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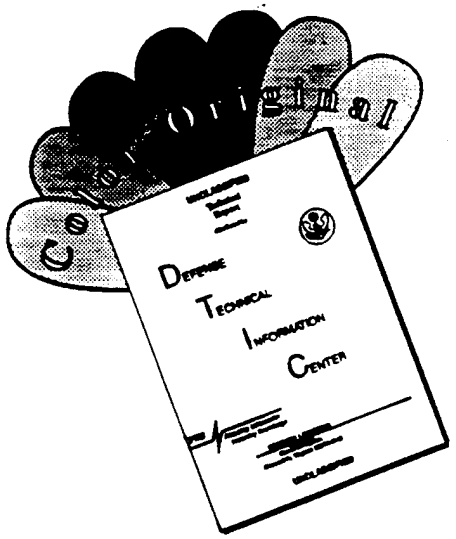

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For breast cancer cells to invade or metastasize, a cascade of proteinases, including the serine proteinase plasmin, must be organized at the cell surface. In the first year of this research program, we demonstrated a unique mechanism whereby breast cancer cells bind plasmin. The putative receptor is cytokeratin 8 (CK 8). CK 8 is a cytoskeletal protein; however, using fluorescence microscopy and immunoelectron microscopy, we demonstrated CK 8 on the external plasma membrane of non-permeablized breast cancer cells. We prepared a monoclonal antibody specific for a synthetic peptide corresponding to the C-terminal 13 amino acids of CK 8. This antibody bound specifically to breast cancer cells and inhibited binding of plasminogen to the same cells. Furthermore, we demonstrate that purified CK 8 not only binds plasminogen, but also tissue-type plasminogen activator. In the resulting ternary complex, the rate of plasminogen activation is significantly increased. This activity is duplicated by CK 8 which is recovered from conditioned medium of breast cancer cell lines. We are now developing the reagents to test the role of CK 8 in breast cancer invasion, using *in vitro* invasion models.

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Introduction: For cancer cells to metastasize, a cascade of proteinases must be organized at the cell surface. Urokinase is a plasminogen activator which can initiate this cascade. A relationship between cellular synthesis of urokinase and tumor invasiveness is well documented in breast cancer. For the proteinase cascade to function, plasminogen must bind to the breast cancer cell-surface. Once activated at the cell surface, plasmin digests glycoprotein components of the extracellular matrix and activates matrix metalloproteinases, with the capacity to digest collagen. These reactions are critical for breast cancer cells to break through basement membranes and penetrate lymphatics or blood vessels.

When this work was initiated, the plasminogen receptor in breast cancer had not been identified. Considerable information was available in the literature to suggest that different plasma membrane proteins might be responsible for plasminogen binding to different cells types. Our laboratory had initiated studies with hepatocytes and demonstrated that the most prominent plasminogen-binding protein in the plasma membrane of this cell type is cytokeratin 8 (CK 8). Hepatocytes are epithelial cells, like the breast epithelial cells from which most malignancies of that organ are derived. Thus, we hypothesized that CK 8 might also play a role in plasminogen immobilization at the cell surface of breast cancer cells. However, when the work proposed in this grant application was undertaken, we had a number of problems to address. First, it was not clear that the CK 8 identified in the plasma membrane of hepatocytes actually had access to the exterior surface of the cell. Furthermore, we did not know whether any CK 8 which was present on the external surface would bind plasminogen or to what extent the CK 8 would contribute to the total plasminogen binding capacity of the cell. Finally, we did not know whether plasma membrane CK 8 would actually influence cellular invasiveness, although we had preliminary data and work in the literature to support this possibility.

Body: Our initial goal was to demonstrate that CK 8 is in fact expressed on the external surfaces of breast cancer cells. The techniques that we used included immunofluorescence microscopy and immunoelectron microscopy. The advantage of these techniques is the ability to determine whether an antigen is expressed homogeneously across an entire population of cells or exclusively by a small subpopulation of cells. To stain only cell surface CK 8, CK 8-specific antibodies were incubated with viable cells at 4°C in physiological buffers containing BSA. Since all incubations were conducted using adherent cells on cover slips, the chance of cellular injury due to culture manipulation was minimized. Cells were not fixed until after all antibody incubations were completed.

Our immunofluorescence microscopy studies demonstrated cell surface exposure of CK 8 on BT 20 and MCF7 breast carcinoma cells. A punctate pattern of CK 8 expression was detected across the entire surface of all of the cells. CK 8 was also detected on breast cancer cell surfaces by immunoelectron microscopy. CK 8-specific antibody in colloidal gold adducts localized to the cell surface without penetrating the plasma membrane in intact cells. Much of the colloidal gold was

observed in association with the bases of microvilli. ***These studies clearly demonstrated that CK 8 is in fact present on the external surfaces of breast cancer cells*** (see publication in appendix).

The next step was to demonstrate whether the cell surface CK 8 contributes in a significant way to the total plasminogen binding capacity of the breast cancer cells. To accomplish this goal, a synthetic peptide corresponding to the C-terminal 13 amino acids of CK 8 was synthesized. This synthetic peptide was coupled to hemocyanin and used to immunize mice. Monoclonal antibodies were then raised and one particular antibody, 1E8, demonstrated high-affinity binding to purified cytokeratin 8 as well as the synthetic peptide coupled to BSA.

Antibody 1E8 binds in a specific and saturable manner to three different breast cancer cell lines which have been studied. Furthermore, the antibody inhibits binding of at least 75% of the plasminogen to the same cell types. We now have available in the laboratory, a full-length cDNA for CK 8. This cDNA was inserted into the expression vector, pBK-CMV, as was the full-length cDNA for CK 18. The two constructs were then co-transfected into fibroblasts. At this time, we have fluorescence microscopy evidence for expression of CK 8 and CK 18 in the fibroblasts. Since fibroblasts do not naturally synthesize these proteins, our monoclonal antibody 1E8 should not block plasminogen binding to these cells. However, an increase in plasminogen binding should be observed in the transfected cells and this binding should be inhibitable with the antibody. These studies will be performed in the upcoming year.

Based on the studies which we have already completed with monoclonal antibody 1E8, we can conclude that cell surface CK 8 is a significant plasminogen receptor in breast cancer cells. Furthermore, antibody 1E8 represents a valuable tool for testing the importance of CK 8 in breast cancer cell invasion and metastasis.

In order to fully characterize the biochemistry of CK 8/plasminogen interactions, we purified CK 8 from hepatocytes. When CK 8 was immobilized in microtiter plates, ^{125}I-plasminogen bound in a specific and saturable manner. The K_D was approximately 0.2 μM. At saturation, one plasminogen was bound per every 2 CK 8s.

An exciting discovery using the immobilized CK 8 system was that the plasminogen activator, tissue-type plasminogen activator (t-PA) also binds to CK 8. The binding sites for plasminogen and t-PA are nonidentical. Thus, CK 8 can bind both activator and substrate. Using fluorescent substrate technology and SDS-PAGE, we determined that CK 8 promotes plasminogen activation by t-PA. The rate of activation is increased at least 50-fold.

In subsequent experiments, we recovered CK 8 from the conditioned medium of breast carcinoma cell lines. This CK 8 retained its ability to bind plasminogen

and promote plasminogen activation. ***Thus, CK 8 has the potential to promote plasminogen activation not only on the cell surface, but also in the pericellular spaces surrounding cancer cells.***

Conclusions: The following is a list of the major conclusions drawn from the work conducted in the first year of this research program.

(1) Cytokeratin 8 is expressed on the external surfaces of breast carcinoma cells.

(2) Cytokeratin 8 on the external surfaces of breast carcinoma cells binds plasminogen and represents the major plasminogen receptor in this cell type.

(3) In addition to binding plasminogen, cytokeratin 8 also binds tissue-type plasminogen activator. The binding sites for the two proteinases are nonidentical.

(4) Binding of plasminogen to t-PA by cytokeratin 8 greatly accelerates plasminogen activation and may thereby enhance the invasive potential of breast cancer cells.

As we move towards year two of this research program, our original plan for investigation is basically intact. One change in strategy is noteworthy. We have determined that an increased emphasis on molecular strategies will greatly enhance our ability to answer the major questions in the research program. Towards that end, we have generated the constructs described in the narrative. We also have utilized site-directed mutagenesis to form a mutant CK 8 that lacks the plasminogen binding site. These constructs will be extremely useful in determining the function of CK 8 in invasion assays.

References: Our immunofluorescence and immunoelectron microscopy studies have been published. The reference is:

Hembrough, TA, Vasudevan, J, Allietta, MM, Glass, WF, Gonias, SL (1995) A Cytokeratin 8-Like Protein with Plasminogen-Binding Activity is Present on the External Surfaces of Hepatocytes, HePG2 cells and breast carcinoma cell lines. *J. Cell Sci.* 108:1071-1082

A copy of this manuscript is included as an appendix.

APPENDIX

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A cytokeratin 8-like protein with plasminogen-binding activity is present on the external surfaces of hepatocytes, HepG2 cells and breast carcinoma cell lines

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SUMMARY

Plasminogen binding to cell surfaces may be important for tumor invasion and other processes that involve cellular migration. In this investigation, the principal plasminogen-binding protein was identified in the plasma membrane fraction of rat hepatocytes. The protein had an apparent mass of 59 kDa, was insoluble in a spectrum of detergents, and was identical to cytokeratin 8 (CK 8) as determined by sequence analysis of nine amino acids at the N terminus of two cyanogen bromide fragments. The 59 kDa protein bound CK 8-specific antibody in western blot analyses. These studies demonstrate that CK 8 or a CK 8-like protein binds plasminogen. Given this newly determined and potentially important CK 8 function, immunofluorescence and immunoelectron microscopy studies were performed to determine whether CK 8 may be present on the external surfaces of unpermeabilized, viable hepatocytes. All of the cells in each preparation were immunopositive with two separate CK 8-specific antibodies. A punctate pattern of immunofluorescence was detected on the cell surface with approximately even intensity from cell to cell. By immunoelectron microscopy, CK 8 was preferentially associated with microvilli. In order to determine whether other epithelial cells express cell-surface CK 8, immunofluorescence and immunoelectron microscopy studies were performed with HepG2 hepatocellular carcinoma cells and with BT20 and MCF-7 breast carcinoma cells. The pattern of antigen expression was equivalent with each cell type and comparable to that observed with hepatocytes. These studies support the hypothesis that CK 8 is associated with the external cell surface where it may express important proteinase receptor function.

Key words: plasminogen, cytokeratin, plasma membrane, intermediate filament

INTRODUCTION

Plasmin is a serine proteinase that plays a central role in fibrinolysis. At the cell surface and in the pericellular spaces, plasmin cleaves many substrates other than fibrin, including: glycoprotein components of the extracellular matrix (Liotta et al., 1981), zymogen forms of certain matrix metalloproteinases (He et al., 1989), glycoprotein IIIa (Pasche et al., 1994), growth factor precursors such as latent transforming growth factor-β (TGF-β) (Lyons et al., 1988), insulin-like growth factor-binding protein (Campbell et al., 1992), interferon-γ (Gonias et al., 1989b), the thrombin receptor (Turner et al., 1994), and the type III TGF-β receptor/betaglycan (LaMarre et al., 1994). These enzyme-substrate reactions suggest multiple mechanisms by which plasmin may regulate cellular growth, differentiation and cellular migration.

The diverse continuum of plasmin substrates emphasizes the importance of regulating this proteinase within the pericellular spaces. Plasmin regulation is accomplished at three levels. The first is activation, which involves the proteolytic conversion of single-chain plasminogen into the active two-chain structure (Ponting et al., 1992). Once activated, plasmin is regulated by proteinase inhibitors, including α_2-antiplasmin and α_2-macroglobulin (Ellis and Dano, 1992; Gonias, 1992; Vassalli et al., 1991). Plasmin is also regulated spatially by noncovalent binding interactions that localize and concentrate the proteinase (and zymogen) in specific microenvironments. Reversible plasmin(ogen) binding to cell surfaces, fibrin clots and specific extracellular matrix proteins is mediated by five homologous plasmin(ogen) kringle domains (Ponting et al., 1992). The kringle domains have binding sites with affinity for lysine residues in other proteins. Association of plasminogen with many proteins depends on the relatively high-affinity interaction of the kringle-1 (K1) domain with a carboxy-terminal lysine residue (Christensen, 1985; Fleury and Anglés-Cano, 1991; Miles et al., 1991).

Plasmin(ogen)-binding proteins, including those present on cell surfaces, not only localize plasmin near potential substrates, but also significantly alter the kinetics of plasminogen activation and plasmin inhibition (Ellis and Dano, 1992;

Gonias, 1992). Therefore, understanding the spectrum of physiologically significant plasminogen-binding proteins is important. Nucleated mammalian cells, including endothelial cells (Hajjar et al., 1986), monocytes/macrophages (Plow et al., 1986), hepatocytes (Gonias et al., 1989a) and tumor cells (Burtin and Fondaneche, 1988; Correc et al., 1990; Hall et al., 1990) express specific plasmin(ogen)-binding sites. The binding affinity is typically low (K_d~1.0 μM); however, the capacity is high (10^5-10^7 sites per cell). Specific plasminogen binding to all cell types can be completely inhibited by lysine analogues such as ε-amino caproic acid (EACA) (Hajjar et al., 1986; Gonias et al., 1989a), confirming the kringle domain-dependency of these interactions.

Plasminogen-cellular interactions may be mediated by different cellular proteins. α-Enolase was identified as a candidate plasminogen receptor in U937 monocytes (Miles et al., 1991). Although α-enolase is primarily an intracellular protein, FACS analysis was used to demonstrate that this antigen is present on cell surfaces. Annexin-2 is another primarily intracellular protein that may be found in association with the external surfaces of endothelial cells where it functions as a receptor for plasminogen and tissue-type plasminogen activator (Hajjar et al., 1994). Amphoterin is a candidate plasminogen receptor in developing brain (Parkkinen and Rauvala, 1991). It has also been shown that purified Heymann Nephritis autoantigen (gp330) binds plasminogen (Kanalas and Makker, 1991).

The present study was undertaken to identify major plasminogen binding proteins in hepatocytes. Similar analyses of epithelial cells have not been previously reported. Our results demonstrate that cytokeratin 8 (CK 8) or a CK 8-like protein is the major plasminogen binding protein in the plasma membrane fraction of rat hepatocytes. CK 8 is a basic-neutral intermediate filament protein, unique amongst cytokeratins in that it has a C-terminal lysine residue (Morita et al., 1988). CK 8 extends from the nucleus to the plasma membrane where it has extensive interactions with the internal leaflet and with various membrane-associated structures, including desmosomes and hemidesmosomes (Garrod, 1993; Owaribe et al., 1991). Epithelial cells, and especially carcinoma cells, secrete CK 8 or CK 8 fragments in vivo; these fragments are a major component of the long-recognized body-fluid complex, Tissue Polypeptide Antigen (TPA) (Leube et al., 1986; Weber et al., 1984). It has been proposed that CK 8 is also present on the external surfaces of epithelial cells (Godfroid et al., 1991; Donald et al., 1991). At least three mechanisms might hypothetically account for this. First, the CK 8 may penetrate through the plasma membrane as part of a multiprotein complex, despite the lack of a transmembrane domain. Second, CK 8 that is secreted by cells may tightly associate with the external surface of the plasma membrane (Riopel et al., 1993). Finally, since CK 8 covalently binds to plasma membrane lipids (Asch et al., 1993), it is conceivable that these complexes are translocated to the outer membrane leaflet.

The previous studies demonstrating cell-surface CK 8 have been contested on the basis that the experimental techniques utilized might detect primarily damaged or permeabilized cells (Riopel et al., 1993). Given the new function of CK 8 demonstrated here, we considered it important to re-evaluate the question of CK 8 exposure on cell surfaces. Therefore, in the second part of this study, immunofluorescence microscopy and immunoelectron microscopy experiments were performed. These studies detected, for the first time, CK 8 or a CK 8-like protein on the external surfaces of rat hepatocytes and HepG2 hepatocellular carcinoma cells. We also present a series of studies confirming the earlier, contested work regarding the presence of CK 8 on the external surfaces of breast carcinoma cells.

MATERIALS AND METHODS

Materials

$Na^{125}I$ was from Amersham International (Arlington Heights, IL). Aprotinin, chloramine T, EACA, phenylmethyl-sulfonyl fluoride (PMSF), bovine serum albumin (BSA), sodium deoxycholate and fetal bovine serum (FBS) were purchased from Sigma (St Louis, MO). Leupeptin, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), and collagenase D were purchased from Boehringer Mannheim. Williams E medium and Earle's balanced salt solution (EBSS) were from Gibco Laboratories (St. Lawrence, MA). D-Val-L-Leu-L-Lys *p*-nitroanilide (S-2251) was from Kabi Vitrum (Stockholm, Sweden).

Murine monoclonal antibody PCK-26, which recognizes CK 5, 6, and 8, and murine monoclonal antibody M20, which is specific for CK 8, were purchased from Sigma. The hybridoma, UCD/AB 6.01, was obtained from ATCC and expanded in ascites using $IRCF_1$ mice (Hilltop Labs, Scottdale, PA). AB 6.01 was purified from the ascites by Protein A-Sepharose chromatography. This antibody is specific for CK 8 but cross-reacts slightly with CK 18 (Chan et al., 1985). In our western blot analyses of purified rat hepatocyte CK, performed as previously described (Wolf et al., 1992), only CK 8 was detected by AB 6.01 (results not shown). A monoclonal IgG_1 specific for GTPase activating protein (p120-GAP) was kindly provided as a gift by Dr Sarah Parsons (University of Virginia).

Cell culture

All cell lines were obtained from ATCC. The human hepatocellular carcinoma cell line, HepG2 (Aden et al., 1979), was cultured in MEM supplemented with 10% FBS and 2 mM glutamine. The human breast carcinoma cell line, BT20 (Lastargues and Ozzello, 1958), was cultured in RPMI 1650 with 10% FBS. MCF-7 (Soule et al., 1973) breast carcinoma cells were cultured in Eagle's minimum essential medium (EMEM) supplemented with 10% FBS, 10 μg/ml insulin, and 2 mM glutamine.

Hepatocytes were isolated from 250-400 g Sprague-Dawley rats (male or female), using the well-described two-step perfusion method (Berry and Friends, 1969; Gonias et al., 1989a). The rats were purchased from Hilltop Laboratories. The hepatic cells were released into Williams E medium containing 10% FBS. The preparations were enriched in hepatocytes by centrifugation so that non-parenchymal cells represented less than 2% of the final populations. Adherent hepatocyte cultures were greater than 98% viable as determined by trypan blue exclusion 2 hours after isolation.

Purified plasminogen and plasminogen-Sepharose

Plasminogen was purified from human plasma by affinity chromatography on lysine-Sepharose (Deutsch and Mertz, 1970). A linear gradient of EACA (0 to 15 mM) was used to elute the plasminogen. Final preparations contained mixtures of the two major glycoforms but were free of detectable plasmin, as determined by SDS-PAGE and S-2251 hydrolysis. Purified plasminogen (100 mg) was coupled to 10 g of CNBr-activated Sepharose CL-4B (Porath et al., 1973). Approximately 60% of the plasminogen bound to the resin.

Plasminogen binding to cells in culture

Isolated rat hepatocytes were plated in collagen-coated 12-well or 24-

well plates at a density of 500,000 cells/ml and cultured in Williams E medium with 10% FBS. After 2 hours, the cultures were washed three times with EBSS, 10 mM Hepes, 0.1% BSA (w/v), pH 7.4 (EHB-buffer). Plasminogen was radioiodinated to a specific activity of 1-2 μCi/μg with Iodobeads (Hall et al., 1990) or chloramine-T (Miles and Plow, 1985). The ^{125}I-plasminogen was then incubated with the hepatocyte cultures in EHB and aprotinin (70 μg/ml) for 4 hours at 4°C. EACA (10 mM) was added to some wells. EACA and excess nonradiolabeled plasminogen are equally effective in inhibiting specific ^{125}I-plasminogen binding to cells in culture (Hajjar et al., 1986; Gonias et al., 1989a). ^{125}I-Plasminogen-binding experiments were terminated by washing the cultures 3 times with EHB. The cells were then lysed overnight in 0.1 M NaOH/1% SDS (w/v). Radioactivity in the lysates was determined in a gamma counter. An identical procedure was used to characterize plasminogen binding to HepG2 or breast carcinoma cells.

Plasma membrane preparation

Plasma membrane-enriched fractions of isolated hepatocytes were prepared according to the method of Fleischer and Kervina (1986) as modified by Wolf et al. (1992). All fractionation steps were performed in medium supplemented with 1 mM PMSF, 70 μg/ml aprotinin, 30 μg/ml leupeptin, 0.2 μg/ml E-64, and 1 mM EDTA. The complete procedure yielded subcellular fractions enriched in nuclei (N), cytoplasm (C), mitochondria (M), and plasma membranes (P). Intermediate filament proteins are typically recovered in the P and N fractions (Hubbard and Ma, 1983).

SDS-PAGE and ligand blotting

Whole-cell homogenates, subcellular fractions, and purified proteins were subjected to SDS-PAGE (Laemmli, 1970). Resolved proteins were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore) using a Hoefer Transphor apparatus (2 hours, 0.5 amp). The membranes were then stained with Coomassie Blue R250 (Bio-Rad, CA) or blocked with 5% nonfat dried milk in 20 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), for ligand blotting or western blot analysis. To insure complete transfer, the original gel slab was stained with Coomassie Blue R250 as well.

Blocked PVDF membranes were rinsed twice with PBS and 0.1% (v/v) Tween-20 (PBS-T). ^{125}I-Plasminogen (10-20 nM) was then incubated with the membranes in PBS-T and 70 μg/ml aprotinin for 45 minutes. The blots were rinsed 3 times for 15 minutes in PBS-T, dried and autoradiographed. In control experiments, ^{125}I-plasminogen was incubated with the PVDF membranes in the presence of EACA.

Plasminogen-affinity chromatography

Enriched plasma membranes (P fraction) were solubilized in 10 mM sodium deoxycholate, pH 8.4, for 2 hours at 4°C and then subjected to centrifugation for 15 minutes at 10^5 *g* in a Beckman airfuge. The insoluble pellet and the supernatant were analyzed by SDS-PAGE and ^{125}I-plasminogen-ligand blotting. The detergent-insoluble pellet was extracted with 6 M urea for 4 hours at 25°C and then dialyzed against 10 mM sodium deoxycholate. The resulting solution and the original detergent-soluble portion of the P-fraction were separately subjected to affinity chromatography on plasminogen-Sepharose. Associated proteins were eluted from plasminogen-Sepharose with 15 mM EACA in 10 mM sodium deoxycholate. Fractions were analyzed by SDS-PAGE with silver staining.

Micro-sequence analysis of the major plasminogen-binding protein

The major plasminogen-binding protein was eluted from PVDF and subjected to amino-terminal sequence analysis using an Applied Biosystems 470A Gas-Phase Sequencer equipped with a model 120A on-line analyzer. Since initial analysis suggested a blocked amino terminus, the binding protein was treated with 150 mM cyanogen bromide (CNBr) in 70% formic acid while adherent to PVDF. Fragments were eluted with 1% (v/v) Triton X-100, 2% SDS, 50 mM Tris, pH 9.5, and then re-subjected to SDS-PAGE and electroblotting. New products were identified by Coomassie staining and subjected to amino-terminal sequence analysis. Results were compared with known sequences in GenBank using the Fasta Program (Pearson, 1994).

Immunofluorescence microscopy

Hepatocytes were adhered to collagen-coated 30 mm glass coverslips for 2 hours at 37°C and then studied immediately in immunofluorescence microscopy experiments. HepG2, BT20, and MCF-7 cells were cultured on coverslips for at least 48 hours. All cultures were washed with EHB buffer and incubated with various primary antibodies at 1/100-1/500 dilution in EHB for 2 hours at 4°C. The cells were then washed three times with EHB, incubated with FITC-labelled goat anti-mouse IgG (1/1000 dilution) for 2 hours at 4°C, rinsed three times again, and fixed in 3.7% paraformaldehyde or freshly prepared, ice-cold buffered-formaldehyde. Cellular immunofluorescence was imaged using an Olympus 3H2 Microscope with a dual wavelength cube. In control experiments, mouse nonimmune IgG was substituted for primary antibody or primary antibody was omitted.

Electron microscopy

Primary hepatocytes, breast carcinoma cells, and HepG2 cells were cultured on 10 mm plastic coverslips, as described for the immunofluorescence microscopy experiments. The cells were brought to 4°C and incubated with primary antibodies for 1 hour. At this point, the cells were incubated with either Protein A adsorbed to colloidal gold (100 Å) or anti-mouse IgG adsorbed to colloidal gold (100 Å) for 2 hours at 4°C. The cells were fixed with 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.5, for 15 minutes. This fixative rapidly reacts with the lipid in plasma membranes and limits plasma membrane vesiculation (Hasty and Hay, 1978). After washing with PBS, the cells were further fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.5, for 8 hours at 4°C. The cells were then serially dehydrated in acetone and embedded in EPON 812 for polymerization at 60°C overnight. Embedded coverslips were thin-sectioned on-side. Sections were transferred to 150 mesh nickel grids and viewed on a Zeiss 902 electron microscope.

RESULTS

Plasminogen binding to cells in culture

In initial studies, we confirmed that hepatocytes in primary culture, HepG2, BT20, and MCF-7 cells bind plasminogen specifically. As expected, EACA displaced greater than 80% of the binding through the entire ^{125}I-plasminogen concentration range with each cell type. Scatchard transformations were linear with correlation coefficients of 0.90-0.98 (not shown). K_d and B_{max} values were derived assuming a single-K_d model (Table 1) and are consistent with previously reported studies of plasminogen binding to cells in culture (as reviewed above).

Characterization of plasminogen-binding proteins in the hepatocyte P-fraction

Plasminogen binds specifically to a number of cellular

Table 1. Specific binding parameters for the interaction of ^{125}I-plasminogen with cells in culture

Cell type	K_d (μM)	B_{max} (per cell)
Rat hepatocytes	0.75(±0.1)	9.7(±0.7)×10^6
HepG2	1.83(±0.6)	4.3(±1.0)×10^6
BT20	1.05(±0.3)	5.9(±1.0)×10^6
MCF-7	0.90(±0.3)	1.9(±0.4)×10^6

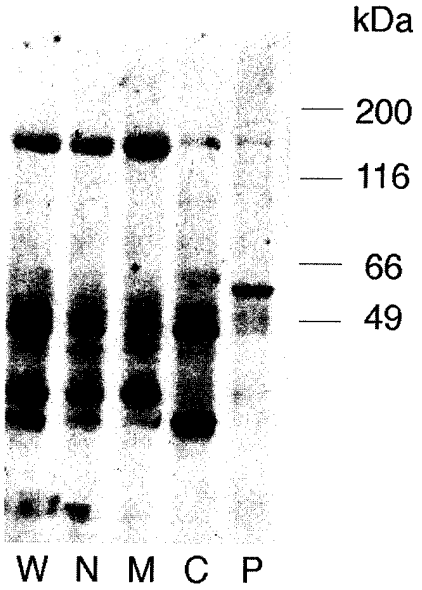

Fig. 1. ^{125}I-Plasminogen ligand blot of rat hepatocyte subcellular fractions. Lanes are labeled as follows: W, whole cell homogenate; N, nuclear fraction; M, mitochondrial fraction; C, cytoplasmic fraction; P, plasma membrane fraction. Each subcellular fraction has a unique set of plasminogen-binding proteins. The major plasminogen-binding species in the P fraction is the 59 kDa protein.

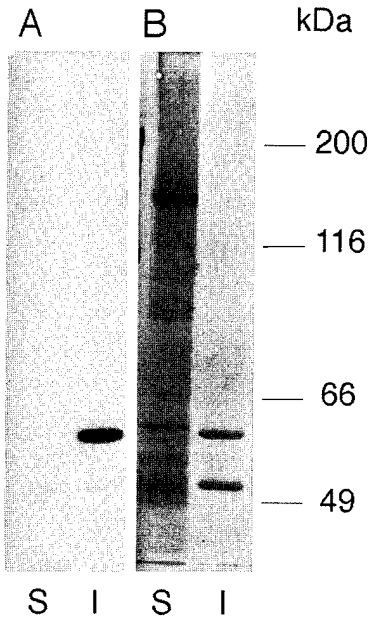

Fig. 2. ^{125}I-Plasminogen ligand blot and Coomassie-staining of detergent extracts of rat hepatocyte plasma membranes. (A) ^{125}I-plasminogen ligand blot of the detergent-soluble (S) and insoluble (I) fractions of the plasma membrane preparation. The detergent was 10 mM sodium deoxycholate. (B) Coomassie-stained blot of the same 'S' and 'I' fractions. The majority of the P-fraction is soluble in sodium deoxycholate. Two major bands remain in the detergent-insoluble fraction, including the 59 kDa plasminogen-binding protein.

proteins, including some that are intracellular and not available unless the cell is permeabilized. To identify plasminogen-binding proteins that may be significant in intact cells, hepatocytes were subjected to subcellular fractionation. Plasminogen-binding proteins in each fraction were compared by ^{125}I-plasminogen ligand blotting. Fig. 1 shows a representative ligand blot in which equal amounts of protein (100 μg) from the various subcellular fractions were compared. Each subcellular fraction demonstrated a distinct and consistent fingerprint of plasminogen-binding proteins. The primary plasminogen-binding protein in the P-fraction had a mass of 59 kDa. ^{125}I-Plasminogen binding to this band was completely inhibited by a 100-fold molar excess of nonradiolabeled plasminogen or 15 mM EACA (results not shown).

The 59 kDa plasminogen-binding protein was not detected in the M or C fractions, in five separate experiments. In whole cell homogenates and N-fractions, the 59 kDa protein either was detected only with prolonged autoradiograph exposure time (as was the case for the preparation shown in Fig. 1) or was present as a distinct but minor band compared with other plasminogen-binding species (as observed in other experiments not shown). These results suggest any of the following possibilities: (i) the 59 kDa protein is selectively recovered in the P-fraction; (ii) the plasminogen-binding activity of the 59 kDa protein is less stable in other fractions; and/or (iii) the other subcellular fractions contain a higher proportion of other plasminogen-binding proteins that are excluded from the P-fraction.

Characterization of the 59 kDa protein

The P-fraction was treated with various detergents, including 1% Triton X-100, 25 mM CHAPS, 35 mM β-D octylglucoside, and 10 mM sodium deoxycholate. Detergent-soluble and -insoluble fractions were incubated in SDS at 95°C for 5 minutes and subjected to SDS-PAGE and ^{125}I-plasminogen ligand blotting. Each of the detergents solubilized the majority of the P-fraction proteins; however, the 59 kDa protein remained in the detergent-insoluble fraction. Fig. 2 shows a representative solubilization experiment with 10 mM sodium deoxycholate. In this experiment, 100 μg of P-fraction were incubated with detergent for 2 hours and then subjected to centrifugation at 10^5 ***g***. The entire contents of the pellet (I) and supernatant (S) were subjected to SDS-PAGE so that the amount of each protein in the gel would approximate the proportion of that protein in the original P-fraction. As shown in the Coomassie-stained PVDF blot (B), the sodium deoxycholate-insoluble fraction contained two major proteins. One of these proteins was the 59 kDa species; this component was the only major ^{125}I-plasminogen-binding protein identified in either detergent solubilization fraction (A). The detergent-soluble fraction included a minor ^{125}I-plasminogen-binding species with an apparent mass of 150 kDa; this species was barely visible in the ligand blot without prolonged autoradiograph exposure time. An equivalently sized species was also apparent in the ligand blot of intact P-fraction shown in Fig. 1.

In ^{125}I-plasminogen ligand blotting experiments, P-fraction components were exposed to SDS. Therefore, we considered the possibility that the P-fraction contains plasminogen-binding proteins that are inactivated by SDS. In eight separate experiments, 5-15 mg of P-fraction were solubilized with the various detergents listed above. The detergent-soluble fractions were subjected to plasminogen-affinity chromato-

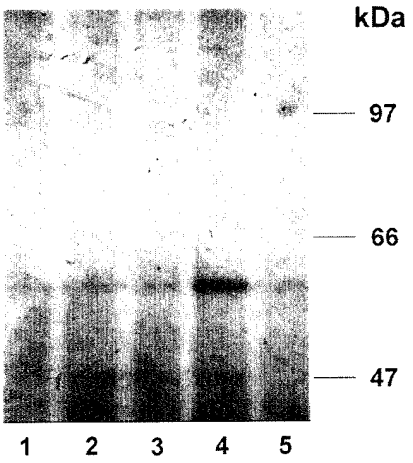

Fig. 3. Plasminogen-binding proteins isolated from the rat hepatocyte P-fraction by affinity chromatography. Lanes are labeled as sequential elution fractions, recovered after adding 15 mM EACA to the running buffer. Fraction 4 contains the major plasminogen-binding protein with a mass of 59 kDa.

graphy. EACA (15 mM) was used to elute bound proteins; elution fractions were analyzed by SDS-PAGE and silver-staining. A significant plasminogen-binding protein was not identified in these experiments.

Affinity chromatography was then performed to identify plasminogen-binding proteins in the detergent-insoluble portion of the P-fraction. Sodium deoxycholate (10 mM) was used to solubilize hepatocyte P-fraction. After centrifugation, the pellet was dissolved in 6 M urea and dialyzed back into 10 mM sodium deoxycholate without significant precipitation. The preparation was then subjected to plasminogen-affinity chromatography. Successive elution fractions, obtained after adding 15 mM EACA to the running buffer, are shown in Fig. 3. The 59 kDa protein bound to plasminogen-Sepharose and was the only major protein recovered in the EACA-eluent. These studies confirm and extend the results of the ligand blotting experiments by demonstrating that the 59 kDa protein binds plasminogen without exposure to SDS or immobilization on PVDF. Furthermore, the results of the chromatography experiments, performed with detergent-soluble and -insoluble fractions, indicate that the 59 kDa protein is the major plasminogen-binding protein in hepatocyte plasma membranes.

Identification of the 59 kDa protein

To identify the 59 kDa protein, sodium deoxycholate-insoluble material was isolated from the P-fraction. The 59 kDa protein was further purified by SDS-PAGE, transferred to PVDF and eluted for amino-terminal sequence analysis. Since the N terminus was apparently blocked, CNBr fragmentation was performed while the protein was adherent to the PVDF. The products were then eluted and subjected to SDS-PAGE and electroblotting. A major band at 12 kDa was isolated and subjected to amino-terminal sequence analysis, yielding a major and a secondary sequence. The major sequence, including the inferred N-terminal methionine residue, was MSTSGPRAFS. Amino acid yield ranged from 1.2 to 2.0 pmol, with the exception of the Ser residues that were recovered at lower yield, as expected. A search for homologous sequences was conducted in GenBank, using the Fasta program (Pearson, 1994). The major sequence was identical with that of mouse CK 8 (residues 12-21). Other significant homologies were detected exclusively with CK 8 from other species, including human (9/10) and potoroo (7/10).

The secondary sequence was present at approximately 25% of the molar yield of the major sequence and consisted of MDxIIAEVRA (a secondary amino acid was not clearly resolved at position three). The secondary sequence was 100% identical with amino acids 263-272 of mouse CK 8 and 89% identical with the comparable region of human CK 8. Significant homologies with proteins other than CK were not identified.

The identification of the plasminogen-binding protein as CK 8, or a CK 8-like protein, is consistent with the mass of the protein determined by SDS-PAGE and with the unique solubility properties of the protein, as determined by incubation with various detergents (Steinert and Roop, 1988). In hepatocytes and in other epithelial cells, CK 8 is typically recovered with CK 18 (Franke et al., 1981a,b), a slightly smaller protein that was observed in the detergent-insoluble fraction in Fig. 2. It is known that some CK 8 will partition into the plasma membrane fraction during subcellular fractionation (Hubbard and Ma, 1983). Furthermore, CK 8 is unique amongst the cytokeratins in that it has a C-terminal lysine that is conserved across species lines (Morita et al., 1988; Leube et al., 1986). As mentioned above, C-terminal lysine residues form a key element of most plasminogen-binding domains. To confirm the results of the microsequencing experiments, the detergent-insoluble portion of the hepatocyte P-fraction was subjected to western blot analysis with PCK-26, as previously described. The 59 kDa band bound antibody; no other immunopositive bands were detected (results not shown).

Immunofluorescence microscopy

The ability of CK 8 or a CK 8-like protein to bind plasminogen is significant, since CK 8 may be released by epithelial cells into the pericellular spaces and into the blood where plasminogen is present (Chan et al., 1986). CK 8 release from cells may be significantly increased in cancer (Bjorklund and Bjorklund, 1983). It has also been suggested that CK 8 is present on the external surfaces of breast carcinoma cells (Godfroid et al., 1991; Donald et al., 1990). If these reports are correct, then cell-surface CK 8 may function as a cellular plasminogen receptor; however, the presence of CK 8 on cell surfaces has been disputed (Riopel et al., 1993). Therefore, we undertook studies to determine whether hepatocytes and other epithelial cells express cell surface CK 8. The techniques used included immunofluorescence microscopy and immunoelectron microscopy. The advantage of these techniques is the ability to determine whether an antigen is distributed homogeneously across an entire population of cells or exclusively by a small subpopulation of cells.

To stain only cell-surface CK 8, CK 8-specific antibodies and secondary antibody were incubated with viable cells at 4°C in physiological buffers that contained BSA. Since all of the incubations were conducted with adherent cells on coverslips, the chance of cellular injury due to culture manipulation was minimized. Cells were not fixed until after all antibody incubations were completed.

Immunofluorescence microscopy studies of hepatocytes

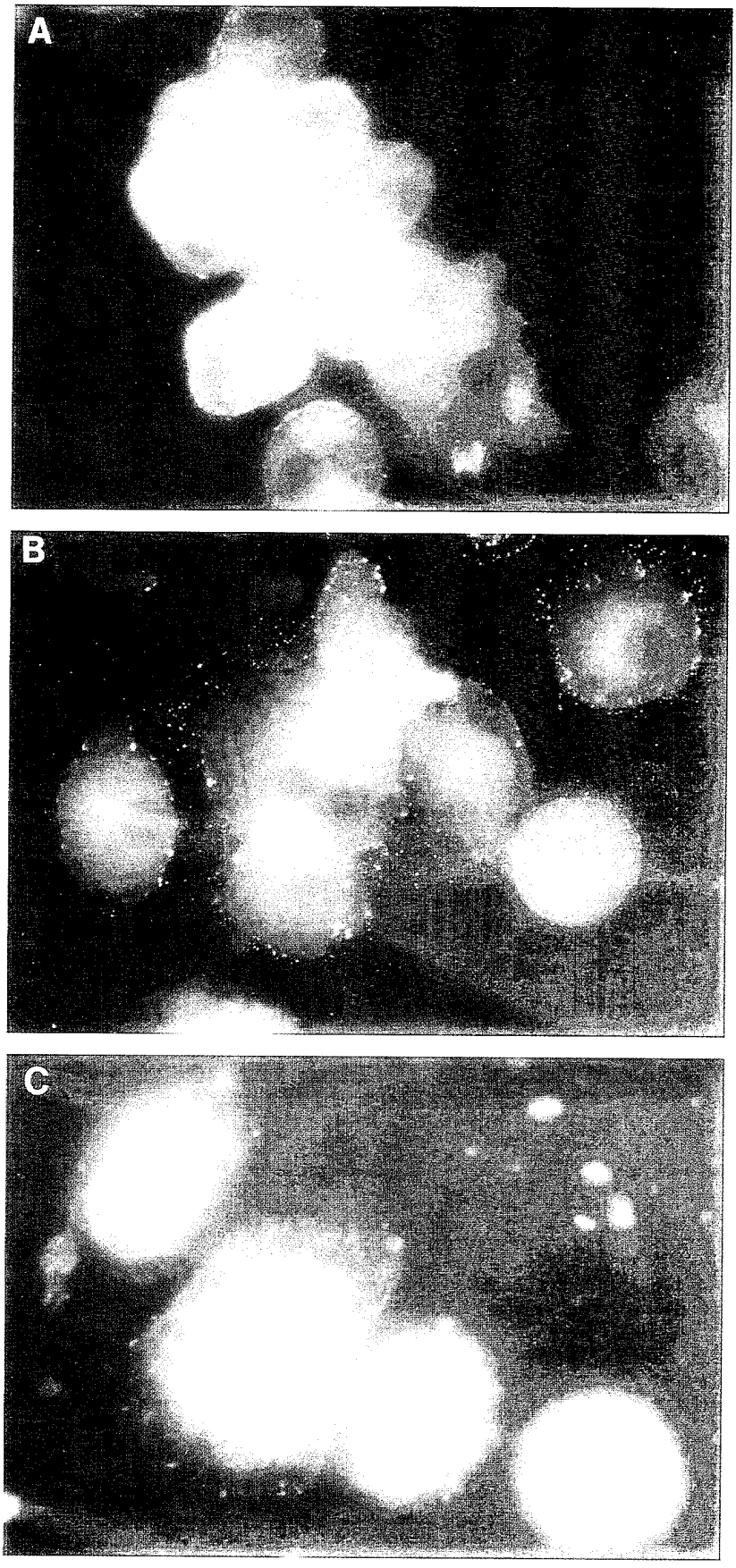

Fig. 4. Detection of CK 8 in viable, unpermeabilized hepatocytes by immunofluorescence microscopy. Primary rat hepatocytes were incubated with AB 6.01, followed by secondary antibody (FITC-anti-mouse IgG). To differentiate antibody binding from hepatocyte autofluorescence, the cells were imaged using a dual wavelength cube, exciting at both FITC and Texas Red wavelengths. Under these conditions, cytoplasmic autofluorescence appears red-white. (A) AB 6.01 labeling of a hepatocyte-cluster. (B) Individual hepatocytes are more prevalent. (C) Incubation with primary antibody was omitted; only cytoplasmic autofluorescence is seen.

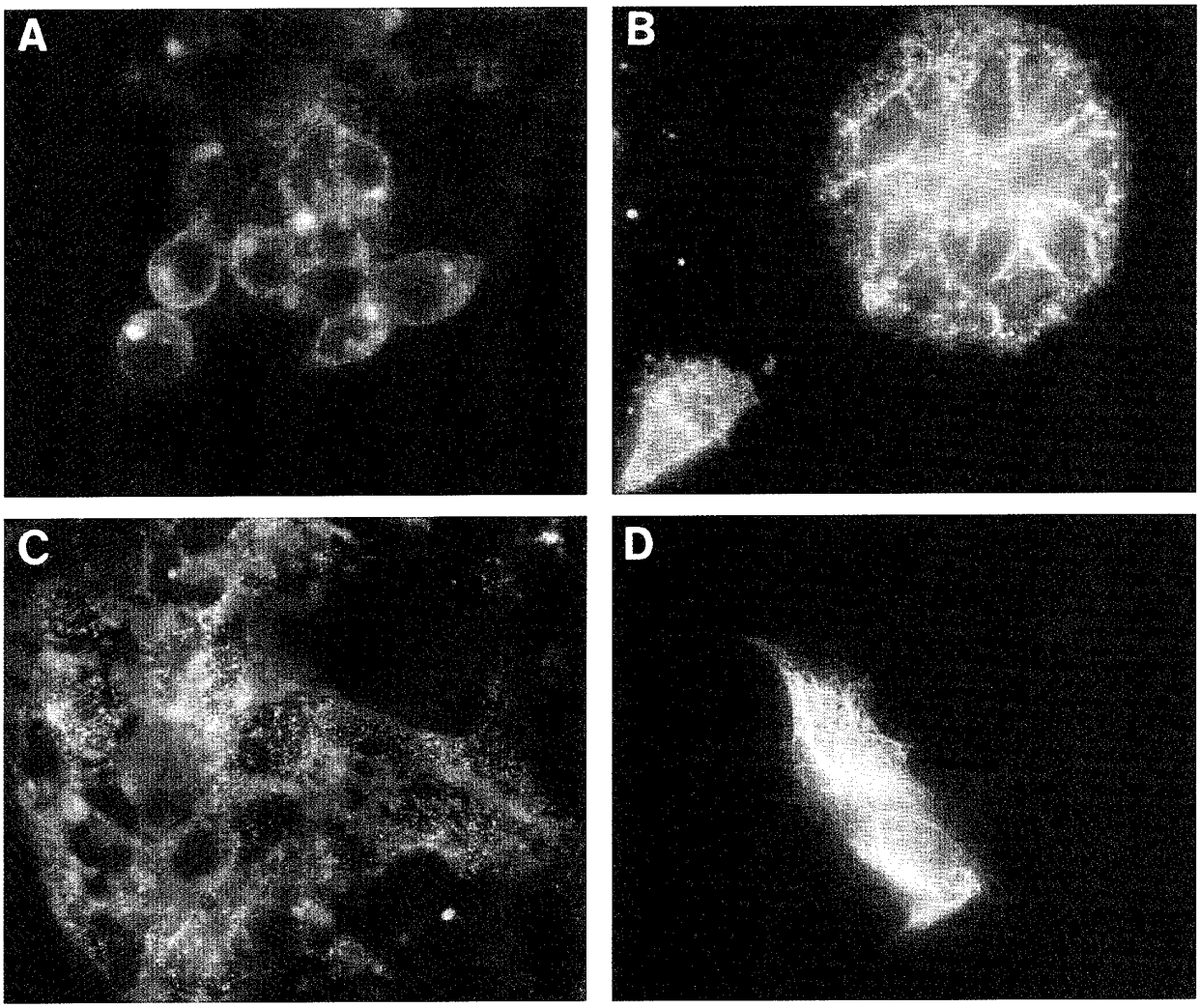

Fig. 5. Detection of CK 8 in viable, unpermeabilized HepG2 human hepatocellular carcinoma cells by immunofluorescence microscopy. (A-C) The cells were incubated with AB 6.01 and secondary antibody prior to fixation. (D) A typical HepG2 cell labeled with AB 6.01 after fixation and permeabilization with 0.02% saponin. The basket-like, perinuclear pattern of intracytoplasmic CK staining in D is readily distinguished from the pattern of cell-surface staining in A-C.

were first performed using AB 6.01 (Fig. 4). A punctate staining pattern was distributed uniformly over the entire surface of every cell. The extent of staining and the pattern were not affected by whether the cells were clustered in aggregates, as in panel A, or isolated, as in panel B. When the primary antibody was omitted, or when nonimmune mouse IgG was substituted for AB 6.01, only the inherent autofluorescence of the hepatocytes was detected (panel C). Similar punctate staining was observed using antibody PCK-26 (results not shown), which is specific for CK 8 in hepatocytes, since these cells do not express CK 5 or CK 6 (Moll et al., 1982; Franke et al., 1981a,b).

Since hepatocytes might be slightly damaged during isolation for primary culture, the immunofluorescence microscopy studies were extended by studying cell lines. Cultures of HepG2 cells were studied 48 hours after transfer to coverslips using AB 6.01. The labeling was similar to that observed with hepatocytes, except for the slightly finer punctate pattern in the HepG2 cells (Fig. 5). In panels A and B, the plane of focus is low near the coverslip. Therefore, the immunofluorescence is seen in association with the edges of the cells, and at cell-cell contacts. In panel C, the plane of focus is near the apical surfaces of some cells, so that the punctate pattern of plasma membrane staining is more apparent. No immunofluorescence was observed when primary antibody was omitted. For comparison, some HepG2 cells were permeabilized with 0.02% saponin prior to incubation with primary antibody. The typical basket-like pattern of intracellular cytokeratin staining (panel D) was significantly different from that observed with viable, unpermeabilized cells.

Many of the previous conflicting studies regarding cell-surface CK 8 were performed with cultures of breast carcinoma cells (Godfroid et al., 1991; Donald et al., 1990). Therefore, we examined surface exposure of CK 8 in BT20 and MCF-7 breast carcinoma cells. Fig. 6 presents a composite of experiments performed with BT20 cells. AB 6.01 was used in panels A and C; M20 was used in panel B. The pattern of staining was comparable to that observed with the HepG2 cells and

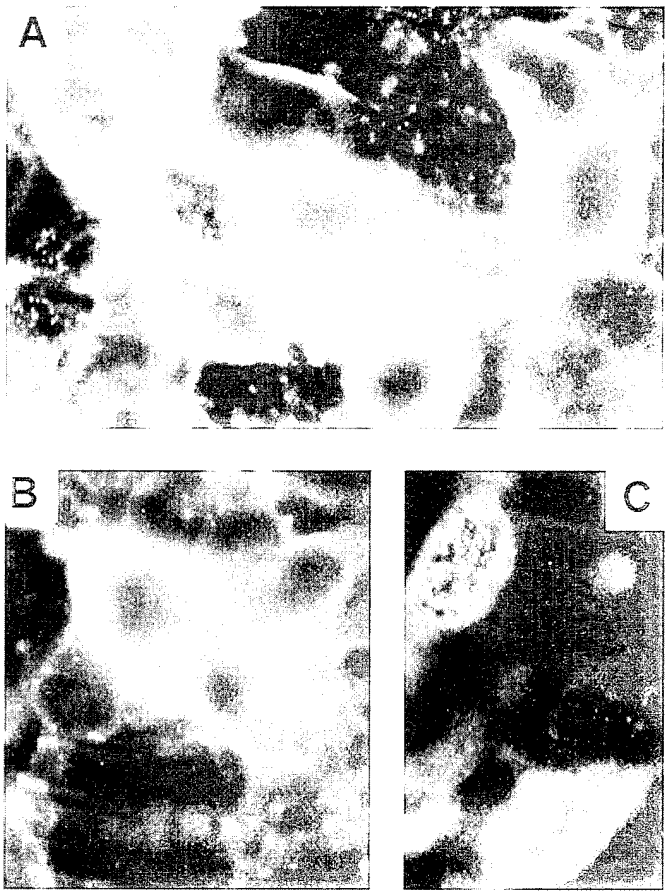

Fig. 6. Detection of CK 8 in viable, unpermeabilized BT20 human breast carcinoma cells by immunofluorescence microscopy. BT20 cells were incubated with AB 6.01 in A and C, or with M20 in B, followed by FITC-conjugated secondary antibody. All cells show diffuse punctate cell surface staining. In A and B, the plane of focus is near the coverslip to demonstrate peripheral, plasma-membrane-associated immunofluorescence. In C, the plane of focus is near the apical surface of the cell and shows the fine punctate pattern of immunofluorescence.

hepatocytes. The plane of focus in panels A and B is low, near the coverslip, demonstrating the plasma membrane distribution of immunofluorescence. Panel C shows punctate staining of apical cell surfaces. Equivalent results were obtained with MCF-7 cells (results not shown). Staining was not observed when primary antibody was omitted or when primary antibody was replaced with nonimmune mouse IgG. Furthermore, the pattern and intensity of staining remained unchanged when the cells were fixed prior to addition of secondary antibody (results not shown).

As a further control for the immunofluorescence microscopy experiments, MCF-7 and BT20 cells were examined using monoclonal antibody directed against the intracellular antigen, p120-GAP. Intense perinuclear fluorescence was observed in cells permeabilized with 0.02% saponin. By contrast, unpermeabilized cells demonstrated no immunofluorescence (results not shown).

Immunoelectron microscopy

To confirm that CK 8 or a CK 8-like antigen is associated with the external surfaces of cells, immunoelectron microscopy experiments were performed. Primary antibody and colloidal gold adducts were incubated with viable cells at 4°C. Under these conditions, cell-associated gold should, hypothetically, indicate the presence of cell surface-bound primary antibody. Fig. 7 presents a composite of imaging studies with hepatocytes, HepG2 cells, BT20 and MCF-7 breast carcinoma cells. In A and B, hepatocytes were incubated with PCK-26. Colloidal gold was observed on the cell surfaces, primarily in association with microvilli and adjacent plasma membranes. No colloidal gold was observed in association with the intracellular spaces of the hepatocytes, suggesting that the cellular membranes were intact and that neither the primary antibody nor colloidal gold had access to the large intracellular pools of CK 8. When hepatocytes were incubated with colloidal gold adduct without prior incubation with PCK-26, essentially no colloidal gold was observed in association with the cell surfaces (results not shown).

The HepG2 cells and breast carcinoma cells were studied using AB 6.01. With each of these epithelial cells, the pattern of immunogold labeling was similar. Colloidal gold was localized exclusively to the cell surface and frequently observed in association with microvilli. As shown in C, large amounts of colloidal gold were observed in association with cellular projections near HepG2 cell-cell junctions. Omission of primary antibody consistently eliminated binding of colloidal gold-adduct to the cell surfaces. Clustering of colloidal gold particles, such as that observed in F, should be interpreted with caution, since the cells were exposed to Protein A-colloidal gold prior to fixation. The colloidal gold-adduct may promote clustering of antigen if the antigen is mobile in the plasma membrane.

In order to demonstrate that large amounts of antigen could be detected by immunoelectron microcopy if the cells were permeabilized, MCF-7 cells were incubated with AB 6.01 and Protein A-colloidal gold, post-embedment (H). The colloidal gold is observed in association with the cells, primarily overlying cytoplasm, as expected.

DISCUSSION

The role of plasmin in tumor invasion and other processes that require cellular migration has been studied extensively (Ellis and Dano, 1992; Vassalli et al., 1991). In this and related systems, association of plasminogen with the cell surface is probably critical. While recent studies have made significant advances in identifying important plasminogen receptors on various cells, epithelial cells have received little attention. These cells are important, since a large variety of common malignancies are of the epithelial cell-derived carcinoma family.

In this investigation, the primary plasminogen-binding protein in the plasma membrane fraction of hepatocytes was identified. This protein was CK 8 or a very closely related homologue of CK 8, as suggested by the molecular mass of the protein, the insolubility of the protein in various detergents, the primary sequence in two different regions of the structure, and the reactivity of the protein with an antibody that should be specific for CK 8 in western blots of hepatocyte extracts. The affinity of CK 8 for plasminogen is predicted by the presence

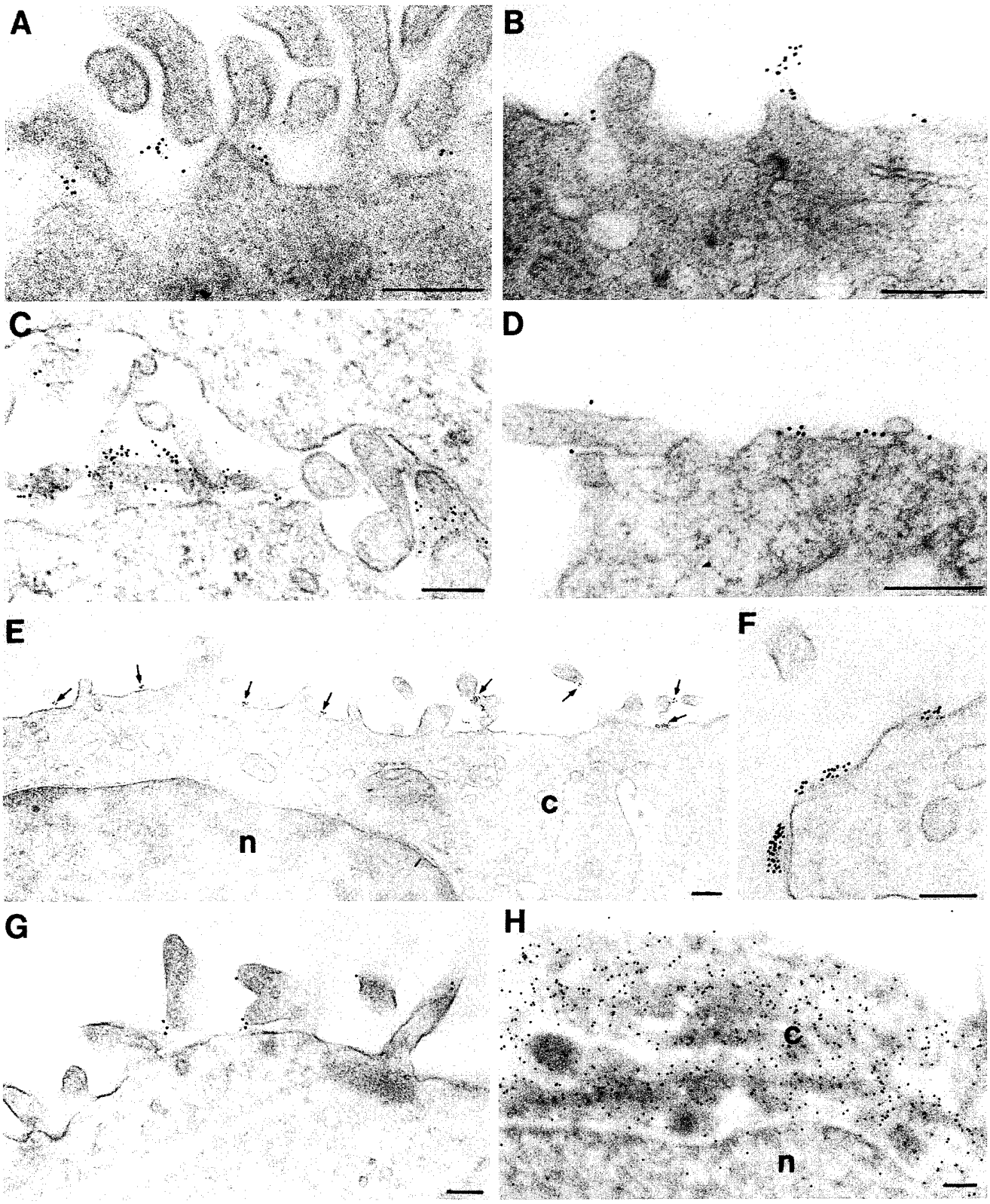

Fig. 7. Immunoelectron microscopy analysis of CK 8 in viable, unpermeabilized rat hepatocytes, HepG2, BT20 and MCF-7 cells. Rat hepatocytes, shown in A and B, were incubated with PCK-26, followed by anti-mouse IgG-colloidal gold adduct. HepG2 cells are shown in C and D, BT20 cells in E and F, and MCF-7 cells are shown in G. The cells in these panels were incubated with AB 6.01, followed by Protein A-colloidal gold adduct. The arrows in E indicate clusters of colloidal gold. In H, MCF-7 cells were incubated with AB 6.01 and Protein A-colloidal gold adduct post-embedment. This procedure exposes intracytoplasmic CK to antibody. c, cytoplasm; n, nucleus. Bars, 200 nm.

of a conserved C-terminal lysine in the CK 8 sequence. Although this is the first description of this novel activity for CK 8, the significance of our observation is dependent on the extent to which plasminogen has access to CK 8.

CK 8 is one of at least 21 related cytokeratins that form intermediate filaments in various epithelial cells and carcinoma cells; however, many cell types express only one acidic (type I) CK and one neutral-basic (type II) CK (Moll et al., 1982). An example is the hepatocyte, which expresses CK 8 and CK 18 (Franke et al., 1981a,b). CK 8-containing intermediate filaments associate extensively with the internal leaflet of the plasma membrane. CK 8 or CK 8 fragments have also been detected in conditioned culture medium (Chan et al., 1986) and in the serum and body fluids of patients with a variety of cancers (Bjorklund and Bjorklund, 1983). It is not clear whether the released CK in vivo is derived primarily from viable or dying cells. Nevertheless, this pool of CK 8 is present in physiological spaces that contain plasminogen. Therefore, it is feasible that CK 8 functions as a plasminogen-binding protein in vivo.

Previous studies have provided evidence for the presence of CK 8 on the external surfaces of cultured breast cancer cell lines (Godfroid et al., 1991; Donald et al., 1991). This association may be accomplished in several ways. It is feasible that CK 8 projects through the plasma membrane as part of a protein complex so the CK 8 does not require a transmembrane domain. CK 8 may associate with the plasma membrane after secretion by cells (Riopel et al., 1993). It has also been demonstrated that CK 8 covalently binds to plasma membrane lipids (Asch et al., 1993); these covalent conjugates may conceivably translocate to the outer membrane leaflet during normal membrane shedding. Our further investigation of this issue was prompted by the work of Riopel et al. (1993), which suggested that detection of cell-surface CK is artifactual. These investigators presented a convincing argument regarding the inadequacy of methods such as cell surface radioiodination for the demonstration of cell surface CK 8. Using such methods, even a rare injured or permeabilized cell might confound the data, since the amount of intracellular CK 8 greatly exceeds that which might be present on the cell surface.

The immunofluorescence microscopy studies presented here are the first of their kind with hepatocytes, HepG2 hepatocellular carcinoma cells and BT-20 breast carcinoma cells. MCF-7 cell immunofluorescence microscopy studies using antibody 6.01 were reported previously by Donald et al. (1991). We chose immunofluorescence microscopy for antibody detection instead of other techniques, such as FACS analysis, so that adherent cultures might be studied without using potentially damaging techniques to release cells. Our results suggest a similar pattern of punctate cell surface CK 8-antigen detection with each of the cell types examined. The protocols used in the immunofluorescence microscopy studies were designed so as to minimize the possibility of cellular injury or death. The uniform pattern of immunofluorescence through the entire population of cells in each preparation demonstrates that the antibody-accessible CK 8 is not contributed by a small subpopulation of damaged cells. Furthermore, the punctate pattern of fluorescence in the intact cells is readily distinguishable from that observed with permeabilized cells. Overall, three separate antibodies were used in the immunofluorescence studies with equivalent results: PCK-26 with hepatocytes; AB 6.01 with hepatocytes, HepG2 cells and breast carcinoma cells; and M20 with breast carcinoma cells. The use of multiple antibodies with well-characterized specificities minimizes the chance that we are detecting a cell-surface protein that is completely unrelated to CK 8. Furthermore, our western blot analyses of whole cell extracts from each of the studied cell types confirmed the expected specificities for each antibody (PCK-26, M20, AB 6.01) (results not shown). Therefore, the immunofluorescence studies strongly suggest the presence of CK 8 or a CK 8-like protein on the surfaces of viable epithelial cells.

Immunoelectron microscopy experiments were performed to confirm that our methods detect cell-surface antigen and not intracytoplasmic antigen. As with the immunofluorescence studies, electron microscopy revealed a similar pattern of antigen expression in all of the epithelial cells studied. Although colloidal gold was distributed across the entire cell surface, we always observed gold preferentially associated with microvilli and the plasma membranes adjacent to microvilli. The pattern of antigen detection by immunogold reported here in hepatocytes, HepG2, and breast carcinoma cells is similar to that reported by Donald et al. (1991) in squamous cell carcinoma.

Our finding that a cytoskeletal protein is located on the cell surface where it may function as an important receptor is not unique. α-Actin has been reported to be present on the surfaces of multiple cell types, including fibroblasts, smooth muscle cells and endothelium (Chen et al., 1978; Jones et al., 1979; Moroianu et al., 1993). Endothelial cell-surface α-actin may function as a receptor for angiogenin and thereby regulate angiogenesis (Moroianu et al., 1993).

The studies presented in this investigation do not permit a determination of the extent to which extracellular CK 8 contributes to the total specific plasminogen-binding capacity of the various cell types. The CK 8-specific antibodies used in this study were of the IgG_1 subtype and therefore all have carboxyl-terminal lysine residues. Although we have performed experiments demonstrating that M20 and PCK-26 substantially inhibit plasminogen binding to each of the cells under investigation (AB 6.01 was a weaker inhibitor, results not shown), Ellis and Dano (1993) demonstrated that IgG_1 antibodies bind plasminogen. Therefore, our results may be explained by antibody binding to plasminogen receptors on the cell surface or by plasminogen-antibody complex formation in solution. To resolve this problem, we are currently attempting to raise monoclonal antibodies specific for the C terminus of CK 8. If an appropriate hybridoma can be obtained, competition binding studies will be performed with Fab fragments.

Numerous studies have shown high levels of CK 8 in malignant cells. In transitional cell carcinoma and squamous cell carcinoma, CK 8 and CK 18 have been detected at increased levels by immunohistochemistry at the tumor invasion front (Schaafsma et al., 1991,1993). The most tumorigenic clones of SW 613-S cells express the highest levels of CK 8 mRNA (Modjtahedi et al., 1992). CK 8 is aberrantly induced in H-*ras* transformed epidermal cells (Diaz-Guerra et al., 1992). Furthermore, mouse L fibroblasts, which lack CK 8 and 18, show increased motility and penetration of Matrigel in vitro after transfection with CK 8 and CK 18 DNAs (Chu et al., 1993). Motility and Matrigel penetration are processes which are dependent on the cell-surface tumor invasion pro-

teinase cascade. Whether cells that express increased levels of CK 8 show an incremental increase in cell surface CK 8 which, by binding plasminogen, supports or accelerates cellular migration and invasion is currently under investigation.

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