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The Interaction of Plasminogen Activator With a Reconstituted Basement Membrane Matrix and Extracellular Macromolecules Produced by Cultured Epithelial Cells

Paul G. McGuire and Nicholas W. Seeds

Department of Biochemistry, Biophysics, and Genetics, University of Colorado Health Sciences Center Denver, Colorado 80262

Laminin and fibronectin are glycoproteins that influence cell behavior and mediate cell/substratum adhesion. We have examined the interaction of these macromolecules with the serine protease plasminogen activator (PA) in two types of extracellular matrices; one produced by the murine Engelbreth-Holm-Swarm (EHS) tumor (Matrigel™), and another by normal kidney epithelial cells in culture. Matrigel™ was found to contain significant quantities of tissue-type PA (tPA). Two of the major components of Matrigel™, laminin and type IV collagen, were also examined. Tissue-type PA was associated with purified preparations of laminin; however, it was not found associated with type IV collagen. Normal kidney epithelial cells in culture secrete large amounts of urokinase (UK) and deposit a subepithelial matrix containing both laminin and fibronectin. These matrix macromolecules were isolated from the deposited matrix by immunoprecipitation, examined by zymography, and found to contain UK. The potential role of this interaction in the mechanisms of cell migration and matrix remodeling is discussed.

Key words: extracellular matrix, cell migration

The extracellular matrix (ECM) influences the growth and phenotypic expression of many cell types, both in vivo and in vitro [1–4]. The most well characterized components of the ECM include the collagens [1,5], the glycosaminoglycans and associated proteoglycans [2,6], and various glycoproteins, the most prominant of which are fibronectin [7–9] and laminin [4,10,11].

Two events that are important in processes such as embryonic development and tumor metastasis are the migration of cells to their final destinations and remodeling of the extracellular matrix. These processes are likely to involve complex interactions between the surfaces of cells and the variety of matrix constituents that they encounter along the migratory pathways. One mechanism that may be involved in migration and matrix turnover is the production of the serine protease plasminogen activator (PA) and its localization or deposition near sites of cell/matrix interaction [12–15].

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Two types of PA have been described, each the product of independent genes and each characterized by differences in function, molecular weight, and immunological characteristics [16–18]. The urokinase-type (UK) plasminogen activator from a variety of human sources is a 50–55 kd glycoprotein [16,17,19], while in other species UK has been shown to have a molecular weight below 50 kd in the range of 35–48 kd [14]. Tissue-type plasminogen activator (tPA) from the human is a two-chain glycoprotein with a molecular weight of approximately 70 kD [16,17,19,20]. Unlike urokinase, tPA requires the presence of fibrin or fibrin-like molecules for full activity. Both UK and tPA possess characteristics of other serine proteases. These enzymes function primarily through the conversion of plasminogen to plasmin and are involved in a number of physiological processes including vascular fibrinolysis [21,22], ovulation [23–25], mammary gland involution [26,27], activation of latent collagenase [28,29], neoplastic metastasis [30–32], and embryonic development [33–41].

The presence of PA in the matrix or bound to the plasma membrane at cell/matrix adhesion sites may help facilitate migration by detachment of the cell from the matrix. Likewise the deposition of PA into the matrix may aid in turnover and remodeling of some of its components. Important regulatory elements in these processes may be the production and deposition of protease inhibitors, the presence of factors that regulate the production and secretion of PA, and the specific interactions of UK and tPA with various elements of the ECM. In this report we describe the presence of PA in basement membrane containing matrices, one produced by a murine tumor and another by primary epithelial cell cultures, and the preferential association of PA with specific components of these matrices.

MATERIALS AND METHODS

Materials

The reconstituted basement membrane matrix Matrigel™ (purified laminin and type IV collagen from the EHS sarcoma [42–44]) human plasma fibronectin, anti-human fibronectin, and ITS+ were obtained from Collaborative Research (Bedford, MA). Anti-mouse laminin antibody was from BRL (Gaithersburg, MD). Protein-A agarose was obtained from Pharmacia (Piscataway, NJ). Goat anti-Rabbit IgG was from Tago (Burlingame, CA). Cell culture reagents were purchased from Gibco (Grand Island, NY). Plasminogen (#P5661), casein (#C3400), and Amiloride were from Sigma (St. Louis, MO). Iodine-^{125} was purchased from Amersham (Arlington Heights, IL).

Cell Culture Conditions

Cultures of primary kidney epithelial cells from both glomerular and tubular origin were prepared from the kidneys of six-day-old C57B/6J mice. Kidneys were decapsulated, cut into small pieces, incubated in a solution of 1 mg/ml collagenase (CLS III, Cooper Biomedical, Freehold, NJ) at 37°C for 30 min, and mechanically dissociated. The tissue fragments were dispensed into tissue culture dishes and incubated at 37°C for 2 hr. This was done to remove the rapidly attaching fibroblasts present in the preparation. Glomerular and tubular fragments remaining unattached after this time were collected and transferred to other dishes and grown in serum-free medium. The serum-free media consisted of Dulbecco-Vogt modified Eagle's medium (DMEM) containing

the ITS$^+$ additives; insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenious acid (6.25 ng/ml), BSA (1.25 mg/ml), and linoleic acid (5.35 μg/ml). After four to six days in culture, a confluent monolayer of cells was formed displaying a distinct epithelial morphology.

For the preparation of radiolabeled matrices, confluent cultures were incubated in DMEM lacking methionine for 1 hr. The cells were subsequently incubated for 24–48 hr with 20 μCi/ml of [^{35}S-]methionine and the cell-free matrices prepared as described below.

Purification of Laminin and Fibronectin From Kidney Cell Cultures

Laminin and fibronectin deposited into the subepithelial matrix were isolated by immunoprecipitation methods. The conditioned media from confluent cultures was collected, centrifuged, and aliquots subjected to zymography (see below). The cells were removed with 0.1% EDTA in calcium- and magnesium-free PBS (CMF-PBS) at 4°C. This procedure effectively removes the cells without lysis thus preventing nonspecific adsorption of intracellular PA to the matrix. The matrices were further incubated with 20 mM NH_4OH for 5 min at room temperature to remove any remaining cellular debris. After rinsing with CMF-PBS, the matrices were solubilized with 4 M urea, in 50 mM Tris-HCl, 0.4 M NaCl, pH 7.5, and dialyzed against 50 mM Tris-HCl (pH 7.5). Samples of solubilized matrix (200 μl) were incubated with 100 μl of appropriate antibody (anti-laminin, anti-fibronectin, or preimmune rabbit serum; 1:100 dilutions) for 2 hr at 22°C. These antibodies were tested in a Western blot and had no cross-reactivity with mouse tPA or urokinase. Protein-A agarose (100 μl of 2 mg/ml) was added and incubation continued for 2 hr. Samples were spun in a microfuge, the supernatant removed, and the pellets washed extensively with 20 mM Tris-HCl containing 0.1% BSA, 0.9% NaCl, 5 mM EDTA, and 0.5% Tween 20. The pellets were then treated with SDS sample buffer and the supernatant was either subjected to zymography (see below) or Western blotting. For Western blotting, aliquots of immunoprecipitated material were electrophoresed in 10% polyacrylamide gels along with known amounts of purified laminin or fibronectin. Following electrophoresis, the proteins were transferred to nitrocellulose, incubated with a 1:100 dilution of anti-laminin or anti-fibronectin, followed by a 1:1000 dilution of ^{125}I-labeled goat anti-rabbit IgG. The laminin and fibronectin bands were observed by exposing the nitrocellulose to Kodak X-Omat AR x-ray film at −70°C. The individual bands were then cut out and the number of ^{125}I-associated counts determined. In this way a standard curve was constructed and the amount of immunoprecipitated laminin and fibronectin was quantitated. Alternatively, after the cells were removed with EDTA, the entire matrix was solubilized in SDS sample buffer and aliquots subjected to zymography.

Deposited matrices were also examined by immunocytochemical techniques for the presence of laminin and fibronectin. Confluent cells on glass or plastic coverslips were rinsed with PBS and removed with EDTA and NH_4OH as described above. Coverslips were subsequently incubated with anti-laminin, anti-fibronectin, or preimmune rabbit serum (1:100 dilution) for 1 hr at room temperature. The coverslips were rinsed extensively in PBS and blocked with normal goat serum (0.1%) for 10 min. This was followed by a 1 hr incubation with a 1:100 dilution of rhodamine-conjugated goat

anti-rabbit IgG. The coverslips were rinsed, mounted on glass slides with 95% glycerol in PBS, and viewed with a Zeiss fluorescence microscope.

Assay for PA Activity Associated With the Extracellular Matrix

The presence and characterization of PA in the various ECM samples was examined by (1) substrate-containing polyacrylamide gel electrophoresis (zymography) and (2) a quantitative amidolytic assay [45].

Matrigel™ (1:100), laminin (8 μg), type IV collagen (8 μg), and aliquots of immunoprecipitated kidney cell laminin and fibronectin were dissolved in sample buffer before electrophoresis in 10% mini-gels (0.75 mm thick). The samples were neither heated nor reduced as both of these treatments destroyed enzyme activity. Plasminogen (5 μg/ml) and casein (1 mg/ml) were crosslinked into the separating portion of the gel [46]. Following electrophoresis, the lane of molecular weight standards was cut off and stained in 0.125% Coomassie Brilliant Blue while the remainder of the gel was incubated for 20 min in 2.5% Triton X-100 to remove SDS. The gels were rinsed with water and incubated at 37°C for 16–18 hr between two pieces of filter paper soaked in 100 mM Tris-HCl, pH 8.1. The zones of casein degradation corresponding to the presence of proteases, including tPA and UK, were visualized by staining gels with 0.125% Coomassie Brilliant Blue. Controls included a comparison of identical samples electrophoresed into gels lacking plasminogen, which distinguishes between the PAs and plasminogen-independent proteases. To confirm the presence of tPA, samples were run in gels containing 50 μg/ml of anti-mouse tPA IgG (see below), while for the identification of urokinase the gels were incubated in buffer containing the drug Amiloride (1 mM) (Sigma, St. Louis, MO), which has previously been shown to specifically inhibit urokinase activity but not that of tPA [47].

To quantitate the PA activity associated with the various matrix samples, a quantitative amidolytic assay was performed as follows. Sample aliquots were incubated for 2 hr in 50 mM Tris-HCl, pH 7.5 containing 0.1% Triton X-100, 250 μg/ml poly-D-lysine, and 2.5 U/ml plasminogen. Following this initial incubation, which allows for the conversion of plasminogen to plasmin by activators in the sample, 35 μl of 50 mM Tris-HCl containing 400 mM L-lysine, and 800 mM NaCl was added followed by 15 μl of chromogenic substrate S2251 (Kabi, Ortho Diagnostics, 600 mM). Incubation was continued for 2 hr. The incubation was terminated by the addition of 250 μl of 10% acetic acid and the absorbance read at 405 nm. Controls included no samples and samples without plasminogen. A standard curve was constructed by use of known amounts of human melanoma tPA (National Institute for Biological Standards and Control, London, England) or human urokinase (Cal Biochem, La Jolla, CA) to activate plasminogen. The amount of PA present in the various samples was expressed as mIU/mg protein or mIU/number of cells. Protein was determined by the method of Lowry [48].

To begin to examine the nature of the tPA and urokinase association with the extracellular matrix, samples of Matrigel™ and kidney cell matrix were treated with the following agents and the amount of PA released was measured by the amidolytic assay described above: PBS, 1 M NaCl, 0.5% Triton X-100, 0.5% NP-40, 0.1 M glycine pH 2.5, 0.5 M aminocaproic acid, 10 mM EDTA, and 4 M urea.

Cell-free radiolabeled matrices were examined for the ability of endogenous UK to activate plasminogen. Triplicate dishes of ^{35}S-labeled matrix were incubated at 37°C in DMEM, DMEM containing plasminogen (2.5 U/ml), or DMEM containing plasminogen and Aprotinin (100 KIU/ml). Following incubation for 16 hr, the conditioned media was removed and an aliquot counted. The cpm associated with the residual matrix was also determined and the percent of total counts in each was calculated.

Isolation of Mouse tPA and the Production of Antisera

Rabbit antibody to murine tPA was the generous gift of S. Verrrall (Ph.D. thesis, University of Colorado, 1989). Murine tPA was isolated from serum-free conditioned media of the neural hybrid cell line NG 108-15 [49] by the procedure of Rijken and Collen [50]. Before injection into rabbits, the purified tPA was inactivated by treatment with diisopropylfluorophosphate (DFP) and crosslinked with glutaraldehyde. Immunoglobulins were isolated from immune serum by affinity chromatography on Protein A-agarose (Pierce, Rockford, IL) and dialyzed against saline. This anti-tPA IgG was tested in Western blots and found to react only with tPA and not UK.

RESULTS

PAs Associated With Commercial Matrix Preparations

The commercially available, reconstituted basement membrane matrix Matrigel™ has been well characterized and consists of laminin, type IV collagen, heparin sulfate proteoglycan, nidogen, and entactin [43]. Diluted samples of Matrigel™ electrophoresed into plasminogen and casein-containing gels revealed bands of caseinolytic activity that migrated with molecular weights of approximately 70 Kd and 35 Kd (Fig. 1A). The identity of these bands as tPA was confirmed when either plasminogen was deleted from the gel (Fig. 1B) or when anti-tPA antibodies were incorporated into the gel (Fig. 1C). In both cases the zones of degradation were absent. An additional band of caseinolytic activity was seen at approximately 90 Kd, which was not related to tPA as it was seen both in gels lacking plasminogen (Fig. 1B) and was not inhibited by the presence of anti-tPA antibodies (Fig. 1C). By the use of a quantitative amidolytic assay, Matrigel™ was found to contain approximately 4.7 IU of PA/mg of protein. To determine whether or not PA preferentially associates with specific components of the basement membrane matrix, laminin and type IV collagen purified from the EHS tumor matrix (two of the major components of Matrigel™) were likewise subjected to zymography. A similar pattern of degradative activity was seen for laminin but not for type IV collagen (Fig. 1A).

PAs Associated With Normal Epithelial Cell Matrices

Primary cultures of mouse kidney epithelial cells secrete both laminin and fibronectin into the culture medium and also produce a subepithelial matrix containing these macromolecules that adheres to the tissue culture dish after the cells are removed with 0.1% EDTA and 20 mM NH_4OH (Fig. 2).

Samples of serum-free conditioned media and deposited matrix were examined for the presence of plasminogen activators. Examination of the conditioned media by zymography revealed bands of proteolytic activity that migrated as a doublet at 48 and

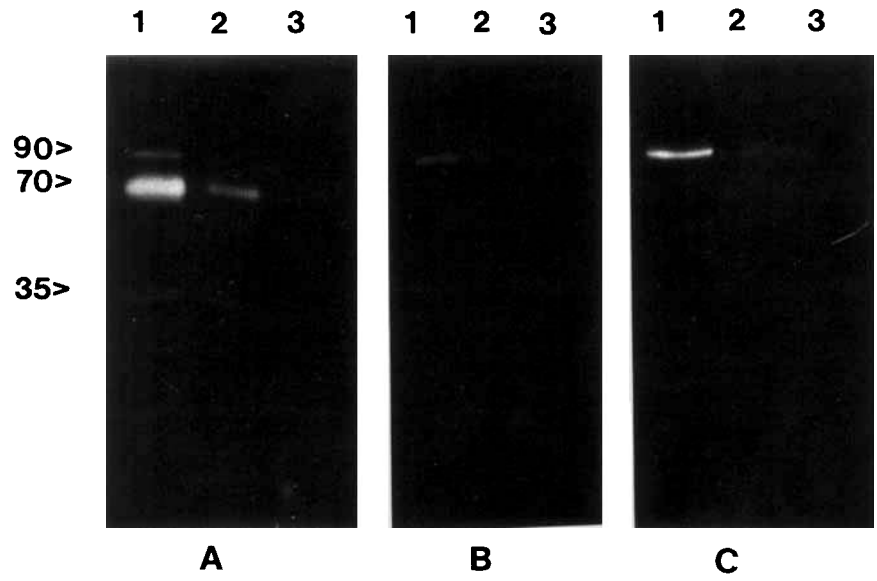

Fig. 1. Plasminogen activator associated with samples of the reconstituted EHS tumor matrix (Matrigel™) and some of its purified components. **A:** Plus plasminogen. **B:** Minus plasminogen. **C:** Plus 50 μg/ml anti-tPA IgG. **Lane 1,** Matrigel™ (5 μl of a 1:100 dilution); **lane 2,** EHS laminin (8 μg); **lane 3,** EHS type IV collagen (8 μg). The molecular weights of specific caseinolytic zones are indicated on the left (MW × 10^{-3}).

51 Kd, as well as a band at 70 Kd (Fig. 3A, lane 2). By the quantitative amidolytic assay, the conditioned media was found to contain approximately 11.5 mIU PA/10^6 cells. Samples of the deposited matrix also contained the doublet at 48 and 51 Kd as well as a higher molecular weight band at 97 Kd (Fig. 3A, lane 1). The matrix was also examined quantitatively and found to contain approximately 3 mIU PA/45 μgprotein in 10^6 cells. All of the proteolytic bands observed were dependent on the presence of plasminogen in the gels (Fig. 3B). In addition, 1 mM amiloride inhibited the bands at 48 and 51 Kd, confirming their identity as urokinase. The high molecular weight band in samples of the matrix was also inhibited by amiloride, suggesting that it represents either a urokinase aggregate or urokinase-inhibitor complex (Fig. 3C). The persistence of the 70 Kd band in samples of the conditioned media treated with amiloride suggests that that these cells also secrete a small amount of tPA into the media.

The urokinase associated with the matrix was present in an active form as it was able to convert plasminogen to plasmin in the amidolytic assay. In addition, when plasminogen was added to the isolated radiolabeled matrix an increase in the number of released counts was seen (Table I), suggesting a conversion of plasminogen to plasmin by the urokinase and subsequent degradation of some of the matrix components. This increased release of radioactive counts could be inhibited when the plasmin inhibitor aprotinin (100 KIU/ml) was added with the plasminogen. We therefore believe that the urokinase present in the matrix is deposited by the cells in an activated as opposed to a proenzyme form of the molecule. In addition, the plasminogen preparation used for these experiments was found to contain less than 0.05 mIU of plasmin activity and therefore was not a source of plasmin that could convert the prourokinase to the active form.

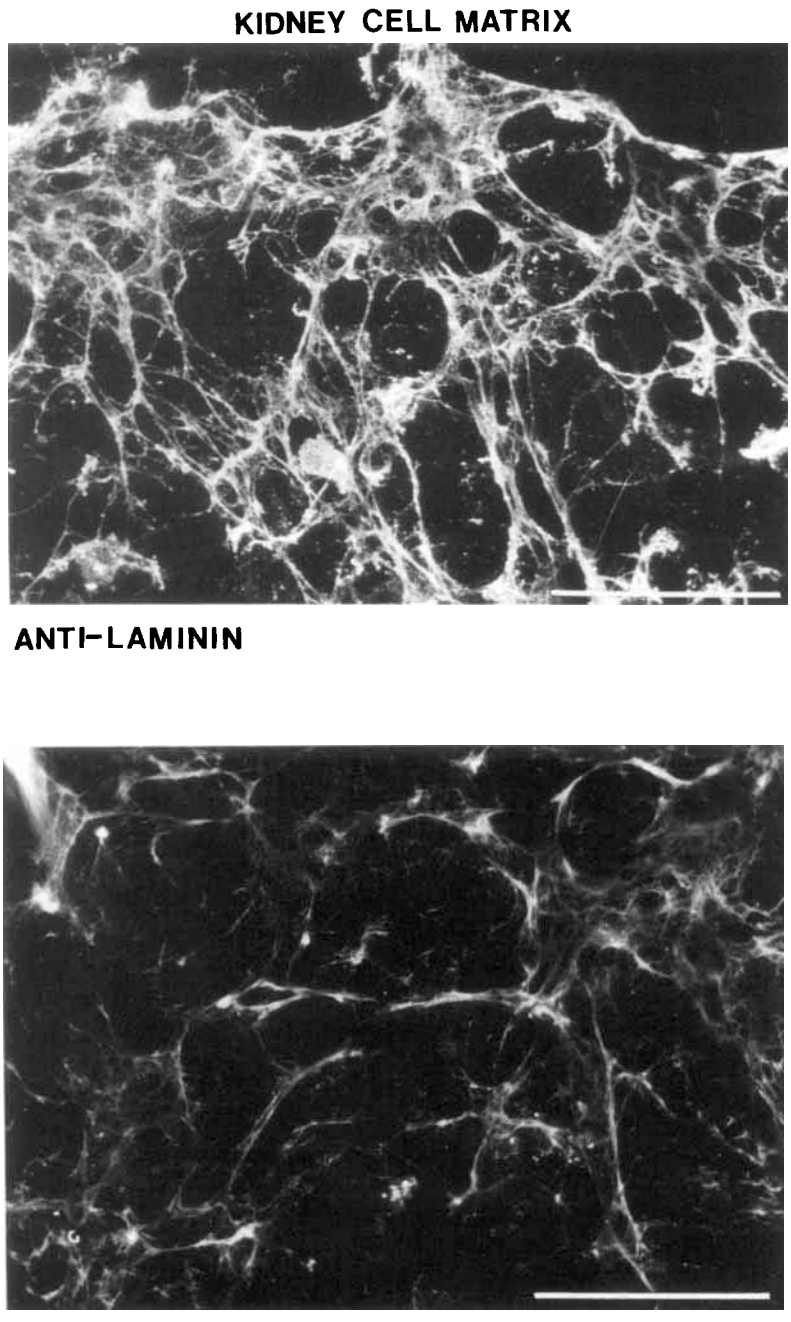

Fig. 2. Immunofluorescent staining of the deposited cell-free matrix from confluent kidney epithelial cells by use of polyclonal rabbit antiserum to laminin and fibronectin. Both laminin and fibronectin are present as fibrillar components of the matrix. Bar = 100 μm.

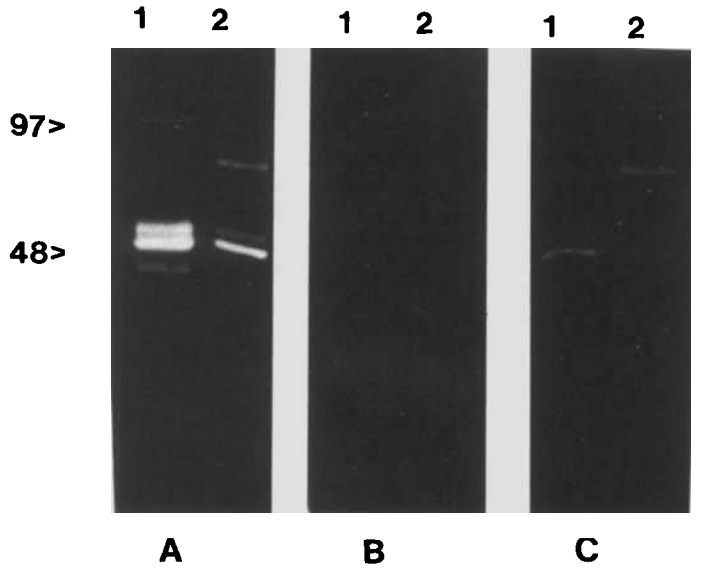

Fig. 3. Detection of PAs in kidney epithelial cell matrix and conditioned media. **A:** Plus plasminogen. **B:** Minus plasminogen. **C:** Plus amiloride (1 mM). **Lane 1,** Mouse kidney cell matrix. **Lane 2,** Mouse kidney cell conditioned media. Molecular weights ($\times 10^{-3}$) are indicated on the left.

TABLE I. Release of Matrix-Associated CPM by Endogenous Urokinase*

	CPM	
Additions	Percent supernatant	Percent matrix
None	29.3 ± 2.5	71.0 ± 2.1
Plasminogen (2.5 U/ml)	56.3 ± 4.6	43.6 ± 3.2
Plasminogen + aprotinin (100K IU/ml)	25.0 ± 3.0	75.0 ± 2.3

*Data based on three experiments for each addition. Data are mean ± SEM.

The association of urokinase with the subepithelial matrix was further examined by treatment of the matrix with various agents and looking for the release of activity into the supernatant (Table II). The use of high salt and low pH had no effect on the release of activity into the supernatant whereas treatment of isolated matrices with the non-ionic detergents Triton X-100 and NP-40 both released significant amounts of urokinase, indicating that the interaction of urokinase with the matrix is not simply ionic in nature. Incubation with 4 M urea caused a complete solubilization of the matrix and therefore the effect of this agent on the UK/matrix interaction was inconclusive. Similar results were found when samples of Matrigel™ were treated with these agents. Once again only Triton X-100 (72.6% release) and NP-40 (83.0% release) were effective in releasing PA activity from the matrix.

Laminin and fibronectin were purified from solubilized matrices by immunoprecipitation and electrophoresed into plasminogen and casein containing gels. These anti-

TABLE II. Release of Matrix-Associated Urokinase Activity*

Treatment	Percent of total activity	
	Matrix	Supernatant
PBS	100	—
1 M NaCl	100	—
0.5% Triton X-100	32	68
0.5% NP-40	48	52
0.1 M glycine pH 2.5	95	5
0.5 M EACA[a]	100	—
4 M urea[b]	—	100

*Isolated matrices were treated with the above reagents for 30 min at room temperature, and the urokinase activity released into the supernatant or remaining in the matrix was determined by the amidolytic assay as described.

[a]Epsilon amino caproic acid.

[b]Entire pellet solubilized by 4 M urea.

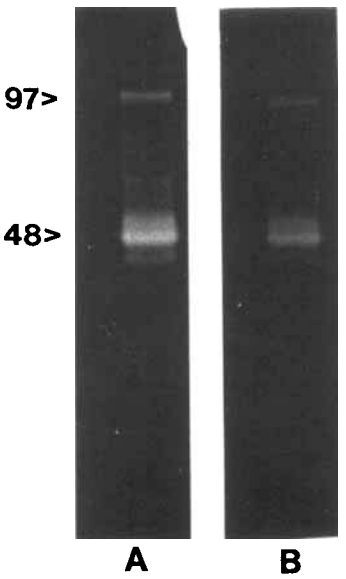

Fig. 4. Demonstration of PAs associated with purified components of the deposited subepithelial matrix. After the cells were removed with EDTA, the residual matrix was extracted and subjected to immunoprecipitation as described. The amount of fibronectin and laminin in immunoprecipitates was quantitated by use of an ^{125}I-labeled second antibody as described. Aliquots were subsequently subjected to zymography. **A:** Fibronectin (0.105 μg) immunoprecipitated from the kidney cell matrix. **B:** Laminin (0.137 μg) immunoprecipitated from the kidney cell matrix. Molecular weights are indicated on the left ($\times 10^{-3}$).

bodies showed no cross-reactivity in Western blots with either murine tPA or UK (not shown), and therefore have been used to specifically immunoprecipitate laminin and fibronectin. The results in Figure 4 show UK activity associated with both laminin and fibronectin, suggesting that these molecules may be possible binding sites for urokinase in the deposited matrix.

DISCUSSION

In this study we have expanded previous observations describing the in vitro binding of PA with matrix components [51–53] and report on the deposition of these proteases into a natural, cell-derived matrix. We have described the presence of the serine protease, plasminogen activator, in a reconstituted basement membrane matrix (Matrigel™) derived from the EHS tumor. This matrix, as well as its individual components, has been used extensively to enhance the growth of many cell types in vitro and has profound effects on both the morphology and phenotypic expression of cells [10]. Madison and coworkers [54] showed that the basement membrane gel, as opposed to a type I collagen gel, significantly enhanced peripheral nerve regeneration in vivo. Since one of the major components of the basement membrane gel is laminin, the authors postulated that this glycoprotein may have provided the stimulatory effect on neurite outgrowth seen in this system. Other investigators have described this effect of laminin on neurite behavior and have localized the "neurite outgrowth" domain of laminin to a specific region of the molecule near the heparin-binding domain [55–59].

The presence of PA's in the tumor matrix is not entirely surprising as others have shown that tumor cells produce large quantities of PA and other proteases and deposit some of these into the substratum [14,16,17,30,60]. The studies presented here show that the PA specifically interacts with components of the matrix even after extraction of the tumor with high salt and urea, which is the basic method used in preparation of the basement membrane matrix [43,44]. Plasminogen activators are bound to laminin but apparently not to type IV collagen (the other major component of Matrigel™). Type IV collagen is routinely purified by a combination of extraction with 2 M guanidine and 2 mM DTT followed by DEAE chromatography [43]. The lack of association of PA with type IV collagen may be due to the different isolation procedures used for this matrix component, which may either inactivate the PA or dissociate it from the collagen molecule. At this point we cannot speculate as to whether or not PA and type IV collagen associate with one another in any specific manner in vivo.

The use of the basement membrane gel or laminin in studies of cell migration, axonal outgrowth, and cellular differentiation should take into account the association of PA with these matrices and its possible effects on cell behavior. Previous studies have shown that PA and its inhibitors may play an important role in the migratory behavior of numerous cell types [15,37,39,61–63]. In addition, the presence of PA in the gel matrix takes on added significance with the recent demonstrations of the ability of cells to bind urokinase and tPA to their surfaces [64–66]. This pool of PA in the substratum may thus provide a convienent source of exogenous PA for the migratory cells.

Previous studies using purified proteins and various types of in vitro binding assays have shown that both plasminogen and PA interact with components of the extracellular matrix [51–53]. In this study we have further shown that a normal epithelial cell type in culture can produce both plasminogen activator and extracellular matrix components, and that these macromolecules interact in a specific way in the deposited subepithelial matrix. The specific interaction of PA with laminin and fibronectin was evident in matrix samples subjected to immunoprecipitation. In both cases urokinase was co-precipitated with the individual matrix components, suggesting a role for these molecules as binding sites for UK in the extracellular matrix.

The deposition of plasminogen activator by various cell types was investigated in previous work from this laboratory. Krystosek and Seeds [14] observed residual PA activity on poly-D-lysine coated coverslips from which cultures of transformed fibroblasts, NG108 cells, and regenerating sensory neurons had been removed by treatment with 3 mM EGTA. The PA activity on these surfaces could not be removed by treatment with Heparitinase (1 unit/ml). Whether this represents nonspecific adsorption of PA to the coverslip surface or specific binding to an ECM component (other than a glycosaminoglycan) deposited by these cells is unknown.

Other components of the PA/plasmin system (specifically plasminogen activator inhibitors) have recently been reported to be associated with cell-derived matrices in vitro. Bovine smooth muscle cells produce an extracellular matrix containing an endothelial-type PA inhibitor [67]. The inhibitor forms a complex with exogenously added tPA and is likely to be important in regulating the PA-mediated proteolysis of the ECM. Levin and Santell [68] describe the presence of PA inhibitor type-1 (PAI-1) in the deposited matrix of human endothelial cells. The specific interaction of this inhibitor with individual matrix components was not investigated, however its release from the matrix was unaffected by treatment with collagenase, heparinase, or chondroitinase ABC. The authors further postulate that the presence of this inhibitor may serve to protect the endothelial basement membrane from degradation by PA-generated enzymes. Similarly, Farrell et al. [69] have described the presence of protease nexin-1 (PN-1) in the matrix produced by cultured fibroblasts. Immunostaining showed that PN-1 co-localized with fibronectin both on the cells and in the deposited matrix.

The interaction of membrane-bound PA with specific extracellular matrix components and its potential ability to directly cleave some of these macromolecules [70] may provide an efficient mechanism whereby cellular processes attach to and detach from the matrix during migration. These specific interactions and the composition of the matrix may also play a crucial role in the process of cellular pathfinding seen in early development [71–74]. In addition, the secretion of PA into the surrounding matrix and its association with specific proteins may target these components for degradation, altering the matrix composition and thus influencing cellular differentiation [1,2,10,75,76].

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