

# Induction of Cell Migration by Pro-urokinase Binding to Its Receptor: Possible Mechanism for Signal Transduction in Human Epithelial Cells

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**Abstract.** A human epithelial cell line, WISH, and a mouse cell line, LB6-uPAR, transfected with the human urokinase receptor (uPAR), both expressed high affinity uPAR but undetectable levels of urokinase (uPA). In two independent assays, binding of exogenous pro-uPA produced an up to threefold enhancement of migration. The migration was time and concentration dependent and did not involve extracellular proteolysis.

This biologic response suggested that uPAR can trigger an intracellular signal. Since this receptor is a glycosyl-phosphatidylinositol-linked protein, we postulated that it must do so by interacting with other proteins, among which, by analogy to other systems, would be a kinase. To test this hypothesis, we carried out a solid phase capture of uPAR from WISH cell lysates using either antibodies against uPAR or pro-uPA adsorbed to plastic wells, followed by *in vitro* phosphorylation of the immobilized proteins. SDS-PAGE and autoradiography revealed two phosphorylated protein bands of 47 and 55 kD. Both proteins were phosphorylated on serine residues. Partial sequence of the two proteins showed a 100% homology to cytokeratin 18 (CK18) and 8 (CK8), respectively. A similar pattern of phosphorylation was obtained with lysates from A459 cells, a lung carcinoma, but not HL60, LB6-uPAR or HEp3 cell lysates, suggesting that the

identified multiprotein uPAR-complex may be specific for simple epithelia. Moreover, immunocapture with antibody to another glycosyl-phosphatidylinositol-linked protein, CD55, which is highly expressed in WISH cells, was ineffective. The kinase was tentatively identified as protein kinase C, because it was inhibited by an analogue of staurosporine more specific for PKC and not by a PKA or tyrosine kinase inhibitors. The kinase was tentatively identified as PKC $\epsilon$  because of its resistance to PMA down-modulation, independence of Ca<sup>2+</sup> for activity, and reaction with a specific anti-PKC $\epsilon$  antibody in Western blots. Cell fractionation into cytosolic and particulate fractions revealed that all four proteins, the kinase, uPAR, CK18, and CK8, were present in the particulate fraction.

*In vivo*, CK8, and to a lesser degree CK18, were found to be phosphorylated on serine residues. Occupation of uPAR elicited a time-dependent increase in the phosphorylation intensity of CK8, a cell shape change and a redistribution of the cytokeratin filaments.

These results strongly suggest that uPAR serves not only as an anchor for uPA but participates in a signal transduction pathway resulting in a pronounced biological response.

**M**IGRATION is a function of both normal cells carrying out physiologic functions such as fertilization, embryogenesis, angiogenesis, and defense mechanisms and of malignant cells during the metastatic process. A substantial body of evidence implicates urokinase-type plasminogen activator (uPA)<sup>1</sup> in cell migration (for review

see Blasi, 1993). uPA is produced and secreted by most cells in the form of a single chain proenzyme (pro-uPA) which is activated by a single proteolytic cleavage. uPA converts plasminogen, a zymogen present in high concentration in plasma and other body fluids, into plasmin (for review see Dano et al., 1985; Vassalli et al., 1991). This latter serine protease can, directly, or indirectly through activation of metalloprotease zymogens, cause the degradation of most extracellular matrix proteins, thought to be a prerequisite for cell migration and tissue remodeling (for review see Mignatti and Rifkin, 1992). Indeed, our initial studies (Ossowski et al., 1975) indicated that PA participated in the migratory process by plasmin generation. Moreover, expression of uPA was found in migrating cells at the edge of wounded monolayers of endothelial cells (Pepper et al., 1987) and at focal

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1. *Abbreviations used in this paper:* Ab, antibody; GPI, glycosyl phosphatidylinositol; KIU, kallikrein international unit; Plg, plasminogen; PKC, protein kinase C; PVDF, polyvinylidene difluoride; pro-uPA, proenzyme uPA; uPA, urokinase-type plasminogen activator; uPAR, receptor for uPA.

adhesion sites in fibroblast and some tumor cell cultures (Pollanen et al., 1988; Hebert and Baker, 1988). Enhanced expression of uPA has been shown in migrating keratinocytes (Morioka et al., 1987). In addition, these cells possess a specific high affinity receptor for uPA (uPAR) which is also modulated during cell migration (McNeill and Jensen, 1990). It is likely that the migratory properties of these cells are conferred, at least in part, through the expression of this receptor.

uPAR has a very high affinity for the growth factor domain of uPA, located in the amino-terminal fragment of uPA A chain, thus binding both pro-uPA and active uPA independently of the catalytic site (Vassalli et al., 1985; Cubellis et al., 1986; Appella et al., 1987). uPAR, a highly glycosylated 313-amino acid polypeptide (Roldan et al., 1990), is covalently attached to the plasma membrane via a COOH-terminal glycosyl-phosphatidylinositol (GPI) anchor (Ploug et al., 1991).

There is published evidence linking uPA binding to its specific receptor with enhanced chemotaxis. Active uPA, uPA inhibited by diisopropylfluorophosphate and/or the amino-terminal fragment of uPA, stimulated the migration of human neutrophils (Gudewicz and Gilboa, 1987), bovine adrenal capillary endothelial cells (Fibbi et al., 1988), and a human epidermal cell line (Del Rosso et al., 1993).

In the present study we showed that in a cell line derived from simple epithelium, ligation of uPAR results in enhancement of chemokinesis. Since this interaction does not involve extracellular proteolysis, and since uPAR is a GPI-linked protein, we postulated that intracellular signal delivery must proceed through interaction with other components. We now describe the identification of some of the proteins which may be a part of the signal-relaying cascade, and the biochemical changes which may be causally linked to induction of migration.

## Materials and Methods

**Materials.** Tissue culture medium, glutamine, and antibiotics were from GIBCO BRL (Grand Island, NY), trypsin from Sigma Chem. Co. (St Louis, MO), FBS from JRH Biosciences (Lenexa, KS), and plasminogen (Plg)-free FBS obtained after two passages of the serum through a lysine-Sepharose column. Tissue culture plates were from Falcon (Lincoln Park, NJ), cytodex 2 microcarrier beads from Pharmacia LKB (Piscataway, NJ), aprotinin from ICN (Irvine, CA), protein kinase C (PKC) inhibitor (GFI09203X) from Calbiochem-Novabiochem (LaJolla, CA), other protease inhibitors from Boehringer-Mannheim Corp. (Indianapolis, IN). Microtiter well strips were from Dynatech Laboratories, Inc. (Chantilly, VA). Recombinant pro-uPA was a gift from Abbott Laboratories (Abbott Park, IL) and EGF was from Collaborative Research, Inc. (Bedford, MA). Murine mAb against pro-uPA (IgG1 and IgG2a) were purified from hybridoma conditioned medium. Murine mAb against human uPAR (R2) was kindly provided by Dr. K. Dano (Finsen Laboratory, Copenhagen, Denmark), murine moAb (IgG2a) against CD55, prepared by M. Davitz (New York University Medical Center, NY) was a generous gift from Dr. M. Fein (Mount Sinai School of Medicine, NY). Goat and mouse anti-human uPAR antibody (Ab) and uPA ELISA were from American Diagnostica Inc. (Greenwich, CT); anti-phosphotyrosine mAb from UBI (Lake Placid, NY); anti-cytokeratin 8 and 18 mAbs, and biotinylated Ab from Sigma Chem Co.; rabbit anti-protein kinase C $\epsilon$  antibody from GIBCO BRL; goat anti-rabbit Fc and IgG from Cappel (West Chester, PA); control mouse Ab (IgG1 and IgG2a) from Coulter Immunology (Hialeah, FL); and streptavidin-FITC and rhodamine-labeled anti mouse Ab from Amersham Corp. (Arlington Heights, IL). Isotopic compounds were from Du Pont-NEN (Boston, MA). Polyvinylidene difluoride (PVDF) and nitrocellulose membranes were from Millipore Corp. (Bedford, MA).

**Iodination of Pro-uPA.** Pro-uPA was iodinated using  $^{125}\text{I}$  and Iodogen to a specific activity of  $5 \times 10^6$ – $10^7$  cpm/ $\mu\text{g}$  of protein.

**Cells.** Mouse LB6 cells transfected with human uPAR cDNA (LB6-uPAR) have been previously described (Roldan et al., 1990; Ossowski et al., 1991). Human amnion WISH cells, A459 lung carcinoma cells, and HL60 cells were obtained from the American Type Culture Collection (Rockville, MD), HEp3 cells were as previously described (Ossowski and Reich, 1980). All cells except HL60, which were grown in RPMI, were grown as monolayers in DME with 10% FBS, streptomycin (200 mg/l), and penicillin ( $5 \times 10^5$  U/l).

**Migration Assays.** Microcarrier beads assay: cytodex 2 microcarrier beads were prepared and the migration assay performed as described by Rosen et al., 1990, with some modifications. Briefly,  $2 \times 10^6$  WISH cells were mixed into a 100-mm plastic Petri dish in 10 ml DME containing 10% FBS with 50 mg of beads and incubated for 3 d. Cell-coated beads were washed twice with DME 5% FBS and once with DME containing 3% of plasminogen-free FBS and 100 kallikrein international units (KIU)/ml aprotinin. Cells on beads were counted as described (Rosen et al., 1990) and the bead suspension adjusted to  $2 \times 10^5$  cells/ml in DME containing 3% Plg-free FBS, 100 KIU/ml aprotinin and pro-uPA where indicated in the figure legend. 0.5-ml aliquots of bead suspensions were incubated in a 24-well plate at 37°C. After 24 h, the plates were washed three times with PBS to remove beads and cells that have migrated from the beads to the plastic of the well were fixed with 1% glutaraldehyde in PBS for 10 min and subsequently stained with 1% crystal violet in water for 10 min. After several washes with H<sub>2</sub>O the colorant associated with the cells was removed with methanol/acetic acid/H<sub>2</sub>O (30:10:60, vol/vol/vol) and the associated optical density read at 570 nm. Wells incubated in parallel but without cells, were stained and washed and served as a background for the determination of optical density. The number of cells/well in the range of 200–1,000 was directly proportional to OD units. Wounding assay: wounding assay was previously described (Ossowski et al., 1975). Briefly, wounded confluent cell cultures were incubated in DME with 3% Plg-depleted FBS and 100 KIU/ml of aprotinin (as described above). The cultures were stained at different times after wounding and the number of migrating cells along a wound edge of 8 to 12 mm was counted by microscopic inspection.

**Determination of Pro-uPA-binding Sites.** Cells plated at  $1 \times 10^5$  per well were grown for 24–48 h, washed in serum-free medium and incubated with increasing concentrations of  $^{125}\text{I}$ -pro-uPA for 90 min at 4°C. Specific binding was determined by subtracting the radioactivity bound in presence of a 100-fold excess of nonradiolabeled pro-uPA. The data were analyzed by Scatchard transformation using the SCATFIT computer program (Munson and Rodbard, 1980).

**uPA Activity and Content.** The uPA activity was determined in conditioned media and cell lysates using chromogenic assay for plasmin as previously described (Mira-y-Lopez and Ossowski, 1987). uPA antigen was measured by ELISA according to manufacturer instructions. Alternatively, cells were metabolically labeled with [ $^3\text{S}$ ]Methionine in 95% methionine-free medium containing 1 mg/ml BSA for 20 h, followed by immunoprecipitation with specific monoclonal anti uPA antibodies and autoradiography.

**Cell Lysate Preparation.** Cells from confluent monolayers were washed with PBS and then scraped in PBS containing 1 mM EDTA. The cell pellets ( $\sim 10^7$  cells) were then resuspended in 1 ml lysis buffer (10 mM Tris-HCl, pH 8.1, 140 mM NaCl, 2 mM EDTA, 1 mM PMSF, 100 KIU/ml aprotinin, 0.1 mg/ml leupeptin, and 1% NP-40 or Triton X-100) and extracted at room temperature. In some experiments, cell suspensions were incubated for 90 min at 4°C in DME containing 20 mM Hepes and 1 mg/ml BSA in the presence or absence of pro-uPA, before cell lysis. For sample preparation for Western blotting with anti-phosphotyrosine mAb, the phosphatase inhibitors NaF and Na<sub>3</sub>VO<sub>4</sub> were added to the lysis buffer at 50 mM and 1 mM final concentrations, respectively. After centrifugation at 3,400 rpm for 10 min the supernatants were collected and assayed. In some experiments the cell lysates were centrifuged for 10 min at 100,000 g and the supernatants were used for in vitro phosphorylation.

**Capture of uPAR.** 100  $\mu\text{l}$  of immune or nonimmune gammaglobulins at 10–50  $\mu\text{g}/\text{ml}$ , or pro-uPA at 50  $\mu\text{g}/\text{ml}$ , in PBS were added to microtiter wells and incubated for 2 h at room temperature, or overnight at 4°C. To block nonspecific protein-binding sites, the wells were then coated with 1% BSA in PBS for 2 h at room temperature. The wells were washed three times with PBS containing 1% NP-40. A 100- $\mu\text{l}$  aliquot of cell lysate was added to each well and incubated for 2 h at room temperature or overnight at 4°C. More consistent results were obtained with a 2-h incubation. The cell lysates were then removed and the wells were washed five times with PBS containing 1% NP-40.

**In Vitro Phosphorylation.** Following uPAR capture, the proteins immobilized in the wells were incubated with 50  $\mu\text{l}$  of a reaction mixture containing 25 mM Hepes pH 7.2, 3 mM MnCl<sub>2</sub>, 0.1% NP-40 and 1–5  $\mu\text{Ci}$  of [ $\gamma$ - $^{32}\text{P}$ ] ATP. After incubation for 5 min at room temperature, proteins

were eluted in sample buffer containing 2% SDS and 10% glycerol and subjected to a 10% SDS-PAGE. The gel was dried and autoradiographed.

**In Vivo Phosphorylation.** Semi-confluent monolayers of WISH cells were washed with phosphate-free medium containing 1 mg/ml BSA and 100 KIU/ml aprotinin, labeled to equilibrium with 100  $\mu\text{Ci/ml}$  [ $^{32}\text{P}$ ]orthophosphate in the same medium, and incubated for 15–120 min before the end of labeling with  $10^{-8}\text{M}$  of pro-uPA. All cultures, including control cultures, were washed extensively, lysed in lysis buffer (prepared as above but supplemented with 50 mM NaF, 20 mM Na pyrophosphate, 1 mM  $\text{Na}_3\text{VO}_4$ , and 2 mM PMSF added immediately before use) for 20 min at room temperature, centrifuged at low speed, and then aliquots of the supernatants, containing equal amounts of radioactivity, were incubated for 2 h at  $4^\circ\text{C}$  with the appropriate antibodies, followed by 30-min incubation with protein A–Sepharose. After extensive washing, the Sepharose beads were eluted in  $2 \times$  sample buffer and heated for 5 min at  $95^\circ\text{C}$ . The samples were electrophoresed in 10% SDS-PAGE, and autoradiographed. The bands were scanned with an LKB Ultrascan XL Enhanced Laser Densitometer.

**[ $^{32}\text{P}$ ]Phosphoamino Acid Analysis.** By superimposing the autoradiogram over the gel, areas of interest were identified and the phosphoproteins were cut out of the dried gel. Gel segments were rehydrated and digested with 0.1 mg/ml trypsin in 50 mM *N*-ethylmorpholine at pH 8. The lyophilized tryptic peptides were hydrolyzed with 6 N HCl, 1 mg/ml phenol for 30 min at  $150^\circ\text{C}$ . [ $^{32}\text{P}$ ]phosphoamino acids were separated by thin layer high voltage electrophoresis at pH 3.5 (Cooper et al., 1983).

**Western-blot Analysis.** 75  $\mu\text{l}$ -samples of cell lysates were electrophoresed on a 10% SDS-PAGE under nonreducing conditions and electroblotted to nitrocellulose sheets which were subsequently blocked with 1% BSA in TBST buffer (10 mM Tris–HCl, pH 8.1, 150 mM NaCl, 0.05% Tween-20). Phosphotyrosyl-containing proteins were detected using anti-phosphotyrosine mAb at 1  $\mu\text{g/ml}$  followed by  $^{125}\text{I}$ -protein A at 0.2  $\mu\text{Ci/ml}$  in TBST buffer. Other proteins were detected using specific mAb at 2  $\mu\text{g/ml}$  and revealed by alkaline phosphatase reaction.

**Cell Fractionation.** Cells were washed with ice-cold PBS and the cytosolic and particulate fractions were prepared by lysing the cells (using  $10^7$  cells/ml buffer) in ice-cold hypotonic buffer containing 20 mM Hepes, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 100 KIU/ml aprotinin, 1 mM PMSF, 0.1 mg/ml leupeptin, and 25 mM sucrose. Cells were homogenized on ice using a Dounce glass homogenizer and the nuclei removed by a low speed spin (1,000 rpm for 5 min). The homogenate was centrifuged at 100,000 *g* for 10 min at  $4^\circ\text{C}$ . The supernatant (cytosolic fraction) was collected and the pellet (particulate fraction) was resuspended in the same volume and the same buffer as the cytosolic fraction.

**Protein Sequence Analysis.** Solid phase immunoprecipitation was performed as described above using 96 wells coated with polyclonal goat anti-uPAR antibodies. Immunoprecipitates from 16 wells were sequentially eluted in sample buffer and loaded in one lane of a 10% polyacrylamide gel (6 lanes in all). After SDS-PAGE the samples were transferred to a PVDF membrane and stained with 0.1% amido black in 10% acetic acid. The stained proteins were cut off and microdigested (Fernandez et al., 1992). Internal sequence analysis was done by automated Edman degradation.

**Immunofluorescence.** WISH cells were plated at  $0.5\text{--}1 \times 10^5$  cells/ml and grown on coverslips for 18–36 h, then fixed briefly with 2% paraformaldehyde in PBS. Aldehyde-induced fluorescence was quenched with 50 mM  $\text{NH}_4\text{Cl}$ . Immunodetection was performed using antibodies diluted in PBS with 0.2% BSA. When required, cells were permeabilized with 0.1% Triton X-100. Specifically, we used as primary antibodies mouse mAb anti-uPA, anti-uPAR, anti-CK8, or CK18, which were visualized in a two step process with biotinylated anti-mouse IgG, followed by streptavidin-FITC. We evaluated in the same cell the relationship between uPAR and/or CKs by performing a second series of immunolocalization steps with the second mAb detected by rhodamine-labeled anti-mouse IgG. Cells were viewed and photographed with Kodak TMAX 3200 using a Zeiss Axiomat microscope equipped for epifluorescence and differential interference contrast (DIC, Nomarsky optics). Alternatively, to evaluate possible colocalization we performed simultaneous double wavelength scanning with a confocal laser scanning microscope (Leica, Inc., Deerfield, IL) at a magnification  $\times 63$  and photographed on Ektachrome 100. Each experimental condition was repeated at least three times.

## Results

### Characterization of uPAR and of uPA Content

An epithelial cell line (WISH), derived from human amnion, was examined for its ability to bind  $^{125}\text{I}$ -labeled pro-uPA.

Based on Scatchard analysis of three individual experiments the  $K_d$  for uPA/uPAR interaction was determined to be  $2.3 (\pm 0.8) \times 10^{-10}$  M (mean  $\pm$  SD). The mean number of receptors per cell was  $7.1 (\pm 1.4) \times 10^4$ . The  $K_d$  and the number of uPAR sites in LB6-uPAR cells was previously shown to be  $1.7 \times 10^{-10}$  M and  $1.2 \times 10^4$ , respectively (Ossowski et al., 1991). Brief treatment with isotonic buffer at pH 3, known to strip receptors of their endogenous ligands (Stoppelli et al., 1986), did not change receptor number or its affinity, thus indicating that all receptors in both cell lines were free of endogenous uPA. Indeed, cell-associated and secreted uPA were below the level of detection by uPA immunoprecipitation of biosynthetically labeled cells, uPA ELISA and PA activity assay or by immunocytochemistry (data not shown). In conclusion, WISH and LB6-uPAR cells, which express uPAR but no detectable uPA, constitute a suitable model to study the effect of exogenously added uPA.

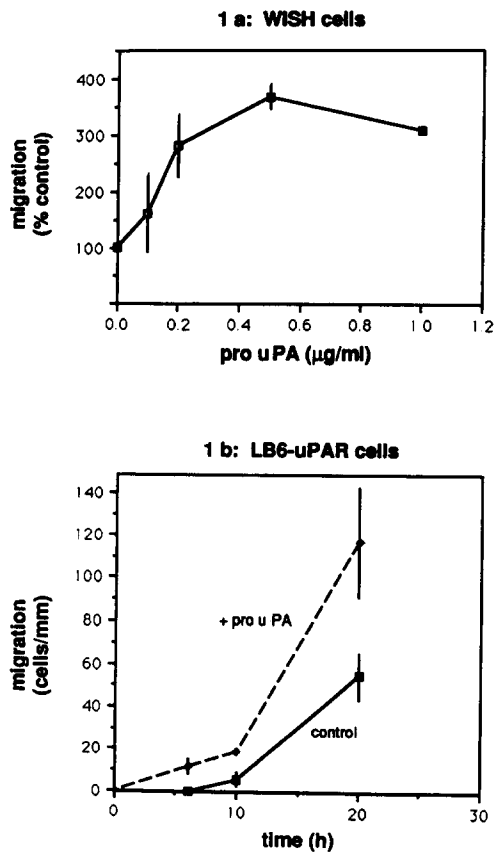
### Effect of Pro-uPA Binding to uPAR on Cell Migration

To examine the effect of exogenous pro-uPA on cell migration we used two cell lines and two different migration assays; migration into an area denuded of cells (“wound”), and migration from the surface of beads to the surface of a culture dish. We noticed that under basal conditions WISH cells migrated four times more efficiently than LB6-uPAR cells when tested in the bead assay (data not shown). In contrast, WISH cells were extremely inefficient in migrating into a wound.

As shown in Fig. 1 *a*, pro-uPA-stimulated migration from beads of WISH cells in a dose-dependent way, reaching a plateau at 0.2  $\mu\text{g/ml}$  with a threefold stimulation. This effect was time and cell number dependent (24 h of incubation and  $10^5$  cells per sample being the best parameters, data not shown). Enhancement of migration required neither active uPA, nor plasmin, since it occurred in medium containing Plg-free serum and aprotinin, a plasmin inhibitor. Moreover, radiolabeled pro-uPA, incubated under conditions of the migration assay, and analyzed in a reduced SDS-PAGE and autoradiography, remained in a single chain form (data not shown). Similar enhancement of migration was obtained in confluent cultures of LB6-uPAR preincubated with medium supplemented with 0.2  $\mu\text{g/ml}$  of pro-uPA, wounded and stained after 6, 10, and 20 h of incubation. As shown in Fig. 1 *b*, cells exposed to pro-uPA migrated more efficiently than controls at every time point examined. In a parallel experiment, exogenous pro-uPA did not affect migration of mouse LB6 cells expressing human uPA (Ossowski et al., 1991), which was  $74.9 (\pm 8)$  cells per mm in control medium and  $72.8 (\pm 10.6)$  cells per mm in medium supplemented with pro-uPA.

uPA has been reported to trigger a mitogenic response in the absence of any uPA-mediated proteolytic activity (Rabani et al., 1992). We excluded the possibility that the increased number of migrating cells was a reflection of an increased cell number due to pro-uPA treatment showing that under conditions determined to be optimal for cell migration, increasing concentrations (up to 0.8  $\mu\text{g/ml}$  for WISH and up to 0.5  $\mu\text{g/ml}$  for LB6-uPAR cells) of pro-uPA did not result in increased DNA synthesis (data not shown).

We judged that WISH cells, in which the receptor, and any protein that might be associated with it, would be of the same species, were better suited for the analysis of signal transduction leading to migration, than were LB6-uPAR cells, which

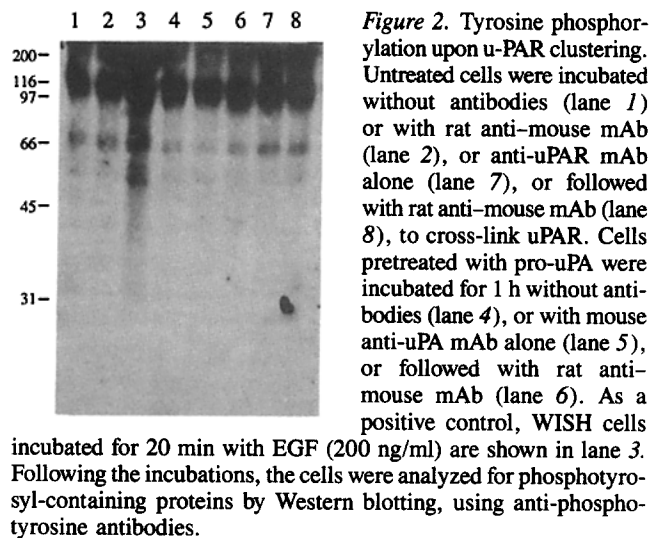


**Figure 1.** Effect of pro-uPA on cell migration. (a) Dose-response curve for pro-uPA-stimulated migration of WISH cells. Migration was measured using the microcarrier bead assay. Cells grown on beads were incubated for 24 h in presence of increasing concentrations of pro-uPA in DB containing 3% plasminogen-free FBS and 100 KIU/ml aprotinin. Results are expressed as % control without pro-uPA (mean  $\pm$  SD of three independent experiments). (b) Time-course of pro-uPA-stimulated migration of LB6-uPAR cells. Migration was measured using the wounding assay. Wounded monolayers were incubated with (broken line) or without (solid line) 0.2  $\mu\text{g/ml}$  of pro-uPA for various length of time. Results are expressed as number of migrating cells/mm (mean  $\pm$  SD of two independent experiments each done in triplicate, and 8–12 mm counted per wound).

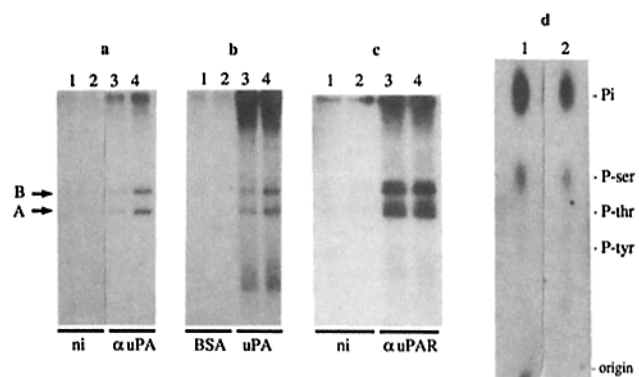
are mouse cells transfected with human uPAR. Thus, WISH cells were used in all subsequent experiments.

### Examination of Tyrosine Phosphorylation Involvement in Signaling Pathways via uPAR

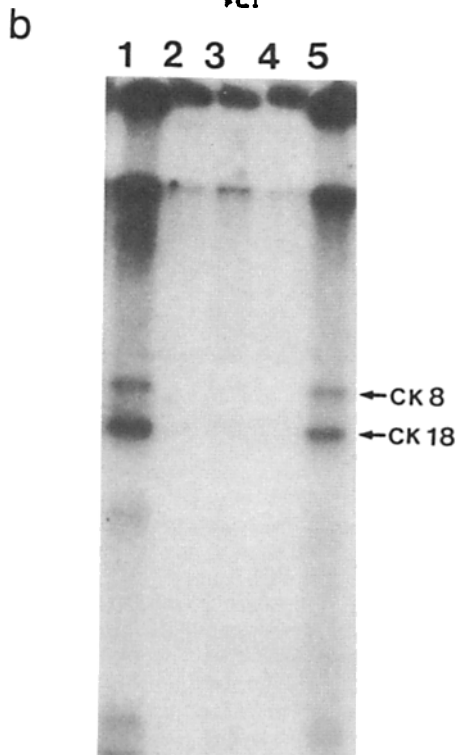
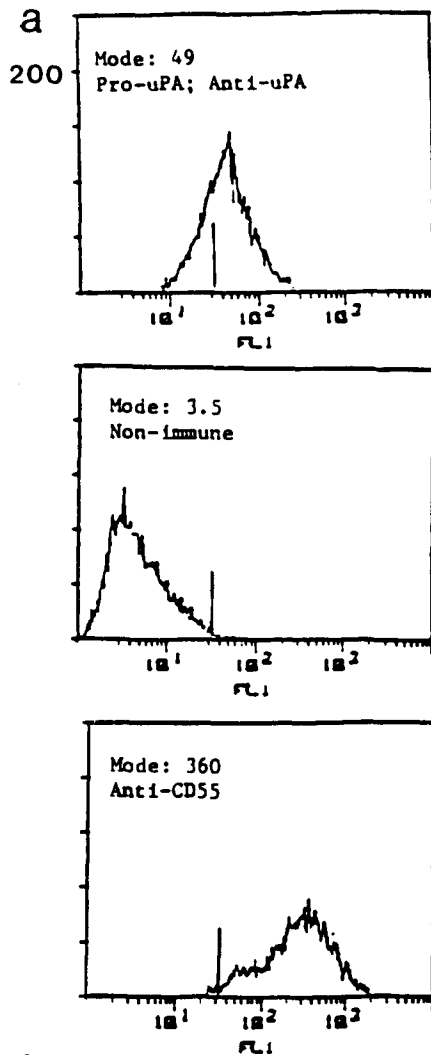
As signal transduction via transmembrane receptors commonly involves tyrosine phosphorylation, including several GPI-anchored proteins (Stefanova et al., 1991; Thomas and Samelson, 1992) and uPAR in the monocytic cell line U937 (Dumler et al., 1993), we tested whether receptor occupancy also induced tyrosine phosphorylation in WISH cells. Cells were preincubated with pro-uPA for various lengths of time (from 5 to 60 minutes) and tyrosine-phosphorylated proteins were visualized by Western blot using anti phosphotyrosine mAb. Binding of pro-uPA was never seen to cause a detectable change in phosphotyrosine levels (Fig. 2, lanes 1 and 4). Receptor clustering, by first exposing the cells to a mouse



uPAR mAb followed by a second, anti-mouse antibody, a condition known to induce tyrosine phosphorylation by several tyrosine kinase-associated receptors (Stefanova et al., 1991), did not consistently affect the pattern of tyrosine-phosphorylated proteins in WISH cells (Fig. 2, lanes 1, 2, and 4–8). By contrast, binding of EGF to its receptor, used as a positive control, led to a pronounced increase of the intensity of phosphotyrosine-containing proteins (compare lanes 1 and 3). Similar results were obtained in three additional experiments in which different methods were used to reveal anti-phosphotyrosine antibody binding (not shown).



are mouse cells transfected with human uPAR. Thus, WISH cells were used in all subsequent experiments.



Inhibition of tyrosine phosphatases by vanadate (Jallal et al., 1992) increased the level of phosphorylated proteins but did not affect the response of WISH cells to either pro-uPA treatment or uPAR cross-linking (not shown).

#### Association of Kinase(s) with uPAR

To capture any kinases and kinase substrates that might be associated with the uPAR, WISH cell lysates were subjected to solid phase immunoprecipitation with polyclonal anti-uPAR antibodies. The precipitates were then reacted with [ $\gamma$ - $^{32}$ P]ATP and the products of this *in vitro* phosphorylation reaction were analyzed by SDS-PAGE and autoradiography. Two bands, one of apparent molecular mass of 47 kD, designated band A, and one of 55 kD, designated band B, were identified (Fig. 3 c, lane 3). Thus, at least one kinase and two substrates were specifically associated with uPAR. Only trace amounts of phosphorylated proteins A and B were seen when lysate proteins, captured with preimmune IgG, were tested (lanes 1 and 2). The association with uPAR was also evident when WISH cells were preincubated with pro-uPA prior to capture with anti-uPAR antibodies which recognize both free and occupied receptors (lane 4), suggesting that, most likely, the association of the kinase with the receptor complex did not depend on receptor occupancy by pro-uPA. Moreover, the same pattern of *in vitro* phosphorylation was obtained when WISH cells were lysed as monolayers or, as has been done in most experiments, first treated with 1 mM EDTA in PBS and detached by scraping, and then lysed.

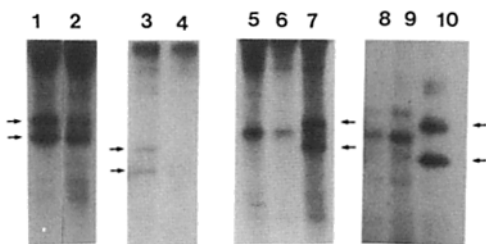
These results were confirmed when anti-uPA antibodies were used to precipitate cell lysates. As expected, since these cells produced no detectable pro-uPA, uPAR and its associated kinase activity were recovered when cells were treated with pro-uPA prior to cell lysate preparation (Fig. 3 a, compare lane 3, without pro-uPA and lane 4, with pro-uPA). While no protein kinase activity was detected in cell lysates incubated in BSA-coated wells (lanes 1 and 2), the pattern of phosphorylated proteins described above was obtained when pro-uPA-coated wells were used to capture the complex (Fig. 3 b). In this latter case, cell lysates from pro-uPA-treated, or -untreated, cells gave similar results (lanes 3 and 4). We hypothesized that some of the pro-uPA may dissociate from the uPAR-complex during cell lysis and incuba-

**Figure 4.** Levels of surface uPAR-bound uPA and CD55 (FACS analysis) in WISH cells and their effectiveness in complex formation by immunocapture with antibodies. (a) Cells detached with PBS/EDTA and preincubated with  $10^{-8}$  M pro-uPA, were washed, resuspended in PBS with 1 mg/ml BSA and  $10 \mu\text{g/ml}$  of anti-uPA IgG2a, nonimmune IgG2a and anti-CD55 IgG2a, incubated for 1 h at  $4^\circ\text{C}$ , washed, and then incubated for 45 min in fluorescein-conjugated rabbit anti-mouse. The cells were washed, resuspended at  $1 \times 10^6$  cells/ml and subjected to cytofluorometric analysis using a FACScan. The data were analyzed by single histogram statistics and the modes are shown for each analysis. Horizontal axes indicate log of fluorescence intensity, and vertical axes cell count. (b) WISH cells incubated with pro-uPA as in a, were lysed and subjected to immunocapture with the following moAbs and *in vitro* phosphorylation. Lane 1, anti-uPA IgG1; lane 2, nonimmune IgG1; lane 3, anti-CD55 IgG2a; lane 4, nonimmune IgG2a; lane 5, anti-uPA IgG2a.

tion, and that the uPAR-complex may then interact with pro-uPA bound to the plastic well. To investigate this, WISH cell lysates (25,000 cpm per well) prepared from cells preincubated with a saturating ( $1.2 \times 10^{-8}$  M) concentration of radioactively labeled pro-uPA were incubated for 2 h in wells coated with either pro-uPA (50  $\mu$ g/ml), goat anti-uPAR, or mouse anti-uPA antibodies; nonimmune goat IgG and BSA served as negative controls. The anti-uPA antibodies captured 6.8% of the radioactive, lysate-associated pro-uPA, the anti-uPAR 1.1%, while the radioactivity associated with pro-uPA-coated wells was equal to background (results not shown). The finding that the anti-uPA-coated wells bind most radioactivity, most likely due to binding of both pro-uPA-uPAR complexes and free radioactive pro-uPA, attests to the fact that some ligand must be dissociating from the complex without substantially affecting the kinase activity.

Phosphoamino acid analysis of proteins A and B indicated that serine was the major labeled residue (Fig. 3 d). Thus, uPAR appeared to be associated with a serine kinase and two potential substrates, proteins A and B.

To test the specificity of the association between uPAR and the multiprotein complex, the phosphorylation pattern produced by antibodies to another GPI-linked protein, CD55, was examined. FACSscan cytofluorometric analysis of WISH cells incubated with pro-uPA, followed either by anti-uPA or anti-CD55 moAbs or nonimmune Ig, and fluorescein-conjugated rabbit anti-mouse IgG, showed that WISH cells expressed on their surface approximately five times more CD55 than uPAR (compare Fig. 4 a, top and bottom). Solid phase immunocapture with these moAbs, followed by in vitro phosphorylation, showed that anti-uPA IgG (Fig. 4 b, lanes 1 IgG1, and 5 IgG2a) produced a pattern of phosphorylation similar to that produced by anti-uPAR antibodies in Fig. 3 c, lanes 3 and 4). In contrast, the intensity of phosphorylation produced by moAbs to CD55, another strongly expressed GPI-linked protein, was only slightly above that of isotype-matched, nonimmune mouse Ig (compare Fig. 4, lanes 3 and 4). Further evidence in support of the specificity of the observed protein phosphorylation pattern was ob-

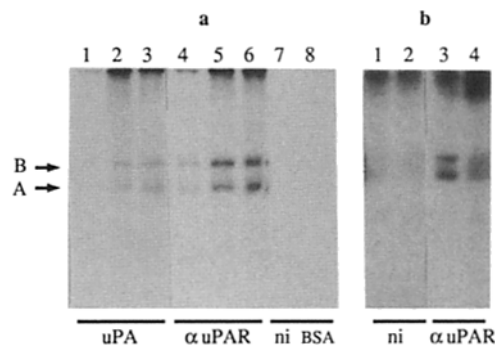


**Figure 5.** In vitro phosphorylation following immunocapture of lysates from different cell lines. In vitro kinase assays were performed on lysates of WISH, LB6-uPAR, A459, HEp3, and HL60 cells. Prior to lysis HL60 cells were incubated in medium with 1 mg/ml BSA without, or with 16 nM PMA. WISH, LB6-uPAR, and HL60 were preincubated for 1.5 h before lysis with  $10^{-8}$  M pro-uPA. HEp3 cells were with and without pro-uPA preincubation. Lanes 2, 3, 7, and 10, WISH cells; lane 1, A459; lane 4, LB6-uPAR; lanes 5 and 6, HL60 without and with PMA; lanes 8, and 9, HEp3 without and with pro-uPA. Antibodies used in immunocapture: lanes 1 and 2, goat anti-uPAR; lanes 3-10, monoclonal mouse anti-uPA IgG1.

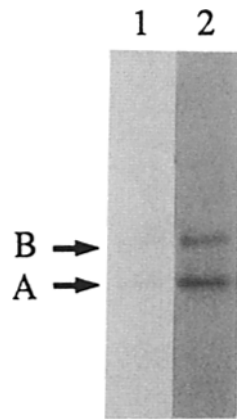
tained from experiments in which uPAR-associated proteins were compared in five different cell lines. Fig. 5 shows that only one other cell line, A459, originating, like WISH cells, in simple epithelium, produced a pattern similar to that of WISH cells. Lysates from squamous epithelial cell line HEp3, LB6-uPAR, and a promyelocytic leukemia HL60, showed different patterns of uPAR moAb-captured, phosphorylated proteins. Incubation of HL60 in PMA (see Fig. 8, lane 6) or HEp3, which produces high levels of endogenous pro-uPA, in absence of pro-uPA (lane 8) did not change the pattern of phosphorylation.

To rule out that uPAR/serine kinase association was not secondary to cell lysis by detergents, cytosolic and particulate fractions of WISH cells were prepared and tested for kinase activity after uPAR antibody capture as before (Fig. 6 a). Phosphorylated A and B proteins were recovered almost exclusively in the particulate fraction (compare cell homogenate prior to fractionation, Fig. 6 a, lanes 3 and 6, with particulate fraction, lanes 2 and 5). The cytosolic fraction contained very little or no phosphorylated proteins A and B (Fig. 6 a, lanes 1 and 4).

Several additional control experiments were performed to confirm the above findings. In one, the importance of uPAR in the complex formation was further tested by degradation of surface uPAR with trypsin. This treatment, known to remove almost all of the uPA-binding sites (Estreicher et al., 1989) resulted in more than 90% decrease of uPA binding in WISH cells (results not shown). As determined by phosphorimager scanning, the radioactivity of bands immunoprecipitated from trypsin-treated cells with anti-uPAR antibodies was reduced by 48% (Fig. 6 b, lane 3 and 4). In another experiment (not shown), in which isolated cell membranes were treated with trypsin, the reduction was 89%. Treatment of the cells with GPI-specific phospholipase C resulted in a



**Figure 6.** (a) Cosegregation of uPAR and a serine kinase activity with membranes. Cytosolic fraction (lanes 1, 4, and 7), particulate fraction (lanes 2, 5, and 8), or cell homogenate prior to fractionation (lanes 3 and 6) were incubated in pro-uPA (lanes 1-3), anti-uPAR antibody (lanes 4-6), preimmune antibody (lane 7), or BSA (lane 8)-coated wells. After several washes, in vitro kination was performed and phosphorylated proteins analyzed by SDS-PAGE followed by autoradiography. (b) Effect of cell surface uPAR removal by trypsin. Cells were treated with 0.025% trypsin for 10 min at 37°C. Proteolysis was stopped by 0.1 mg/ml of soybean trypsin inhibitor. Cell extracts from untreated (lanes 1 and 3) or trypsin-treated cells (lanes 2 and 4) were incubated in wells coated with preimmune (lanes 1 and 2) or anti-uPAR (lanes 3 and 4) antibodies and processed as above.



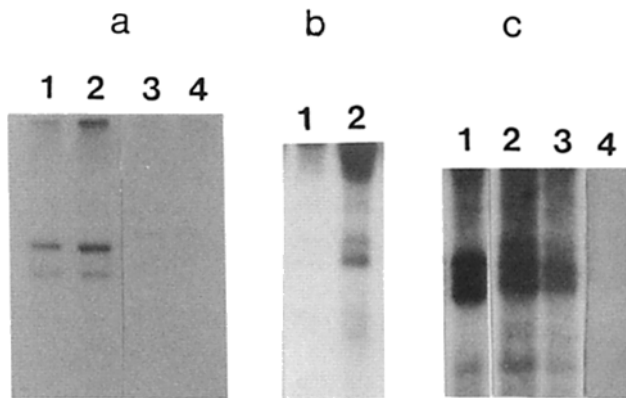
**Figure 7.** Isolation and microsequencing of proteins A and B. Lane 1, WISH cell lysates were immunoprecipitated by solid phase immunocapture using goat anti-uPAR antibodies. Proteins bound to 16 individual wells, pooled by consecutive transfer of sample buffer from well to well, were electrophoresed under non-reducing conditions in one lane of a 10% SDS-PAGE and blotted to a PVDF membrane. Proteins were stained with amido black. Six such lanes were combined, the stained

proteins were cut out, microdigested, and used for determination of internal amino acid sequences. Lane 2, in vitro kinase assay was performed on an immunoprecipitate from a single anti-uPAR-coated well. Phosphorylated proteins were analyzed by SDS-PAGE and autoradiography. Note that the stained and the radioactive bands comigrate.

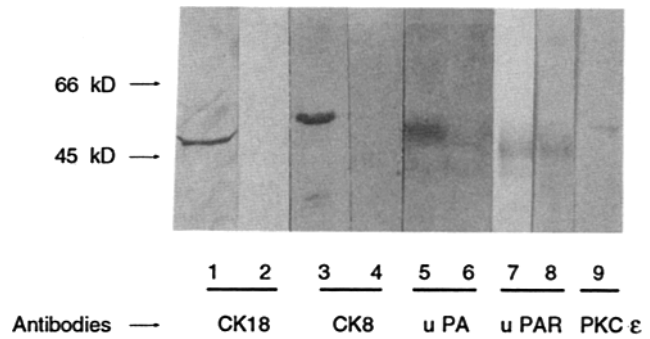
less marked decrease in both uPA binding and phosphorylation of the uPAR immunoprecipitate (data not shown).

#### Identification of the Components of the uPAR Complex

Proteins associated with uPAR were coprecipitated along with uPAR using a polyclonal anti-uPAR antibody. Immunoprecipitates collected by consecutive transferring of sample buffer through 16 individual wells, were submitted to SDS-PAGE and electroblotted. Staining of the membrane revealed two major uPAR-associated proteins (Fig. 7, lane 1) which comigrated with the phosphoproteins A and B (lane 2). Pooling of six stained lanes (or lysates of ~50 million



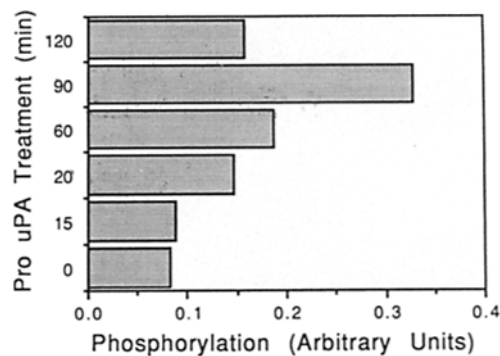
**Figure 8.** Characterization of the uPAR-associated kinase. WISH cells were incubated with, or without,  $10^{-7}$  M PMA for 24 h, and processed for solid phase immunocapture and in vitro phosphorylation. (a) Lanes 1 and 3, control; lanes 2 and 4, PMA treated. Lanes 1 and 2, goat anti-uPAR antibody; lanes 3 and 4, goat anti-rabbit IgG. (b) Lane 1, staurosporine analogue (GF109203X) at  $10^{-4}$  M; lane 2, control. (c) Lane 1, control with 0.5% DMSO in the phosphorylation mixture; lane 2, PKI (1  $\mu$ g/ml); lane 3, genistein  $10^{-5}$  M; lane 4, GF109203X at  $10^{-5}$  M.



**Figure 9.** Detection of the uPAR complex proteins in lysates of WISH cells by Western blotting. Cell lysates were prepared as described in Materials and Methods, electrophoresed on a 10% SDS-PAGE, transferred to nitrocellulose filter paper and blotted with the appropriate antibodies. Lanes 1, 3, 6, 8, and 9 are lysates of untreated WISH cells; lane 5 is a lysate from pro-uPA treated cells; lane 2 and 4, lysates of HEp3 cells; lane 7, 10 ng of purified uPAR from CHO cells transfected with a truncated uPAR cDNA. Western blots were revealed using: lanes 1 and 2, anti-CK18 moAb; lanes 3 and 4, anti-CK8 moAb; lanes 5 and 6, anti-uPA moAb; lanes 7 and 8, anti-uPAR moAb; lane 9, anti-PKC $\epsilon$  polyclonal antibodies.

cells) provided sufficient material for obtaining an internal sequence from A and B proteins. The sequence obtained for A (MQSLNDRRLASYLDRVR) and B (QRASLEAAIADA-EQRGELAIAK) showed 100% homology with cytokeratins 18 and 8, respectively.

Since these two bands were shown to be phosphorylated on serines (Fig. 3 d), we wondered whether members of kinase C or A may be responsible for this reaction. Addition of an analogue of staurosporine (GF109203X), a more specific PKC inhibitor (Toullec et al., 1991) at  $10^{-4}$  M (Fig. 8 b, lane 1) or  $10^{-5}$  M (Fig. 8 c, lane 4) to the in vitro phosphorylation reaction reduced the phosphorylation intensity of CK8 and CK18 to background levels. A high concentra-



**Figure 10.** In vivo phosphorylation of CK8; effect of uPAR occupancy. WISH cells, washed and labeled with  $^{32}$ P (100  $\mu$ Ci/culture) for a total of 4 h (see Materials and Methods) were incubated for 15–120 min before the end of labeling, with  $10^{-8}$  M pro-uPA, then washed, lysates prepared, uPAR captured as described in Materials and Methods, and then analyzed by SDS-PAGE and autoradiography. The gel was exposed for 2 h, scanned, and the relative intensity of the CK bands measured. This experiment was repeated three times with essentially similar results.



tion of PKA inhibitor (synthetic peptide, PKI), or genistein, a tyrosine kinase inhibitor, had only minor effects (Fig. 8 c, lanes 2 and 3). To further characterize the kinase, lysates of WISH cells incubated for 24 h without or with  $10^{-7}$  M PMA were subjected to immunocapture and phosphorylation. A member of the PKC family, PKC  $\epsilon$ , known to be associated with CK8 and CK18 (Omary et al., 1992), was shown to be resistant to down-regulation by PMA treatment (Baxter et al., 1992). As seen in Fig. 8 a, PMA treatment for 24 h yielded the same pattern and intensity of phosphorylated bands as controls (compare lanes 1 and 2).

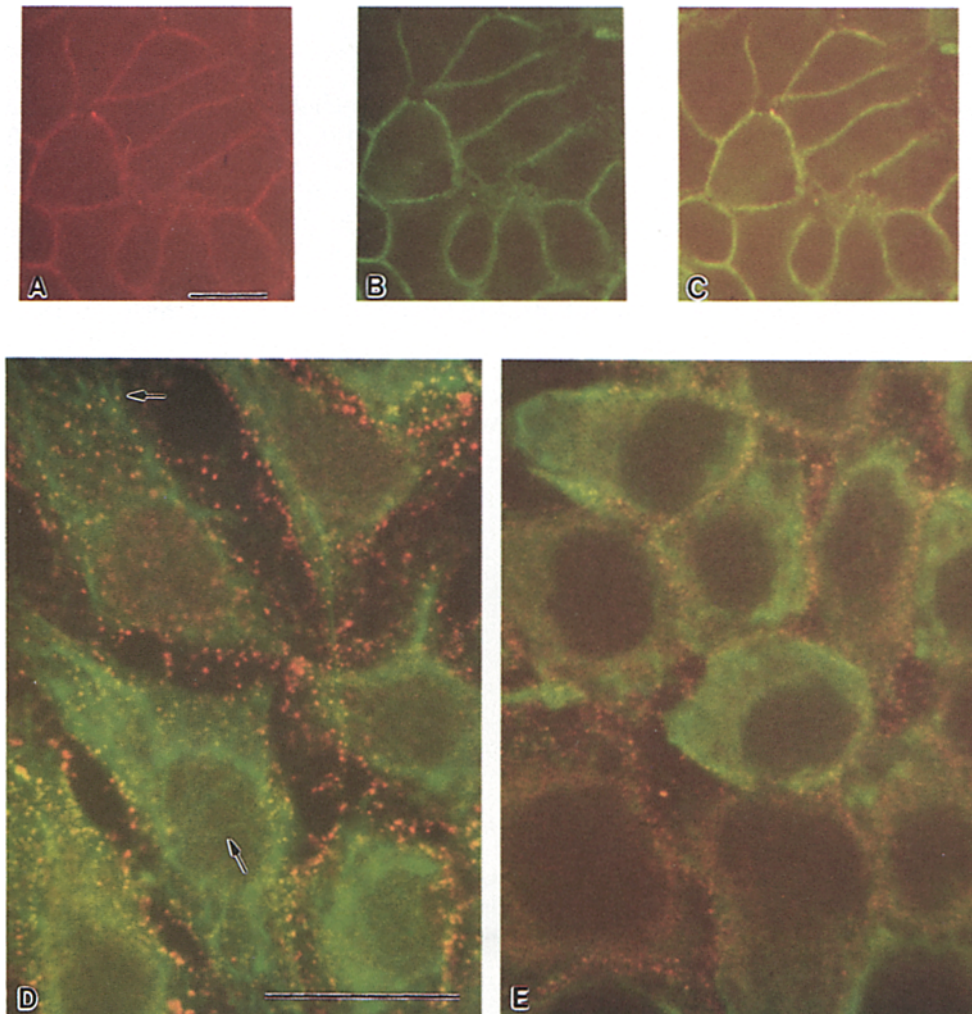
In Western blot, CK8 and CK18 were easily detectable in WISH cells, in which they participate in complex formation with uPAR (Fig. 9, lanes 1 and 3, respectively), but not in Hep3 cells (lanes 2 and 4), in which this complex was absent. As expected, uPA was present only in WISH cells preincubated with pro-uPA (Fig. 9, lane 5), but not in untreated cells (lane 6), confirming the results obtained in ELISA and in uPA activity assays. A band comigrating with purified uPAR (Fig. 9, lane 7) was also detected in WISH cell lysates (lane 8). Anti-PKC $\epsilon$  antibodies revealed a 50-kD band which may correspond to the catalytic fragment of PKC $\epsilon$  (Strulovic et al., 1991, Baxter et al., 1992), but this band was not

diminished by preincubation of the antibody with the immunizing peptide at twice the concentration recommended by the manufacturer. Concentrations of the peptide 20-fold greater than that used here were shown to block the binding of anti-PKC $\epsilon$  antibody in Western blots (Baxter et al., 1992).

The results of Western blotting confirmed the presence in WISH cells of the proteins participating in the uPAR-complex formation and established the validity of the antibodies used as appropriate reagents for immunocytochemical analysis (see below).

#### *In Vivo Effects of Pro-uPA Binding to uPAR on Cytokeratin 8 and 18 Phosphorylation and Cytokeratin Filament Organization*

To examine the effect of pro-uPA binding to uPAR complex in vivo WISH cells were labeled with  $^{32}$ P, incubated with and without pro-uPA for 15–120 min, and the pattern and the intensity of phosphorylation of the immunocaptured uPAR complexes was compared using anti-CK8 mAb. Two phosphorylated proteins of mol wt corresponding to those of CK8 and CK18 were detected. CK8 was more intensely phosphorylated than CK18 under all conditions. Scanning of the



**Figure 11.** Immunocytochemical localization of uPA, uPAR and cytokeratins in WISH cells incubated with and without pro-uPA. Cells were grown 18–36 h on coverslips and then incubated for 20 min with  $10^{-8}$  M pro-uPA (A–C and E) or with no addition (D) in medium with 10 mM HEPES, pH 7.4, and 1 mg/ml BSA and 100 KIU/ml aprotinin. All coverslips were fixed and then stained without (A–C) or after permeabilization (D and E). Staining for uPAR was with mouse anti-uPAR mAb followed by biotinylated anti-mouse IgG and rhodamine-conjugated streptavidin (A and C–E). A second mouse mAb, anti-uPA (B) or anti-CK8 (D and E) was then applied and detected with fluorescein-conjugated anti-mouse IgG. The cultures were examined with confocal microscope and scanned with rhodamine optics at 525 nm and fluorescein optics at 475 nm. Coincidence of the two fluorophores is seen in C (uPAR and uPA), and D and E (uPAR, CK). Staining with anti-CK18 mAb gave identical results to anti-CK8 (not shown). The experiment was repeated four times with essentially identical results. Bar, 20  $\mu$ m.



bands revealed that the intensity of CK phosphorylation almost doubled after 20 min of incubation with pro-uPA, and reached a maximum at 90 min (Fig. 10).

An additional approach, aimed at testing the localization and spatial relations between the uPAR complex proteins and the effect of pro-uPA binding utilized immunocytochemistry with double label fluorescence and confocal microscopy. WISH cells were incubated for 20 (Fig. 11) or 60 min (Fig. 12), without or with  $10^{-8}$  M (or  $10^{-7}$  M, respectively) pro-uPA in medium with no serum. In nonpermeabilized pro-uPA-treated cells, mAb to uPAR (Fig. 11 A) or to uPA (Fig. 11 B) produced a homogenous staining of the outer cell membrane, which at higher magnification appeared punctate (D and E). The images obtained by anti-uPA and anti-uPAR moAbs were entirely superimposable, indicating a full colocalization of these proteins (Fig. 11, A-C). WISH cells incubated without pro-uPA were completely negative when tested with mAb to uPA (results not shown). The flat morphology of control cells (Fig. 12 A) was dramatically altered after pro-uPA treatment (Fig. 12 B). The cells rounded up and their cross-sectional diameters were reduced. An identical cell shape change was observed after only 20 min of pro-uPA treatment, but the proportion of cells affected was somewhat smaller (not shown). Confocal microscopy of cells incubated in absence of pro-uPA revealed that CK8 and CK18 formed filaments often organized in a lattice parallel to the surface of the coverslip and distributed evenly throughout the cytoplasm (Fig. 11 D, arrows). In contrast, in similarly oriented through-sections of pro-uPA-treated cells, very few CK8 and CK18 filaments were resolvable (Fig. 11 E). The distribution of uPAR did not appear to be affected by pro-uPA treatment (Fig. 11 D and E). Transmission fluorescent microscopy revealed that pro-uPA treatment did not result in disruption of the CK filaments (results not shown), suggesting that the main change was their redistribution. Whether this change was the cause or the result of the cell shape change and how it was related to the CK phosphorylation is at present unknown.

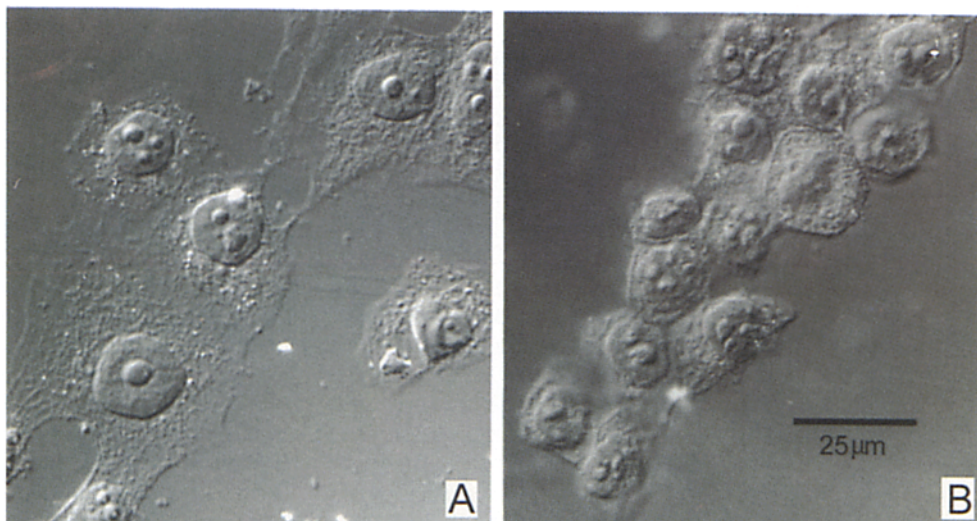
The localization of PKC $\epsilon$  was examined using polyclonal anti PKC $\epsilon$  antibodies which in Western blots of WISH cells recognized a band of  $\sim 50$  kD (Fig. 9, lane 9). Immuno-

cytochemistry revealed only a small number of intracellular (found only in permeabilized cells) particles which did not redistribute nor showed a specific cellular localization upon pro-uPA treatment (not shown). The observed pattern was different from the one described, in which PKC $\epsilon$  was shown to be a membrane-associated protein (Omary et al., 1992). New reagents will be required to critically assess the role of PKC $\epsilon$ .

## Discussion

The experiments described here were designed to confirm the observation that binding of pro-uPA to uPAR stimulates cell migration, and to identify those proteins which, together with uPAR, participate in the transduction of the external signal and in the propagation of the specific intracellular responses.

Using two cell lines, LB6-uPAR and WISH, expressing high affinity uPAR but no detectable levels of uPA, and two types of assays, migration into a wound (Ossowski et al., 1975) and from cyto beads (Rosen et al., 1990), we showed that binding of exogenous pro-uPA to uPAR increased cell migration. Proteolytically active uPA was not required as the enhancement was observed in absence of plasminogen and in presence of aprotinin. A qualitatively similar, but quantitatively modest effect on migration into a wound of pro-uPA or amino-terminal fragment-treated endothelial cells has been previously reported (Odekun et al., 1992). Stimulation of chemokinesis, similar in magnitude to that observed in our experiments, was reported in neutrophils (Gudewicz and Gilboa, 1987) and bovine endothelium (Fibbi et al., 1988). Chemotaxis was also shown to be potently stimulated by uPA and its inactive forms in several cell types (Gudewicz and Gilboa, 1987; Fibbi et al., 1988; Del Rosso et al., 1993). Combined, these findings indicate that both chemotaxis and chemokinesis may proceed in the absence of proteolytically active uPA or plasmin, and that binding of pro-uPA elicits a specific biologic response which, by its very nature, must involve intracellular signaling. Considering that uPA is secreted by most cells in the inactive, proenzyme form, the ability of pro-uPA and its receptor to elicit an intracellular



**Figure 12.** The effect of pro-uPA treatment on cell shape changes. Cells were grown and incubated as in legend to Fig. 11, but then treated for 60 min without (A) or with  $10^{-7}$  M pro-uPA (B). After fixation, cells were viewed with Nomarsky optics and photographed at the same magnification. The control cells (A) were flat and thus had larger diameter than the pro-uPA-treated cells (B). Similar changes were observed in cells treated for 20 min or in cells treated with  $10^{-8}$  M pro-uPA (not shown).

signal may be physiologically important in migration of normal and tumor cells.

Since uPAR is a GPI-linked protein anchored in the lipid bilayer and lacking a cytoplasmic domain, it must be assumed that to transduce a signal, it has to associate with other proteins which extend into the intracellular space. We sought to isolate these proteins by capturing the receptor-containing complex with either surface-immobilized anti-uPAR antibodies, or pro-uPA, or with surface-immobilized anti-uPA antibodies in the case of lysates of cells preexposed to pro-uPA. The lysates were prepared by extracting cells for 20 min at room temperature with 1% nonionic detergent because it was shown that some GPI-linked proteins were insoluble in 1% Triton X-100 at low temperature (Brown and Rose, 1991). Under the conditions used, most of the uPAR and, as previously shown (Omary et al., 1992), most cytokeratins were solubilized, thus reducing the possibility of the complex being a mixture of accidentally entrapped proteins. This conclusion was further strengthened by the finding that supernatant of cell lysates spun at 100,000 g for 10 min yielded the same assembly of proteins, when captured with anti-uPAR antibodies and phosphorylation, as the supernatant from low speed (3,400 rpm) centrifugation. Also, extraction of WISH cells at 0°C, room temperature, or 37°C, followed by a 100,000 g, 10-min centrifugation and Western blotting of the supernatants, produced uPAR bands of identical intensity (results not shown). This shows that uPAR is solubilized and that the complex with other proteins is stable under the conditions used. The results of *in vitro* phosphorylation of the proteins captured by this approach, combined with an analysis of the phosphorylated residues and microsequencing of the two <sup>32</sup>P-labeled proteins allowed the conclusion that uPAR formed a complex with cytokeratins 8 and 18, and a serine kinase. A similar complex was observed in membranes of cells fractionated in absence of detergents. The following results provide additional confirmation for the specificity of the observed interaction between uPAR, the CKs, and the kinase. A goat polyclonal anti-uPAR, a mouse moAb anti-uPAR, two different anti-uPA moAbs, and anti-CK8 moAb all produced identical patterns of phosphorylated proteins (Figs. 3–6 and 8), while goat anti-rabbit Fc, goat anti-rabbit Ig, and a mouse IgG1 and IgG2a, or BSA were ineffective in immunocapture (Figs. 3–6 and 8). The role of uPAR in formation of the complex was further strengthened by the observation that *in vitro* phosphorylation was decreased by the reduction in cell surface uPAR either by phospholipase C (not shown) or trypsin (up to 90% reduction) treatment, (Fig. 6 *b*). Moreover, antibodies to CD55, another GPI-linked protein which is highly expressed on the surface of WISH cells, behaved like nonimmune antibodies.

While the two phosphorylated bands were positively identified as CK8 and CK18 by amino acid sequence, the definitive identity of the uPAR-associated kinase has not been established. The following considerations, however, suggest that it belongs to the PKC family and is possibly a PKC $\epsilon$  related isoform: (*a*) PKC-specific inhibitor (a staurosporine analogue), and not PKA or tyrosine kinase inhibitors, blocked the *in vitro* phosphorylation of CK8 and CK18 (Fig. 8). (*b*) The *in vitro* phosphorylation of the uPAR complex could proceed in absence of exogenous calcium or allosteric activators of PKC. This is in agreement with published re-

sults showing calcium independence of this enzyme (Schaap et al., 1989; Chou and Omary, 1991; Strulovici et al., 1991). It is of interest to note that treatment of cells with pro-uPA results in an increase in diacylglycerol, most likely due to *de novo* synthesis, but no mobilization of intracellular pools of calcium (Del Rosso et al., 1993). Moreover, in analogy to our finding, a 40-kD form of PKC $\epsilon$ , which is the main form associated with cytokeratins (Omary et al., 1992), was shown to be independent of allosteric activators (Baxter et al., 1992). (*c*) The activity of uPAR associated kinase from WISH cells pretreated for 24 h with 10<sup>-7</sup> M phorbol myristate acetate was not down-regulated as compared to control (Fig. 8). The resistance to down-regulation by phorbol myristate acetate is a previously described property of PKC $\epsilon$  (Strulovici et al., 1991). (*d*) In permeabilized WISH cells, polyclonal antibodies recognizing the catalytic domain of PKC $\epsilon$  produced a specific pattern of immunostaining (not shown) and in Western blots identified a single band of ~50 kD (Fig. 9). Our results, therefore, together with the evidence for a tight physical association of PKC $\epsilon$  with cytokeratins 8 and 18 and phosphorylation of these cytokeratins by this enzyme (Omary et al., 1992), strongly suggest that the enzyme in the uPAR complex may be a PKC $\epsilon$ -related kinase.

The finding of a serine kinase in a complex with a GPI-linked protein was unexpected, since only an association with tyrosine kinases was previously demonstrated (Stefanova et al., 1991; Thomas and Samelson, 1992; Dumler et al., 1993). Our attempts to show phosphorylation on tyrosine, either in response to pro-uPA binding to uPAR or to receptor clustering by mouse anti-uPAR followed by rat anti-mouse antibodies, were unsuccessful both in Western blots (Fig. 2) and *in vivo*-labeled <sup>32</sup>P immunoprecipitates (not shown). The same anti-phosphotyrosine antibodies used in the above experiments were effective in detecting an increase in EGF-dependent tyrosine phosphorylation in WISH cells. Thus, the signal transducing assembly in WISH cells may be unique and specific to epithelium, and possibly even simple epithelium. This is also suggested by the finding of an identical pattern of phosphorylation in lysates of human lung carcinoma cells (A459), which express cytokeratins 8 and 18 (Ledinko and Constantino, 1990) but entirely different patterns in human squamous cell carcinoma (HEp3) and promyelocytic leukemia (HL-60) cell lysates (Fig. 5).

A direct implication of uPAR in signal transduction requires evidence linking its ligation to a dynamic, biochemical intracellular event which is detectable earlier than increased migration or increased DNA synthesis. We used two approaches to examine whether such events ensued as a result of exposure to pro-uPA: a change in phosphorylation pattern or intensity, and a change in localization or distribution of the proteins identified as members of the uPAR complex. As evidenced from Figs. 10–12 both events followed. In addition, incubation of cells with pro-uPA produced a striking change in cell shape. Although, increase in phosphorylation and in redistribution of cytokeratins were most pronounced after 60 min of treatment, we do not yet have a sufficiently detailed time course which would allow us to conclude whether temporally, phosphorylation of cytokeratin 8 preceded the change in its cellular distribution and how the two events related to the cell shape change. However, phosphorylation appears to play a role in intermediate filaments dynamics, inducing, for instance, reversible disas-

sembly of both lamins and vimentin intermediate filaments during mitosis (see for review Steiner and Liem, 1990). The confocal immunofluorescence analysis revealed that, exposure of cells to pro-uPA, under conditions which prevented proteolysis, caused a cell shape change (Figs. 11 and 12) associated with a redistribution of cytokeratin filaments (Fig. 11, *D* and *E*). Phosphorylation of cytokeratins in EGF-treated rat hepatocytes was shown to be associated with their redistribution (Baribault et al., 1989). In colonic carcinoma cells, the cytokeratins were found near the inner phase of the cell membrane (Omary et al., 1992), resembling the effect observed in pro-uPA treated WISH cells. Since colonic carcinoma cells continuously produce uPA, it is possible that the receptor in these cells is in a state of permanent "activation."

The link between the change in cytokeratin phosphorylation and their redistribution, and enhanced migration is at present not understood. There is evidence that properly assembled cytokeratins may be essential for cells to migrate and to invade. For example, transfection of a human melanoma with a mutated cytokeratin CK18, resulted in disrupted keratin filaments and reduced migration (Hendrix et al., 1992). Likewise, retinoid-induced down regulation of cytokeratin 18 in epithelial cell line, A459, was accompanied by a suppression of its *in vitro* invasive abilities (Ledinko and Constantino, 1990). In contrast, transfection of cytokeratins 8 and 18 into L cells resulted in filament formation and increased migratory activity (Chu et al., 1993).

Taken together, our data show that uPAR forms a complex with several proteins, including a PKC, and that at least some members of this complex undergo chemical changes as a result of its occupation by pro-uPA. Whether these changes are causally linked to migration in WISH cells will have to await the identification of all the members of this complex, most importantly the transmembrane protein, if such exists, and testing the effects of inactivation or down-regulation of each of the identified proteins.

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