Cytokeratin 8 released by breast carcinoma cells *in vitro* binds plasminogen and tissue-type plasminogen activator and promotes plasminogen activation

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Cell-surface activation of plasminogen may be important in diseases that involve cellular migration, including atherosclerosis and tumour invasion/metastasis. Cytokeratin 8 (CK 8) has been identified as a plasminogen-binding protein expressed on the external surfaces of hepatocytes and breast carcinoma cells [Hembrough, Vasudevan, Allietta, Glass and Gonias (1995) J. Cell Sci. **108**, 1071–1082]. In this investigation, we demonstrate that a soluble form of CK 8 is released into the culture medium of breast cancer cell lines. The released CK 8 is in the form of variably sized polymers that bind plasminogen and promote the activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen by single-chain tissue-type plasminogen activator (sct-PA). To assess the mechanism by which CK 8 promotes plasminogen activation, CK 8 was purified from rat hepatocytes and immobilized in microtitre plates. Immobilized CK 8 bound ^{125}I-plasminogen and ^{125}I-sct-PA in a specific and saturable manner. The K_Ds were 160 ± 40 nM and 250 ± 48 nM, respectively. Activation of plasminogen bound to immobilized CK 8 was accelerated compared with plasminogen in solution, as determined using a coupled-substrate fluorescence assay and SDS/PAGE. The ability of CK 8 to promote plasminogen activation may be important in the pericellular spaces surrounding breast cancer cells and at the cell surface.

INTRODUCTION

Tissue-type plasminogen activator (t-PA) is one of the two major plasminogen activators *in vivo*; however, in the absence of protein cofactors, the efficiency of plasminogen activation by t-PA is poor. Proteins that bind t-PA and/or plasminogen have been identified in the plasma and basement membranes [1,2]. The most important of these, from the standpoint of haemostasis, is fibrin, which substantially increases the catalytic efficiency of plasminogen activation [3].

Many cells in culture express specific and saturable binding sites for t-PA and plasminogen. The affinity of cellular receptors for plasminogen is typically low; however, the total cellular plasminogen binding capacity is high (10^5–10^7 sites per cell) [4]. Binding of plasminogen to certain cells, including endothelial cells [5] and hepatocytes [6], promotes activation by t-PA. Like other plasminogen-binding interactions, those involving cellular receptors utilize the five tandem plasminogen kringle domains, three of which (K1, K4 and K5) demonstrate affinity for lysine residues in other proteins [1,2]. The kringle-1 domain contains the highest-affinity lysine-binding site and interacts preferentially with C-terminal lysines [7–10]. Thus, plasma membrane proteins with extracellular C-terminal lysines are most likely to function as plasminogen receptors.

Several cellular proteins have been identified as candidate plasminogen receptors, including α-enolase, on monocytoid cells, and annexin II, on endothelial cells [10,11]. Although these proteins are primarily intracellular in location, each has been identified on the external cell surface, using immunofluorescent antibody-based techniques [10,11]. The structure of α-enolase includes a C-terminal lysine [10]; annexin II does not have a C-terminal lysine and is thus dependent on modification by plasmin or another lysine-specific proteinase in order to acquire plasminogen-binding activity [11].

Recently, we identified cytokeratin 8 (CK 8) as a candidate epithelial cell plasminogen receptor [12]. CK 8 is an intermediate filament protein [13]; however, using fluorescence and immunoelectron microscopy, we demonstrated CK 8 or a CK 8-like protein on the external surfaces of hepatocytes, breast carcinoma cells and hepatocellular carcinoma cells. CK 8 contains a C-terminal lysine residue [14]. Thus, CK 8 has intrinsic plasminogen binding capacity and does not require prior proteolytic modification in order to bind plasminogen.

The interaction of plasminogen with CK 8 may not be restricted to the cell surface since cytokeratins are released in significant quantities by cancer cells *in vitro* and by carcinomas *in vivo*. CK 8 has been identified as a major component of Tissue Polypeptide Antigen, a tumour-associated marker, found in the serum and body fluids of cancer patients [15,16]. Levels of Tissue Polypeptide Antigen have been used to monitor the progression of malignancies [17]. CK 8 is also released into the culture medium of breast cancer cells *in vitro* [18,19]. Whether the released forms of CK 8 retain the ability to bind plasminogen is unknown.

In this investigation, we confirm the results of previous studies [18] by demonstrating the presence of CK 8 in conditioned medium of breast cancer cells *in vitro*. We then demonstrate, for the first time, that the soluble form of CK 8, recovered in cancer

Abbreviations used: t-PA, tissue-type plasminogen activator; sct-PA, single-chain tissue plasminogen activator; CK 8, cytokeratin 8; [Glu^1]plasminogen, native plasminogen; [Lys^{78}]plasminogen, proteolytically modified plasminogen with Lys^{78} as its N-terminus; EBSS, Earle's balanced salt solution; EHB, EBSS, 10 mM Hepes, 10 mg/ml BSA; S-2251, D-valyl-L-leucyl-L-lysine-*p*-nitroanilide; S-2288, D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide; VLK-AMC, D-valyl-L-leucyl-L-lysine-5-aminomethylcoumarin; PPACK, phenylprolylarginylchloromethane; BCA, bicinchoninic acid; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanido)butane; ϵACA, ϵ-aminohexanoic acid; u-PA, urokinase; tcu-PA, two-chain urokinase; DTT, dithiothreitol.

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cell-conditioned medium, retains plasminogen-binding activity. Purified CK 8 also binds t-PA and enhances the efficiency of plasminogen activation by t-PA. Thus, soluble CK 8, released from cancer cells *in vivo*, may promote plasminogen activation in malignancy.

MATERIALS AND METHODS

Materials

D-Valyl-L-leucyl-L-lysine-*p*-nitroanilide (S-2251) and D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide (S-2288) were from Kabi Vitrum (Stockholm, Sweden). D-Valyl-L-leucyl-L-lysine-5-aminomethylcoumarin (VLK-AMC) was from Enzyme Systems Products (Livermore, CA, U.S.A.). Phenylprolylarginylchloromethane (PPACK) was from Calbiochem (San Diego, CA, U.S.A.). Leupeptin and L-*trans*-epoxysuccinyl-leucylamido(4-guanido)butane (E-64) were from Boehringer Mannheim. Aprotinin, chloramine T, ϵ-aminohexanoic acid (ϵACA), PMSF, BSA and bicinchoninic acid solution (BCA) were from Sigma Chemicals (St. Louis, MO, U.S.A.). $Na^{125}I$ was from Amersham International (Arlington Heights, IL, U.S.A.). Iodobeads were purchased from Pierce (Rockford, IL, U.S.A.). Earle's balanced salt solution (EBSS) was from Gibco Laboratories (St. Lawrence, MA, U.S.A.). Cell culture plasticware was from Costar (Cambridge, MA, U.S.A.).

Proteins and antibodies

[Glu^1]Plasminogen (native plasminogen) was purified from human plasma by the method of Deutsch and Mertz [20]. [Glu^1]Plasminogen was converted into [Lys^{78}]plasminogen (proteolytically modified plasminogen with Lys^{78} as its N-terminus) by incubation with plasmin–Sepharose. Single-chain t-PA (sct-PA) was from American Diagnostica. The concentration of active sct-PA was determined by the velocity of S-2288 hydrolysis, using the kinetic constants ($K_m \sim 1.0$ mM, $k_{cat} \sim 26\ s^{-1}$) provided by the manufacturer. Recombinant single-chain urokinase (u-PA) was generously provided by Dr. Jack Henkin (Abbott Laboratories) and converted into two-chain urokinase (tcu-PA) by incubation with plasmin–Sepharose. Monoclonal antibody PCK-26, which recognizes CK 5, CK 6 and CK 8, was from Sigma. Of these three cytokeratins, only CK 8 is expressed by breast carcinoma cells [13].

Preparation of BT20 cell-conditioned medium

BT20 breast carcinoma cells were allowed to reach 60–75% confluency and then cultured in serum-free medium (RPMI 1640 with 100 units/ml penicillin and 100 μg/ml streptomycin) for 4 days. The conditioned medium was collected and centrifuged at 1000 ***g*** for 10 min to pellet cellular debris and then concentrated 10-fold using an Amicon concentrator with a YM-10 membrane.

Concentrated BT20 cell-conditioned medium (0.5 ml) was subjected to molecular exclusion chromatography on a Superose 12 column, using a flow rate of 0.4 ml/min. Elution of proteins was detected by constant monitoring of absorbance at 280 nm. Fractions were collected at 1 min intervals. CK 8 was detected in chromatography fractions by immunoblot analysis using antibody PCK-26. In order to calibrate the column, the following proteins were subjected to chromatography under identical conditions: α_2-macroglobulin (720 kDa), mouse IgG (150 kDa), BSA (66 kDa) and cytochrome *c* (14 kDa).

Effects of BT20 cell-conditioned medium on plasminogen activation by t-PA

Activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen (0.05–1.0 μM) by sct-PA (2 nM), in the presence and absence of BT20 cell-conditioned medium, was studied using a continuous assay and the plasmin-specific substrate, S-2251 (0.5 mM). The BT20 cell-conditioned medium was preincubated with plasminogen in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer for 30 min at 22 °C. sct-PA and S-2251 were added to start the reaction. In the final incubation mixture, the BT20 cell-conditioned medium was diluted 1:2. Absorbance at 406 nm was measured at 5 s intervals for 300 s. To determine rates of plasminogen activation, absorbance measurements were transformed using the first derivative function (dA_{406}/dt). Kinetic constants for the hydrolysis of S-2251 by plasmin ($K_m = 180\ \mu$M, $k_{cat} = 12\ s^{-1}$) were used to convert data into plots of active plasmin concentration against time. In control experiments, we analysed S-2251 hydrolysis in the presence of BT20 cell-conditioned medium and plasminogen (no sct-PA) or in the presence of BT20 cell-conditioned medium and sct-PA (no plasminogen). In both cases, significant substrate hydrolysis was not observed within 600 s. Finally, unconditioned cell culture medium, at the dilution used in our experiments, did not significantly affect the kinetics of activation of [Glu^1]plasminogen or [Lys^{78}]plasminogen by sct-PA.

CK 8 purification

CK 8 was purified by the method of Achtstaetter et al. [21]. Rat hepatocytes were collected as previously described [12] and homogenized in EBSS with 10 mM Hepes, 0.1 mM dithiothreitol (DTT), 1.0 mM PMSF, 2.0 μM E-64, 10 mM EDTA and 10 μM aprotinin. The homogenate was centrifuged at 1250 ***g*** for 15 min. The resulting pellet was extracted with 1.0% Triton X-100 for 1 h at 4 °C and then subjected to centrifugation at 2500 ***g*** for 5 min. The pellet was resuspended in 1.5 M KCl, 0.5% Triton X-100, 10 mM Hepes, 5 mM EDTA, 5 mM DTT at 4 °C and forced through a syringe to shear DNA. Centrifugation was performed again. The pellet was rinsed with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, and solubilized in 9.5 M urea, 10 mM Tris/HCl, 5 mM DTT, pH 8.8, for 2 h. This solution, which includes CK 8, CK 18 and some contaminating proteins, was clarified by centrifugation at 14000 ***g*** for 20 min and dialysed against 8 M urea, 30 mM Tris/HCl, 5 mM DTT, pH 8.8. The preparation was then subjected to DEAE-Sephacel ion-exchange chromatography. The column was equilibrated in 8 M urea, 30 mM Tris/HCl, pH 8.0, and eluted with a linear gradient of 0 to 100 mM guanidine hydrochloride. Samples were screened by absorbance at 280 nm and by SDS/PAGE/Western blot analysis using antibody PCK-26. Fractions containing purified CK 8 were pooled and stored at room temperature.

Ligand blotting

BT20 cell-conditioned medium or purified CK 8 was subjected to SDS/PAGE [22] and electrotransferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore) using a Hoefer Transphor apparatus (2 h, 0.5 A). In some studies, the transferred proteins were stained with 0.2% (w/v) Coomassie Blue R250 (Bio-Rad, CA, U.S.A.). Otherwise, the membranes were blocked with 5% non-fat dried milk, rinsed twice with 20 mM sodium phosphate, 150 mM NaCl, 0.1% (v/v) Tween-20, pH 7.4 (PBS-T), and then incubated with ^{125}I-plasminogen (10 nM) and aprotinin (10 μM), in the presence and absence of 10 mM ϵACA or a 50-fold molar

excess of non-radiolabelled plasminogen. To determine radioligand binding, the blots were rinsed three times for 15 min in PBS-T, dried and autoradiographed, or analysed with a Phosphorimager.

Binding of plasminogen and t-PA to immobilized CK 8

Purified CK 8 (100 μg/ml) in 8 M urea was diluted 1:3 with 20 mM sodium phosphate, 150 mM NaCl, pH 7.4, in 48-well cell culture plates and incubated for 1.5 h at 37 °C. The plates were rinsed three times and then blocked by the addition of BSA (10 mg/ml) in EBSS, 10 mM Hepes, pH 7.4, (EHB) for 1.5 h at 37 °C. Control plates were blocked with BSA without prior adsorption of CK 8. All plates were stored at 4 °C until use. The amount of CK 8 immobilized per well was 0.43 ± 0.07 μg ($n = 6$), as determined by BCA assay of SDS-extracted samples.

Plasminogen and sct-PA were radioiodinated using Iodobeads (Pierce). The specific activities of both proteins ranged from 1–2 μCi/μg. Increasing concentrations of either ^{125}I-plasminogen or ^{125}I-sct-PA were incubated in CK 8-coated and control wells for 1 h at 22 °C. Plates were then washed three times with EHB. Bound radioligand was recovered by incubation with 1% SDS, 0.1 M NaOH and quantified in a gamma counter. Non-specific ^{125}I-plasminogen binding was defined as residual binding in the presence of 10 mM ϵACA or a 50-fold molar excess of plasminogen (either method yielded equivalent results). For ^{125}I-sct-PA concentrations up to 0.25 μM, non-specific binding was determined using a 50-fold molar excess of non-radiolabelled t-PA. Non-specific binding of ^{125}I-sct-PA was plotted as a function of ^{125}I-sct-PA concentration and extrapolated to higher concentrations, assuming a continuously linear relationship, in order to conserve material.

Activation of plasminogen bound to immobilized CK 8 as determined by fluorescent substrate hydrolysis

Various concentrations of [Glu^1]plasminogen were incubated in CK 8-coated wells for 1 h at 22 °C. The plates were rinsed three times with EHB to remove unbound plasminogen. sct-PA (10 nM) and the fluorescent plasmin substrate, VLK-AMC (0.5 mM), were then added. Initial rates of substrate hydrolysis were detected by measuring fluorescence emission at 5 s intervals using a Cytofluor 2350 fluorescence plate reader (Millipore). The excitation wavelength was 380 nm and the emission wavelength was 480 nm (5 nm slit widths). Levels of CK 8-associated plasminogen, available for activation by sct-PA, were determined in separate ^{125}I-plasminogen binding studies. Equivalent amounts of plasminogen, in solution, were activated by 10 nM sct-PA in BSA-blocked wells (no CK 8).

In control experiments, hydrolysis of VLK-AMC (0.5 mM) by equal amounts of free (pre-activated) plasmin and CK 8-associated plasmin were compared. The velocity of VLK-AMC hydrolysis was 97 ± 6% higher for free plasmin ($n = 6$).

Activation of plasminogen bound to immobilized CK 8 as determined by SDS/PAGE

^{125}I-[Glu^1]plasminogen (1.0 μM) was incubated in wells with immobilized CK 8 for 1 h at 22 °C. Aprotinin (10 μM) was included in the incubation mixture. After washing the plates three times with EHB to remove unbound plasminogen, 10 nM sct-PA and 10 μM aprotinin were added. At various times, the sct-PA was inhibited by the addition of 10 μM PPACK. Laemmli SDS sample buffer with reductant (7 mg/ml DTT) was then added to the wells. Samples were subjected to SDS/PAGE and autoradiography. Plasminogen activation was detected by conversion of the single-chain zymogen (90 kDa) into the two-chain active species.

When 1.0 μM ^{125}I-[Glu^1]plasminogen was incubated in the CK 8-coated wells, 2.0 ± 0.3 pmol ($n = 5$) associated with the immobilized phase. In control experiments, the equivalent amount of ^{125}I-plasminogen was added to BSA-blocked wells, without CK 8. sct-PA and aprotinin were then added and plasminogen activation was studied, as a function of time, by SDS/PAGE and autoradiography.

RESULTS

Identification of CK 8 in BT20 cell-conditioned medium

BT20 cell-conditioned medium was concentrated 10-fold and subjected to SDS/PAGE and Western blot analysis with antibody PCK-26. A prominent species with an apparent mass of 56 kDa was detected, together with a second less-intense band with an apparent mass of 53 kDa (Figure 1, lane B). The molecular mass of human CK 8 is about 55 kDa [13]. The Western blot analysis suggests that the BT20 cell-conditioned medium contains intact CK 8 (apparent mass of 56 kDa) and a partially degraded form of CK 8. Analysis of three separate preparations demonstrated the same two bands; the amount of the 53 kDa species was slightly increased in one of the three (results not shown).

Plasminogen-binding proteins in the BT20 cell-conditioned medium were identified by ligand blotting. A single protein, with identical electrophoretic mobility to intact CK 8, bound ^{125}I-plasminogen (Figure 1, lane C). No other plasminogen-binding proteins were detected. The 53 kDa band did not bind ^{125}I-plasminogen. This result suggests that the 53 kDa species may be CK 8, proteolytically modified at the C-terminus, so that the C-terminal Lys residue is deleted.

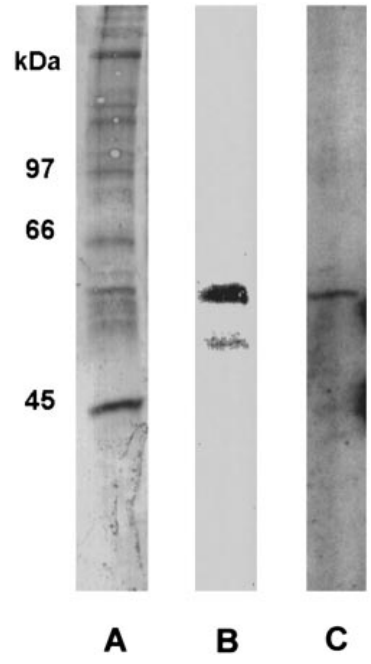

Figure 1 SDS/PAGE analysis of BT20 cell-conditioned medium

Concentrated BT20 cell-conditioned medium (100 μg of protein) was subjected to SDS/PAGE on 10% acrylamide slabs and transferred to poly(vinylidene difluoride) membranes. Resolved proteins were stained with Coomassie Blue (**A**); subjected to Western blot analysis with monoclonal antibody PCK-26 (**B**); or ligand blotted with 10 nM ^{125}I-plasminogen (**C**).

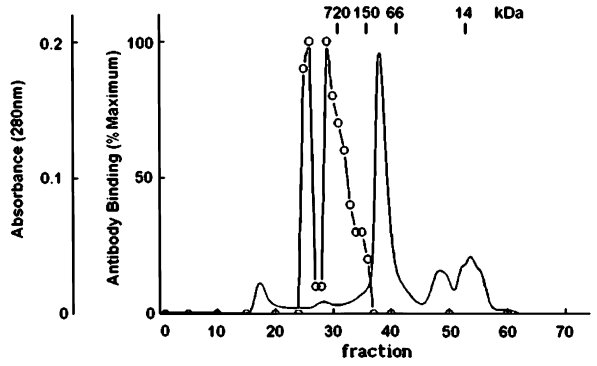

Figure 2 Superose 12 chromatography of BT20 cell-conditioned medium

BT20 cell-conditioned medium (500 μg of protein) was subjected to molecular exclusion chromatography on a Superose 12 column. Protein elution was detected by continuous monitoring of absorbance at 280 nm (unbroken curve). CK 8 was detected by dot–blot analysis with antibody PCK-26 (○).

Characterization of CK 8 in BT20 cell-conditioned medium

BT20 cell-conditioned medium was subjected to molecular exclusion chromatography on a Superose 12 column. CK 8 was detected in elution fractions by dot–blot analysis with antibody PCK-26. Figure 2 shows that the CK 8 was recovered over a wide range of fractions which, by comparison with the chromatography standards, correspond in apparent molecular mass from 150 kDa to over 1000 kDa. These results suggest that the CK 8 in BT20 cell-conditioned culture medium is in the form of variably sized polymers or complexes. The chromatography standards were globular proteins; if the CK-8-containing complexes were highly asymmetric, then the apparent masses are overestimated by comparison with the standards.

Effect of soluble CK 8 on plasminogen activation by sct-PA

Some plasminogen-binding proteins enhance the rate of plasminogen activation by t-PA, especially when the proteins bind plasminogen activator as well [2]. Thus, we tested the ability of BT20 cell-conditioned medium to promote plasminogen activation by sct-PA. It was assumed that any changes in the kinetics of plasminogen activation would be due to CK 8, since CK 8 was the only plasminogen-binding protein in BT20 cell-conditioned medium. Figure 3 shows a representative experiment in which [Glu^1]plasminogen or [Lys^{78}]plasminogen was activated by sct-PA in the presence of S-2251. Initial rates of activation were determined and plotted against plasminogen concentration. In the absence of BT20 cell-conditioned medium, [Lys^{78}]plasminogen was activated more rapidly than [Glu^1]plasminogen, as expected. Removal of the 77-amino-acid N-terminal pre-activation peptide, in [Lys^{78}]plasminogen, causes the zymogen to adopt a more open, readily activated conformation [23,24]. In the presence of conditioned medium, both plasminogens were activated at increased rates. When the BT20 cell-conditioned medium was passed through a sterile Acrodisc 0.2 μm filter (Gelman), the ability of the preparation to promote plasminogen activation was unchanged.

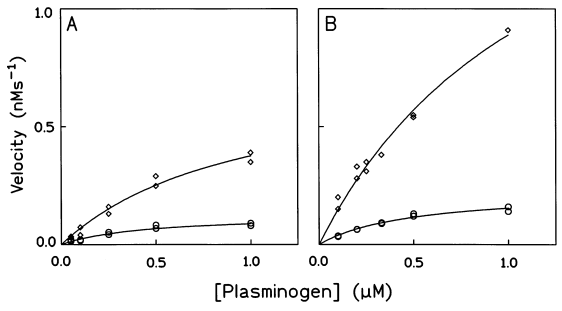

Figure 3 Effects of BT20 cell-conditioned medium on the activation of [Glu^1]plasminogen and [Lys^{78}]plasminogen by sct-PA

Increasing concentrations of [Glu^1]plasminogen (**A**) or [Lys^{78}]plasminogen (**B**) (0.05–1.0 μM) were activated with 2 nM sct-PA in the presence (◇) and absence (○) of a 1:2 dilution of BT20 cell-conditioned medium. The initial velocity of plasminogen activation is plotted against plasminogen concentration.

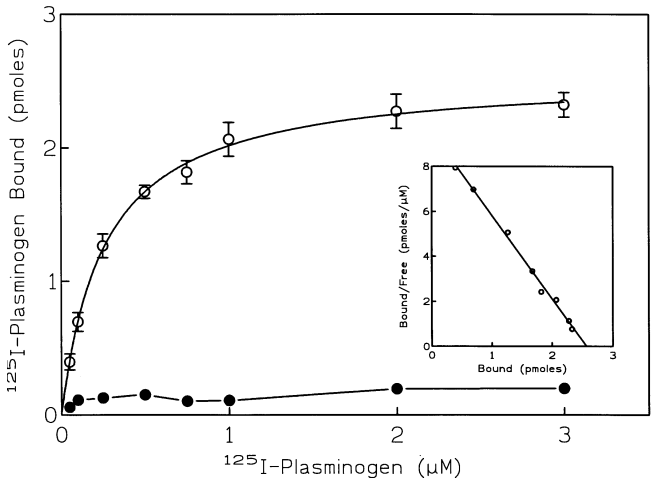

Figure 4 ^{125}I-plasminogen binding to immobilized CK 8

A representative study showing specific binding of ^{125}I-plasminogen to immobilized CK 8 (○) and to BSA-blocked wells (●). Each point represents the average of four replicate determinations ($\pm$S.E.M.) in one of four separate experiments. The Scatchard transformation of the data for plasminogen binding to CK 8 is shown in the inset.

The increased rate of activation of [Lys^{78}]plasminogen by sct-PA, in the presence of BT20 cell-conditioned medium, suggests that the mechanism is not limited to plasminogen conformational change. To better address the mechanism by which CK 8 stimulates plasminogen activation, CK 8 was purified from hepatocytes. The effects of purified CK 8 on plasminogen activation were then studied as a model of interactions that may occur with tumour cell-released CK 8.

In separate experiments, the activation of [Glu^1]plasminogen (1.0 μM) by 5 nM tcu-PA was assessed in the presence and absence of BT20 cell-conditioned medium. The [Glu^1]plasminogen was pre-incubated with the conditioned medium for 30 min, according to the standard protocol followed with sct-PA; however, the velocity of plasminogen activation by tcu-PA was not significantly affected by the conditioned medium (the rate of plasminogen activation was decreased by $3 \pm 10\,\%$ in the presence of the medium, $n = 4$).

Binding of plasminogen and sct-PA to immobilized CK 8

^{125}I-[Glu^1]plasminogen bound to immobilized CK 8 at 22 °C. Figure 4 shows that binding was specific and saturable. By

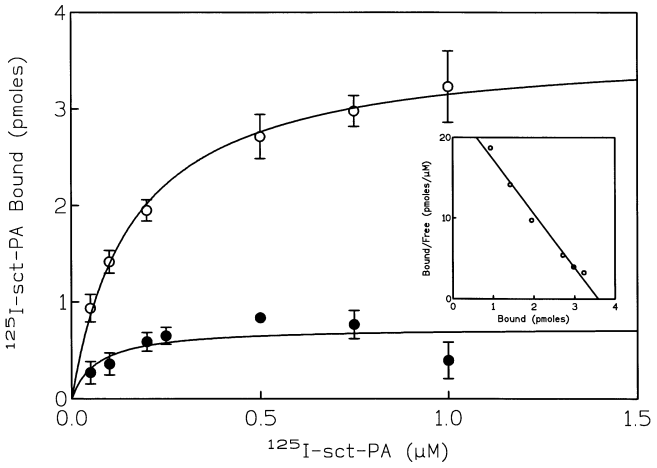

Figure 5 ^{125}I-sct-PA binding to immobilized CK 8

A representative study showing specific binding of ^{125}I-sct-PA to immobilized CK 8 (○) and BSA-blocked wells (●). Each point represents the average of four replicate determinations in one of three separate experiments. The Scatchard transformation of the data for sct-PA binding to CK 8 is shown in the inset.

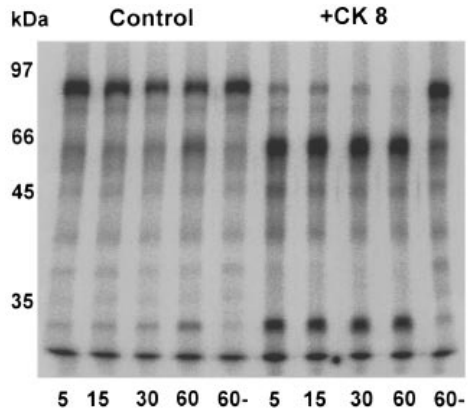

Figure 7 SDS/PAGE analysis of the activation of CK 8-associated ^{125}I-plasminogen by sct-PA

^{125}I-plasminogen, in association with immobilized CK 8 (+CK 8) or free in solution (Control), was activated with 10 nM sct-PA at 22 °C. Reactions were terminated by the addition of 10 μM PPACK at the times (in min) indicated at the bottom of each lane. The contents of each well were recovered in SDS and subjected to SDS/PAGE on 8% gels. Radioactive gels were imaged with a Phosphorimager. Incubations conducted for 60 min in the absence of sct-PA are labelled 60—. Aprotinin (10 μM) was present in each well through the entire incubation.

Scatchard analysis, the K_D was 160±40 nM and the B_{max} was 3.6±0.7 pmol/well ($n = 4$). At saturation, 0.5 mol of plasminogen was bound per mol of immobilized CK 8 monomer. Non-specific plasminogen binding accounted for less than 15% of total binding through the entire plasminogen concentration range. Plasminogen binding to BSA-coated wells was negligible.

^{125}I-sct-PA also bound to immobilized CK 8 at 22 °C (Figure 5). Binding was specific and saturable with a K_D of 250±48 nM, and a B_{max} of 4.0±0.7 pmol/well ($n = 3$). Non-specific binding accounted for less than 10% of total binding for ^{125}I-sct-PA concentrations up to 0.25 μM.

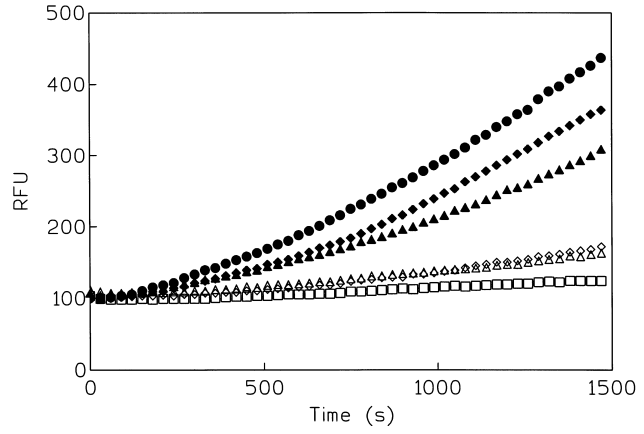

Figure 6 Activation of CK 8-associated plasminogen by sct-PA as determined by fluorescent substrate hydrolysis

CK 8-associated plasminogen [2.0 pmol (●), 1.3 pmol (◆), 1.0 pmol (▲)] was activated with 10 nM sct-PA in the presence of 0.5 mM VLK-AMC. The identical protocol was followed using plasminogen in solution [2.0 pmol (◇), 1.3 pmol (△), 1.0 pmol (□)], in BSA-coated wells. Fluorescent substrate hydrolysis was monitored continuously at 480 nm.

Activation of immobilized CK 8-associated plasminogen as determined by fluorescent substrate hydrolysis

The effects of CK 8 on plasminogen activation were characterized using immobilized CK 8 and the fluorescent plasmin substrate, VLK-AMC. In these experiments, various concentrations of plasminogen were incubated with immobilized CK 8 for 1 h at 22 °C. The wells were washed and activation was initiated by the addition of sct-PA (10 nM) and VLK-AMC (5 mM). The amount of plasminogen bound to the CK 8 had been determined in previous radioligand binding experiments. Thus, we could compare the rates of activation of identical amounts of CK 8-associated plasminogen and plasminogen in solution, in BSA-coated wells. Figure 6 shows that plasminogen activation by sct-PA was accelerated when the plasminogen was CK 8-associated. For the three concentrations of plasminogen studied, the velocity of VLK-AMC hydrolysis was 5–10 times greater for plasminogen that was initially CK 8-associated, compared with plasminogen in solution. Since plasmin that is CK 8-associated hydrolyses VLK-AMC at a decreased rate, the rate of plasminogen activation may have been as much as 20 times greater in the CK 8-containing wells.

SDS/PAGE analysis of ^{125}I-plasminogen activation by sct-PA

In continuous assays (activator, plasminogen and plasmin substrate present in the same solution), plasmin substrates can affect the rate of plasminogen activation [25,26]. Thus, SDS/PAGE experiments were performed to confirm that CK 8 accelerates the rate of [Glu^1]plasminogen activation by sct-PA. ^{125}I-[Glu^1]plasminogen (1 μM) was incubated with immobilized CK 8 for 1 h at 22 °C. After washing the wells, 10 nM sct-PA and 10 μM aprotinin were added. At various times, the sct-PA inhibitor, PPACK (10 μM), was added to stop the reaction. The contents of the wells were solubilized with SDS and subjected to SDS/PAGE. ^{125}I-[Glu^1]plasminogen activation was detected as conversion of the single-chain form of the protein into the two-chain form (apparent masses of 60 and 35 kDa). Figure 7 shows that the majority of the CK 8-associated ^{125}I-[Glu^1]plasminogen

was converted into plasmin within 5 min. By contrast, the identical amount of ^{125}I-[Glu^1]plasminogen remained primarily in the zymogen form when incubated with 10 nM sct-PA in BSA-coated wells. No activation was observed when CK 8-associated plasminogen was incubated for 1 h at 22 °C without sct-PA (Figure 7, lane 60−).

Equivalent experiments were performed with [Lys^{78}]plasminogen and sct-PA. In the absence of CK 8, the [Lys^{78}]plasminogen was activated at a more rapid rate compared with [Glu^1]plasminogen, as expected. When the [Lys^{78}]plasminogen was initially CK 8-associated, the rate of activation was substantially increased. Almost all of the plasminogen was converted into the two-chain form in 5 min (results not shown). These studies and the experiments with BT20 cell-conditioned medium demonstrate that the effects of CK 8 on plasminogen activation are not restricted to the closed plasminogen conformation adopted selectively by [Glu^1]plasminogen.

DISCUSSION

Plasmin activity is controlled locally by the action of plasminogen activators, such as t-PA and u-PA, and by plasmin inhibitors, including α_2-antiplasmin and α_2-macroglobulin [1,2]. A second level of control is imposed by proteins which bind plasminogen via kringle domain lysine-binding sites. These proteins, which include soluble factors, extracellular matrix components and plasma membrane receptors, alter the rate of plasminogen activation by t-PA and u-PA [1,2,27,28]. The same factors also decrease the reactivity of bound plasmin with inhibitors. Thus, kringle domain-dependent interactions profoundly influence the concentration of available plasmin in various micro-environments.

In the present study, we demonstrated the presence of soluble CK 8-containing complexes in the conditioned medium of breast carcinoma cells, confirming previous studies [18]. The soluble CK 8 bound plasminogen and enhanced the rate of plasminogen activation by t-PA. In experiments with purified immobilized CK 8, we demonstrated that CK 8 has the capacity to bind both t-PA and plasminogen. Thus, CK 8 may promote plasminogen activation on the cell surface and in the pericellular spaces surrounding breast cancer cells, *in vivo*.

Plasminogen exists in at least two conformations that are differentially activated [1,23,24]. In the presence of physiological concentrations of Cl^-, [Glu^1]plasminogen exists primarily in the 'closed' conformation which is relatively resistant to activation [29]. [Glu^1]Plasminogen is converted into the more readily activated 'open' conformation by plasmin-mediated cleavage of the N-terminal peptide to yield [Lys^{78}]plasminogen or by binding ω-amino acids such as ϵACA [2,24,29]. Proteins that bind to the kringle domains may also convert [Glu^1]plasminogen into the open conformation and thereby promote [Glu^1]plasminogen activation [30]. By contrast, the conformation of [Lys^{78}]plasminogen is minimally affected by lysine-binding interactions [1,24]. In our experiments, with BT20 cell-conditioned medium and purified CK 8, an increased rate of plasminogen activation was observed with both [Glu^1]- and [Lys^{78}]plasminogen, suggesting that plasminogen conformational change is not exclusively responsible for the increased rate of activation. An alternative model is that which has been proposed for fibrin [3]. t-PA and plasminogen bind to fibrin, forming a ternary complex in which the plasminogen is activated more readily. We hypothesize that a similar ternary complex forms with CK 8; however, based on the studies completed to date, we cannot rule out the possibility that CK 8 functions primarily as an enzyme (t-PA) modulator without requiring plasminogen binding for enhanced activation.

Recently, we identified CK 8 as a candidate plasminogen receptor on several epithelial and carcinoma cell lines [12]. While most of the cellular CK 8 is contained within highly insoluble filaments, CK 8 may also exist as a component of soluble complexes intracellularly and in the extracellular spaces [15,18,19]. Intracellular soluble cytokeratin is in the form of heteropolymers; little or no cytokeratin exists as complexes smaller than a tetramer [31]. Bachant and Klymkowsky [32] subjected soluble cytokeratins from *Xenopus* oocytes to molecular exclusion chromatography and found that the majority of the protein eluted with an apparent mass of 750 kDa, while a smaller amount eluted at 150 kDa. Our chromatography analysis of CK 8 released by tumour cells showed similarly sized complexes, although some of the CK 8 also eluted in the void volume. At the present time, we do not understand the relationship of released CK 8 to soluble and insoluble forms of intracellular cytokeratin; however, the CK 8-containing complexes released from the BT20 cells are probably equivalent to Tissue Polypeptide Antigen. The ability of tumour-secreted, soluble CK 8 to enhance plasminogen activation suggests that Tissue Polypeptide Antigen may influence fibrinolysis *in vivo*.

Expression of CK 8 is usually restricted to simple epithelia; however, numerous studies have shown that CK 8 is aberrantly expressed in malignant cells [33–35]. Aberrant expression of CK 8 may correlate with an invasive phenotype. In transitional cell carcinoma and squamous cell carcinoma, aberrant expression of CK 8 is localized to the tumour invasion front [36,37]. In malignant melanoma, aberrant expression of CK 8 is correlated with an increase in tumour invasiveness, *in vitro* [33]. Most significantly, mouse L fibroblasts, which lack CK 8, show increased motility and penetration of Matrigel after transfection with CK 8/CK 18 [38]. Motility and cellular invasion through Matrigel are processes that are promoted by cell-surface-associated plasmin. The studies presented here suggest a model that may partially explain the correlation of CK 8 expression with cellular invasiveness. We propose that the small fraction of total cellular CK 8, which is expressed on the outer cell surface, may promote cellular invasiveness by enhancing proteinase activation in the pericellular spaces.

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REFERENCES

1 Ponting, C. P., Marchall, J. M. and Cederholm-Williams, S. A. (1992) Blood Coagulation Fibrinolysis **3**, 605–614
2 Henkin, J., Marcotte, P. and Yang, H. (1991) Prog. Cardiovasc. Dis. **34**, 135–164
3 Hoylaerts, M., Rijken, D. C., Lijnen, H. R. and Collen, D. (1982) J. Biol. Chem. **257**, 2912–2919
4 Plow, E. F., Herren, T., Redlitz, A., Miles, L. A. and Hoover-Plow, J. L. (1995) FASEB J. **9**, 939–945
5 Hajjar, K. A., Harpel, P., Jaffe, E. and Nachman, R. L. (1986) J. Biol. Chem. **261**, 11656–11662
6 Gonias, S. L., Braud, L. L., Geary, W. A. and VandenBerg, S. R. (1989) Blood **74**, 729–736
7 Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T. E. and Magnusson, S. (1978) Prog. Chem. Fibrinolysis Thrombolysis **3**, 191–209
8 Christensen, U. (1985) FEBS Lett. **182**, 43–46
9 Fleury, V. and Angles-Cano, E. (1991) Biochemistry **30**, 7630–7638
10 Miles, L. A., Dahlberg, C. M., Plescia, J., Felez, J., Kato, K. and Plow, E. F. (1991) Biochemistry **30**, 1682–1691
11 Hajjar, K. A., Jacovina, A. T. and Chacko, J. (1994) J. Biol. Chem. **269**, 21191–21197
12 Hembrough, T. A., Vasudevan, J., Allietta, M. M., Glass, W. F. and Gonias, S. L. (1995) J. Cell Sci. **108**, 1071–1082

13 Moll, R., Franke, W. W. and Schiller, D. L. (1982) Cell **31**, 11–24
14 Morita, T., Tondella, M. L., Takemoto, Y., Hashido, K., Ichinose, Y., Nozaki, M. and Matsushiro, A. (1988) Gene **68**, 109–117
15 Leube, R. F., Bosch, F. X., Romano, V., Zimblemann, R., Hofler, H. and Franke, W. W. (1986) Differentiation **33**, 69–85
16 Weber, K., Osborn, M., Moll, R., Wiklund, B. and Luning, B. (1984) EMBO J. **3**, 2707–2714
17 Bjorklund, B. and Bjorklund, V. (1983) Cancer Detect. Prevent. **6**, 41–50
18 Chan, R., Rossitto, P. V., Edwards, B. F. and Cardiff, R. D. (1986) Cancer Res. **46**, 6353–6359
19 Brabon, A. C., Williams, J. F. and Cardiff, R. D. (1984) Cancer Res. **44**, 2704–2710
20 Deutsch, D. G. and Mertz, E. T. (1970) Science **170**, 1095–1096
21 Achtstaetter, T., Hatzfeld, M., Quinlan, R. A., Parmelee, D. C. and Franke, W. W. (1986) Methods Enzymol. **134**, 355–371
22 Laemmli, U. K. (1970) Nature (London) **227**, 199–205
23 Mangel, W., Lin, B. and Ramakrishnan, V. (1990) Science **248**, 69–73
24 Violand, B. N., Byrne, R. and Castellino, F. J. (1978) J. Biol. Chem. **253**, 5395–5401
25 Kolev, K., Owen, W. G. and Machovich, R. (1995) Biochim. Biophys. Acta **1247**, 239–245
26 Machovich, R. and Owen, W. G. (1993) Thromb. Haemostasis **70**, 864–866
27 Knudsen, B. S., Silverstein, R. L., Leung, L. L., Harpel, P. C. and Nachman, R. L. (1986) J. Biol. Chem. **261**, 10765–10771
28 Stack, S., Gonzalez-Gronow, M. and Pizzo, S. V. (1990) Biochemistry **29**, 4966–4970
29 Urano, T., Chibber, B. and Castellino, F. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 4031–4034
30 Pannell, R. and Gurewich, V. (1986) Blood **67**, 1215–1223
31 Fuchs, E. and Weber, K. (1994) Annu. Rev. Biochem. **63**, 345–382
32 Bachant, J. B. and Klymkowsky, M. W. (1996) J. Cell Biol. **132**, 153–165
33 Hendrix, M. J. C., Seftor, E. A., Chu, Y.-W., Seftor, R. E. B., Nagle, R. B., McDaniel, K. M., Leong, S. P. L., Yohem, K. H., Leibovitz, A. M., Meyskens, F. L., Conaway, D. H., Welch, D. R., Liotta, L. A. and Stetler-Stevenson, W. (1993) J. Natl. Cancer Inst. **84**, 165–174
34 Knapp, A. C., Bosch, F. X., Hergt, M., Kuhn, C., Winter-Simanowski, S., Schmid, E., Regauer, S., Bartek, J. and Franke, W. W. (1989) Differentiation **42**, 81–102
35 Franke, F. E., Schachenmayr, W., Osborn, M. and Altmannsbergen, M. (1991) Am. J. Pathol. **139**, 67–79
36 Schaafsma, H. E., Ramaekers, F. C. S., van Muijen, G. N. P., Robben, H., Lane, E. B., Leigh, I. M., Ooms, E. C. M., Schalken, J. A., van Moorselaar, R. J. A. and Ruiter, D. J. (1991) Am. J. Pathol. **139**, 1389–1400
37 Schaafsma, H. E., van der Velden, L. A., Manni, J. J., Peters, H., Link, M., Ruiter, D. J. and Ramaekers, F. C. S. (1993) J. Pathol. **170**, 77–86
38 Chu, Y.-W., Runyan, R. B., Oshima, R. G. and Hendrix, M. J. C. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 4261–4265

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