand interactions with t-PA-R. \(^{125}\text{I}\)-t-PA. Microtiter wells were coated with t-PA-R \((10.0\) ng/μl), washed, and incubated with \(^{125}\text{I}\)-t-PA \((384,540\) cpm/pmol, 5 nM) for the indicated times. Wells were washed three times rapidly, and bound radioactivity quantified. At 30 min, additional wells were emptied and then filled with 400 μl Tris/Tween to approximate "infinite dilution." After a second incubation for the indicated time period, wells were emptied, washed rapidly three times, and bound radioactivity quantified. Non-specific binding at 60 min is indicated by an asterisk (*) for each ligand. B, \(^{125}\text{I}\)-Lys-PLG. T-PA-R-coated wells were incubated with \(^{125}\text{I}\)-Lys-PLG \((519,740\) cpm/pmol, 5 nM) for the indicated times, and processed as described for A.

**Fig. 4.** Co-competition studies. Microtiter wells were coated with t-PA-R \((5.3\) ng/μl), and exposed to \(^{125}\text{I}\)-DFP-t-PA \((5\) nM; 143,968 cpm/pmol; A, △) or \(^{125}\text{I}\)-Lys-PLG \((10\) nM; 888,470 cpm/pmol; □, ▼) in the presence of increasing concentrations of unlabeled DFP-t-PA (■, ◆) or unlabeled Lys-PLG (◆, □) for 1 h at 37 °C. Bound radioactivity was quantified as described under "Experimental Procedures." The 100% values represented 39.4 fmol/well for DFP-\(^{125}\text{I}\)-t-PA and 6.8 fmol/well for \(^{125}\text{I}\)-Lys-PLG. Asterisks (*) indicate the labeled ligand for each experimental group. Mean values for quadruplicate determinations are shown.
to each well, and the plates were incubated for 1 h, 37°C. t-PA-R
binding to wells coated with buffer alone (20% and 12% of maximum
value for 125I-t-PA and 125I-Lys-PLG, respectively). The 100% values
represented 104 fmol/well for 125I-t-PA and 55 fmol/well for 125I-Lys-
PLG. The values shown represent the means of quadruplicate deter-
ninations.

Maximal inhibition of specific binding was estimated as that present after subtracting precipitated from EC membrane extracts with either DFP-t-
PA-Sepharose or Lys-PLG-Sepharose also reacted with IgG directed against the
placental t-PA-R in immunoblot analyses (Fig. 7B). These results indicated that the endothelial cell t-PA-R could be precipitated from EC membrane extracts with either DFP-t-PA-
or Lys-PLG-Sepharose, that this protein was synthesized by these cells, and that it cross-reacted with antibody to the purified placental protein.

125I-Lys-PLG binding was achieved at low molar ratios of fluid-phase t-PA-R to ligand (2:1 and 3:5:1, or 100 nM and 350 nM, respectively). Maximal inhibition of specific binding was achieved at a t-PA-R concentration of 850 and 900 nM for 125I-t-PA and 125I-Lys-PLG, respectively. This represented molar ratios of 17:1 and 9:1 of t-PA-R:t-PA or Lys-PLG, respectively. In contrast, an irrelevant protein, bovine serum albumin, dissolved in the same buffer, blocked only 12–23% of binding of 125I-t-PA and 125I-Lys-PLG, respectively, to immobilized t-PA-R. These data suggested that both ligands could interact efficiently with either fluid-phase or insolubilized t-PA-R, and that nearly all of the observed specific binding could be attributed to sites on this protein.

Relationship of Placental t-PA-R to Endothelial Cell Binding Sites for t-PA and Plasminogen—To determine whether the putative t-PA-R on EC was synthesized by these cells rather

than adsorbed to the cell surface from the growth medium, biosynthetic labeling studies were carried out (Fig. 7A). A membrane fraction was prepared as previously described from EC cultured in the presence of [35S]methionine. As shown in lane 1, metabolically labeled membrane proteins varied in apparent molecular mass from 10 to 200 kDa. When this material was precipitated with DFP-t-PA- or Lys-PLG-conjugated Sepharose, two major proteins with apparent Mr, 46,000 and 42,000 were detected by SDS gel fluorography under reducing conditions. Of these two bands, only the Mr, 42,000 band coprecipitated with DFP-t-PA-Sepharose (lane 2), as well as Lys-PLG-Sepharose (lane 3), but un conjugated Sepharose (lane 4). The Mr, 46,000 band was detected under all three conditions, indicating a nonspecific precipitation product. The Mr, 42,000 band corresponded in apparent mass to the reduced form of the putative t-PA binding site on EC, as previously described (12). In addition, the Mr, 42,000 band precipitated with either DFP-t-PA-Sepharose or Lys-
PLG-Sepharose also reacted with IgG directed against the placental t-PA-R in immunoblot analyses (Fig. 7B). These results indicated that the endothelial cell t-PA-R could be precipitated from EC membrane extracts with either DFP-t-PA-
or Lys-PLG-Sepharose, that this protein was synthesized by these cells, and that it cross-reacted with antibody to the purified placental protein.

125I-Lys-PLG binding was achieved at low molar ratios of fluid-phase t-PA-R to ligand (2:1 and 3:5:1, or 100 nM and 350 nM, respectively). Maximal inhibition of specific binding was achieved at a t-PA-R concentration of 850 and 900 nM for 125I-t-PA and 125I-Lys-PLG, respectively. This represented molar ratios of 17:1 and 9:1 of t-PA-R:t-PA or Lys-PLG, respectively. In contrast, an irrelevant protein, bovine serum albumin, dissolved in the same buffer, blocked only 12–23% of binding of 125I-t-PA and 125I-Lys-PLG, respectively, to immobilized t-PA-R. These data suggested that both ligands could interact efficiently with either fluid-phase or insolubilized t-PA-R, and that nearly all of the observed specific binding could be attributed to sites on this protein.

Relationship of Placental t-PA-R to Endothelial Cell Binding Sites for t-PA and Plasminogen—To determine whether the putative t-PA-R on EC was synthesized by these cells rather

than adsorbed to the cell surface from the growth medium, biosynthetic labeling studies were carried out (Fig. 7A). A membrane fraction was prepared as previously described from EC cultured in the presence of [35S]methionine. As shown in lane 1, metabolically labeled membrane proteins varied in apparent molecular mass from 10 to 200 kDa. When this material was precipitated with DFP-t-PA- or Lys-PLG-conjugated Sepharose, two major proteins with apparent Mr, 46,000 and 42,000 were detected by SDS gel fluorography under reducing conditions. Of these two bands, only the Mr, 42,000 band coprecipitated with DFP-t-PA-Sepharose (lane 2), as well as Lys-PLG-Sepharose (lane 3), but un conjugated Sepharose (lane 4). The Mr, 46,000 band was detected under all three conditions, indicating a nonspecific precipitation product. The Mr, 42,000 band corresponded in apparent mass to the reduced form of the putative t-PA binding site on EC, as previously described (12). In addition, the Mr, 42,000 band precipitated with either DFP-t-PA-Sepharose or Lys-
PLG-Sepharose also reacted with IgG directed against the placental t-PA-R in immunoblot analyses (Fig. 7B). These results indicated that the endothelial cell t-PA-R could be precipitated from EC membrane extracts with either DFP-t-PA-
or Lys-PLG-Sepharose, that this protein was synthesized by these cells, and that it cross-reacted with antibody to the purified placental protein.

125I-Lys-PLG binding was achieved at low molar ratios of fluid-phase t-PA-R to ligand (2:1 and 3:5:1, or 100 nM and 350 nM, respectively). Maximal inhibition of specific binding was achieved at a t-PA-R concentration of 850 and 900 nM for 125I-t-PA and 125I-Lys-PLG, respectively. This represented molar ratios of 17:1 and 9:1 of t-PA-R:t-PA or Lys-PLG, respectively. In contrast, an irrelevant protein, bovine serum albumin, dissolved in the same buffer, blocked only 12–23% of binding of 125I-t-PA and 125I-Lys-PLG, respectively, to immobilized t-PA-R. These data suggested that both ligands could interact efficiently with either fluid-phase or insolubilized t-PA-R, and that nearly all of the observed specific binding could be attributed to sites on this protein.

Relationship of Placental t-PA-R to Endothelial Cell Binding Sites for t-PA and Plasminogen—To determine whether the putative t-PA-R on EC was synthesized by these cells rather

than adsorbed to the cell surface from the growth medium, biosynthetic labeling studies were carried out (Fig. 7A). A membrane fraction was prepared as previously described from EC cultured in the presence of [35S]methionine. As shown in lane 1, metabolically labeled membrane proteins varied in apparent molecular mass from 10 to 200 kDa. When this material was precipitated with DFP-t-PA- or Lys-PLG-conjugated Sepharose, two major proteins with apparent Mr, 46,000 and 42,000 were detected by SDS gel fluorography under reducing conditions. Of these two bands, only the Mr, 42,000 band coprecipitated with DFP-t-PA-Sepharose (lane 2), as well as Lys-PLG-Sepharose (lane 3), but un conjugated Sepharose (lane 4). The Mr, 46,000 band was detected under all three conditions, indicating a nonspecific precipitation product. The Mr, 42,000 band corresponded in apparent mass to the reduced form of the putative t-PA binding site on EC, as previously described (12). In addition, the Mr, 42,000 band precipitated with either DFP-t-PA-Sepharose or Lys-
PLG-Sepharose also reacted with IgG directed against the placental t-PA-R in immunoblot analyses (Fig. 7B). These results indicated that the endothelial cell t-PA-R could be precipitated from EC membrane extracts with either DFP-t-PA-
or Lys-PLG-Sepharose, that this protein was synthesized by these cells, and that it cross-reacted with antibody to the purified placental protein.
To further delineate the binding characteristics of placental t-PA-R, several experiments were carried out. When \(^{125}\)I-Lys-PLG was added to immobilized t-PA-R in the presence of increasing concentrations of epsilon-aminoacaproic acid (EACA, 1-100 mM), 50% inhibition of binding was observed at 17 mM EACA, and maximal inhibition of binding (83.5%) was noted at 50-100 mM EACA (Fig. 8). In contrast, the interaction between \(^{125}\)I-t-PA and immobilized t-PA-R was relatively resistant to EACA since doses of 1-50 mM blocked no more than 15% of total specific binding, and the highest dose tested (100 mM EACA) inhibited only half of the specific binding. These data suggested that the interaction of Lys-PLG, but not t-PA, with t-PA-R involved “kringle”-associated lysine binding sites. These results mimicked those previously observed at the cell surface (2, 5).

Similarly, lipoprotein(a) (Lp(a)), a low density lipoprotein-like particle whose apoprotein shares structural homology with plasminogen (15), selectively inhibited binding of Lys-PLG to t-PA-R (Fig. 9). When a fixed concentration of \(^{125}\)I-Lys-PLG was added to t-PA-R-coated wells in the presence of increasing molar excess amounts of Lp(a), specific binding was strongly inhibited. Eighty percent of total specific binding was inhibited by 20 to 100:1 molar ratio (Lp(a):Lys-PLG) (200-1000 nM), and 50% inhibition was observed at 7.5:1 molar excess (75 nM). In sharp contrast, binding of \(^{125}\)I-t-PA to t-PA-R was not affected by up to a 100:1 molar ratio of Lp(a):t-PA (1000 nM). Similar results were obtained with recombinant apo(a), suggesting that both Lp(a) and apo(a) can selectively inhibit binding of Lys-PLG to t-PA-R, while exerting no discernible effect on t-PA binding. These findings mimic the behavior of Lp(a) at the endothelial cell surface (9-11) and suggest that separate mechanisms support binding of t-PA and Lys-PLG to t-PA-R.

To investigate whether the t-PA receptor on endothelial cells might also possess dual-ligand capacity, ligand blotting experiments were carried out with endothelial cell membrane extract (Fig. 10). Test ligands included t-PA (lane 1), Lys-PLG (lanes 2 and 3), and Lp(a) (lanes 4 and 5). All three ligands interacted specifically with an M, 40,000 polypeptide, whereas urokinase, as previously reported (12), did not (not shown). Interaction of Lys-PLG with this protein was inhibited in the presence of 10 mM EACA (lane 3), while the interaction of Lp(a) was only slightly affected (lane 5). In experiments not shown, EC t-PA-R reacted with recombinant apo(a) in an identical fashion. Thus, endothelial cell t-PA-R, like placental t-PA-R, interacted with both t-PA and Lys-PLG, as well as the PLG-like Lp(a).

Further ligand blotting experiments (not shown) demonstrated that the 42-kDa placental protein, like the previously described 40-kDa EC protein, was unrelated to the physiologic inhibitor of t-PA, plasminogen activator inhibitor type 1 (PAI-1). First, interaction of placental t-PA-R, like its endothelial cell counterpart (12), was unaffected by active-site blockade of t-PA with DFP. Second, placental t-PA-R, like the endothelial cell protein (12), failed to react by Western blotting with antibody to PAI-1. Third, reduction of the placental protein with dithiothreitol (530 mM, 100 °C, 5 min) resulted in a 2-kDa increment in apparent molecular mass as judged by ligand blotting. PAI-1 is a cysteine-less protein which is not susceptible to chemical reduction (16). In addition, neither protein reacted either by ELISA or by immunoblot analysis with antibody directed against human actin.

\(\alpha\)-Enolase (M, 54,000) has recently been identified as a cell surface plasminogen-binding protein (17). However, when the purified t-PA-R was assayed for enolase activity, as assessed by the ability to convert 2-phospho-D-glycerate to phosphoenolpyruvate using rabbit muscle enolase as a standard (18), no activity was detected. In immunoblot analyses, furthermore, neither placental nor endothelial cell forms of t-PA-R reacted with two anti-\(\alpha\)-enolase antibodies, kindly provided by Dr. Kanefusa Kato (Institute for Developmental Research, Aichi Prefectural Colony, Aichi, Japan) and Dr. Agata Giallongo (Istituto di Biologia dello Sviluppo, Palermo, Italy). Thus, the isolated t-PA-R could be distinguished from \(\alpha\)-enolase based on these criteria.

Immunologic cross-reactivity between placental t-PA-R and the endothelial cell t-PA-R was demonstrated using polyclonal anti-placental t-PA-R antibodies. Anti-t-PA-R IgG (half-maximal titer 1:3000), but not preimmune IgG, reacted strongly with nonpermeabilized confluent EC monolayers in a cell ELISA (2). This antibody, furthermore, specifically recognized an M, 40,000-42,000 doublet in the 1 M NaCl DFP-t-PA column eluate (Fig. 11A, lane 2), and also reacted with...
Dependent fashion in the presence of fluid phase placental extract. To polyvinylidene difluoride. After blocking with bovine serum albumin IgG) blocked no more than 15% of binding of either t-PA and Lys-PLG. Anti-t-PA-R inhibited binding of t-PA and Lys-PLG to confluent human endothelial cell monolayers (Fig. 11B). T-PA and Lys-PLG were closely related immunologically. Half-maximal inhibition of specific t-PA binding was observed at 20 nM fluid-phase t-PA-R, while inhibition of Lys-PLG binding was half-maximal at ~40 nM t-PA-R. Maximal inhibition of specific binding of both ligands (~60%) occurred with 200–500 nM fluid phase t-PA-R. These results

Fig. 10. Immunoligand blotting of endothelial cell membrane extract. Detergent extract from a plasma membrane-enriched fraction from cultured human umbilical vein endothelial cells was prepared as described under "Experimental Procedures." Proteins in the resulting mixture were resolved by SDS-PAGE and transferred to polyvinylidene difluoride. After blocking with bovine serum albumin (50 mg/ml) and 0.2% nonfat dry milk, the blots were incubated with various ligands including t-PA (lane 1, 10 μg/ml), Lys-PLG (lanes 2 and 3, 0.5 μg/ml), and Lp(a) (lanes 4 and 5, 1.0 μg/ml) in the absence (lanes 1, 2, and 4) or presence (lanes 3 and 5) of 10 mM EACA. Binding of t-PA and Lys-PLG were detected with alkaline phosphatase-conjugated rabbit anti-t-PA or alkaline phosphatase-conjugated goat anti-rabbit IgG at 1:1500, lane 3), or alkaline phosphatase-conjugated goat anti-rabbit IgG (1:500, lanes 1, 2, 4, and 5). The blots were developed with p-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate p-toluidine (12). Immunoinhibition studies. Confluent EC monolayers in 24-well cluster plates were equilibrated to 4°C, washed twice with IB(5) (IB(5), 11 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 137 mM NaCl, 4 mM KCl, 3 mM CaCl2, 1 mM MgCl2, 11 mM glucose, pH 7.4, 5 mg/ml bovine serum albumin), once with IB(5) containing 10 mM EACA, and once more with IB(5). EC were preincubated with rabbit anti-human albumin (140 μg/ml), rabbit anti-t-PA-R (88 μg/ml), or IB(5) alone (30 min, 4°C) as indicated. Following three washes with IB(5), T-PA-R (1 nM; 580,000 cpm/pmol) or Lys-PLG (1 nM; 588,500 cpm/pmol) were added and incubated at 4°C, 30 min. After sampling unbound radioactivity, the monolayers were washed three times and solubilized for quantification of bound radioactivity. Specific binding represented 83.6% of total binding for t-PA and 90.1% for Lys-PLG.
serum albumin (10 μg/ml), or carbonate buffer, washed, exposed to t-PA fluorogenic plasmin substrate to the t-PA-R-coated wells based on concurrent radioligand studies. Plasmin generation was estimated as relative fluorescence units per min as described under "Experimental Procedures." Shown are mean values (±S.E.) for three separate experiments.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Plasmin formed</th>
<th>t-PA/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-PA-R</td>
<td>0.257 ± 0.063</td>
<td>550</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.063 ± 0.012</td>
<td>127</td>
</tr>
<tr>
<td>Buffer</td>
<td>0.061 ± 0.039</td>
<td>166</td>
</tr>
<tr>
<td>Fluid-phase t-PA</td>
<td>0.040 ± 0.008</td>
<td>500</td>
</tr>
</tbody>
</table>

* Relative fluorescence units.

strongly suggested that both ligands could interact with fluid phase t-PA-R in a manner which blocks binding to the endothelial cell surface.

Functional Properties of t-PA-R-associated t-PA—Since previous studies have demonstrated that t-PA associated with the endothelial cell surface (5) or with its isolated receptor (12) retains its catalytic activity, we investigated whether isolated placental t-PA-R would support the plasmin-generating activity of t-PA (Table II). When microtiter wells were coated with purified t-PA-R, exposed to t-PA (200 nM), washed, and then tested for the ability to generate plasmin in the presence of Lys-PLG and a fluorescent plasmin substrate, bound t-PA was found to retain its activity. On average, t-PA-R-associated t-PA generated 6.4-fold more plasmin than an equivalent amount of fluid-phase t-PA. In wells coated with either bovine serum albumin or buffer alone, plasminogen activating activity was slightly enhanced (1.5- to 2.1-fold, respectively) over the fluid phase control. These results suggested that t-PA-R preserved the catalytic activity of t-PA.

DISCUSSION

This study describes the isolation and characterization of a membrane protein, common to endothelial cells and placenta, which specifically binds both plasminogen and tissue plasminogen activator. Several lines of evidence indicate that this protein, isolated from placenta and synthesized by cultured endothelial cells, is functionally and immunologically related to the previously described EC receptor for t-PA (12). Polyclonal antibodies raised against placental t-PA-R recognized an EC membrane protein which comigrated with the EC t-PA-R identified by ligand blotting. These antibodies inhibited ~66 and ~69% of specific t-PA and Lys-PLG binding to EC, respectively. In addition, fluid phase t-PA-R inhibited ~60% of specific binding of both t-PA and Lys-PLG to endothelial cells, and both t-PA- and PLG-Sepharose precipitated the putative receptor from biosynthetically labeled EC membrane extract. In addition, specific binding activity for t-PA and Lys-PLG copurified through DFP-t-PA affinity chromatography and preparative SDS-PAGE, suggesting a common binding activity. We conclude, therefore, that t-PA-R may represent a unique double-ligand EC receptor, functioning to support plasmin generation at the cell surface.

The specificity of binding of both t-PA and Lys-PLG to placental t-PA-R was demonstrated in several experiments. First, binding of both radioligands could be specifically competed with excess quantities of the same but not dissimilar unlabeled ligand (Fig. 4). Second, binding was directly proportional to the quantity of t-PA-R present on microtiter wells (Fig. 5), and binding of radiolabeled ligands to empty wells represented a small fraction (12-18%) of binding to t-PA-R-coated wells. Third, binding of both ligands to t-PA-R-coated wells could be largely (75-85%) eliminated in the presence of excess molar quantities of fluid phase t-PA-R (Fig. 6). Finally, the increment in catalytic activity observed for t-PA associated with t-PA-R-coated wells was not observed for wells coated with buffer or an irrelevant protein (Table 2).

Although t-PA-R specifically bound both t-PA and Lys-PLG, its interaction with these two proteins could be differentiated in several ways. As shown in Fig. 2, the affinity of t-PA-R for t-PA was several fold higher ($K_d = 30$ nM) than its affinity for Lys-PLG ($K_d = 114$ nM). In addition, the lysine analog, EACA, was much more effective in inhibiting the interaction of t-PA-R with Lys-PLG than with t-PA, implicating a role for the lysine-binding sites of Lys-PLG, but not t-PA (Fig. 8). Third, while binding of Lys-PLG was effectively competed by Lp(a) and its apoprotein apo(a), these agents had no effect on t-PA binding to t-PA-R (Fig. 8).

Interestingly, the interactions of t-PA and Lys-PLG with t-PA-R closely mimicked their behavior at the endothelial cell surface. In a purified system, t-PA-R bound both t-PA and Lys-PLG with high affinity (Fig. 2). $K_d$ values (30 nM and 114 nM, respectively) estimated in these experiments approximated those reported previously for the endothelial cell surface ($K_d = 18$ and 120 nM, respectively) (5, 6). Like the cell surface (5) and the previously characterized endothelial cell t-PA receptor (12), placental t-PA-R supported the catalytic activity of t-PA and increased plasmin generation by about 6.4 times. Furthermore, binding of Lys-PLG, but not t-PA, was significantly blocked by either EACA (2) or Lp(a) (10), as previously reported for EC.

Together, these data suggest that t-PA-R represents a unique binding site for both t-PA and Lys-PLG on the endothelial cell surface. This protein is clearly distinguishable from other known t-PA or PLG-binding proteins such as PAI-1 and α2-Plasminogen activator, as well as from other known t-PA or PLG-binding proteins such as PAI-1 and α2-Plasminogen activator, in a way which would promote plasmin generation, and we propose the abbreviation t-PA/PLG-R in referring to this dual-ligand binding protein.

There are several examples of enzyme-substrate binding proteins in the literature. Fibrin forms a ternary complex with plasminogen and t-PA, leading to a several log order increase in the efficiency of plasmin generation (19, 20). The adhesive glycoprotein, thrombospondin, moreover, found in extracellular matrices and platelet α-granules, binds both t-PA and plasminogen in vitro (21), leading to a 40-fold enhancement of plasminogen generation over the fluid phase (22). Thrombomodulin, an EC membrane protein, binds thrombin (23) as well as its substrate, protein C (24–26). Membrane-associated factor VIIIa may support assembly of factor IXa and its substrate factor X to form prothrombinase, while membrane-associated factor Va may support the assembly of prothrombin and factor Xa during generation of thrombin (27). Formation of these trimolecular complexes may represent modulating events which control vascular fluidity at a site of blood vessel injury (27).

Plasmin is a serine protease with broad substrate specific-
ity. In addition to degrading fibrin, plasmin may play a role in accelerating its own generation by converting Glu-PLG to the more readily cleaved Lys-PLG (29-30), and by catalyzing specific cleavage events leading to the formation of two-chain plasminogen activators which possess enhanced biologic activity (31-33). Recent evidence suggests, furthermore, that plasmin can activate pro-collagenase (34) and convert transforming growth factor β from latent to active form (35, 36). Plasmin may also regulate a variety of cellular processes involving cell migration or pericellular matrix degradation. These include inflammation (37), tumor cell implantation and invasion (38), embryogenesis (39), ovulation (40), prohormone activation (41), and neurodevelopment (42, 43). Thus, another potential role for t-PA/PLG-R might be to facilitate these highly specialized functions by promoting plasmin generation on a spectrum of cell surfaces.

Acknowledgments—I gratefully acknowledge the technical assistance of Giles Shih and Louise Burgaud. I am indebted to Ralph Nachman, Aaron Marcus, and David Hajjar for helpful discussions.

Note Added in Proof—Since submission of this manuscript a M, D. P., and Boyle, M. D. P. (1991) J. Biol. Chem. 266, 4922-4928.

REFERENCES

6. Deleted in proof