

The Urokinase-Type Plasminogen Activator Receptor, a GPI-linked Protein, Is Localized in Caveolae

Andreas Stahl and Barbara M. Mueller

Department of Immunology, The Scripps Research Institute, La Jolla, California 92037

Abstract. The urokinase plasminogen activator receptor (uPAR), a glycosylphosphatidylinositol-linked glycoprotein, plays a central role in the regulation of pericellular proteolysis and participates in events leading to cell activation. Here, we demonstrate that uPAR, on a human melanoma cell line, is localized in caveolae, flask-shaped microinvaginations of the plasma membrane found in a variety of cell types. Indirect immunofluorescence with anti-uPAR antibodies on the melanoma cells showed a punctated staining pattern that accumulated to stretches along sides of cell-cell contact and membrane ruffles. uPAR colocalized with caveolin, a characteristic protein in the coat of caveolae, as demonstrated by double staining with specific antibodies. Further, uPAR could be directly localized in caveolae by *in vivo* immunoelectron microscopy. Both uPAR and its ligand, uPA, were

present in caveolae enriched low density Triton X-100 insoluble complexes, as shown by immunoblotting. From such complexes, caveolin could be coprecipitated with uPAR-specific antibodies suggesting a close spatial association between uPAR and caveolin that might have implications for the signal transduction mediated by uPAR. Further, functional studies indicated that the localization of uPAR and its ligand in caveolae enhances pericellular plasminogen activation, since treatment of the cells with drugs that interfere with the structural integrity of caveolae, such as nystatin, markedly reduced cell surface plasmin generation. Thus, caveolae promote efficient cell surface plasminogen activation by clustering uPAR, uPA, and possibly other protease receptors in one membrane compartment.

TIGHTLY regulated extracellular proteolytic systems are involved in the dissolution of extracellular matrix and in the modulation of cellular interactions in such processes as leukocyte recruitment to sites of inflammation or tumor cell invasion and metastasis. For example, plasminogen activators specifically convert the latent ubiquitous zymogen plasminogen into plasmin, a protease which can degrade a wide spectrum of glycoproteins. Plasmin generation, particularly through the urokinase-type plasminogen activator (uPA)¹, contributes significantly to pericellular proteolysis and is likely modulated by cell surface receptors (for review see Ellis et al., 1992; Blasi, 1993).

A specific uPA-receptor (uPAR) has been detected on many different cell types including monocytes (Stoppeli et al., 1985; Min et al., 1992), neutrophils (Plesner et al., 1994), endothelial cells (Miles et al., 1988; Pepper et al.,

1993), keratinocytes (McNeill and Jensen, 1990; Del Rosso et al., 1990), as well as various tumor cell lines (Stoppeli et al., 1986; Boyd et al., 1988; Hollas et al., 1992; Mohanam et al., 1993). The biochemical characterization of uPAR identified a single chain, 55–60-kD glycoprotein (Nielsen et al., 1988) that is anchored in the cell membrane by a glycosylphosphatidylinositol (GPI) moiety (Ploug et al., 1991). Primarily, uPAR is thought to enhance and direct uPA proteolytic activity (Ellis et al., 1989; Estreicher et al., 1990; Manchanda and Schwartz, 1991). However, uPAR has also been implicated in mediating the internalization of uPA-plasminogen activator inhibitor complexes (Olson et al., 1992; Li et al., 1994). Moreover, the binding of catalytically active or inactive uPA to uPAR can stimulate cell proliferation (Kirchheimer et al., 1989; Rabbani et al., 1990), as well as chemotactic and haptotactic cell migration (Odekon et al., 1992; Gyetko et al., 1994; Stahl and Mueller, 1994; Busso et al., 1994). Together, these findings demonstrate that the GPI-linked uPAR functions not merely as a uPA-binding protein, but is also involved in the regulation of cell-mediated proteolysis and cell activation.

GPI-linked proteins have been described to cluster in plasma membrane conformations known as caveolae. Caveolae were originally described as membrane invaginations on the cell surface that were later found to have a characteristic

Please address all correspondence to Dr. B. M. Mueller, Department of Immunology, The Scripps Research Institute, 10666 N. Torrey Pines Rd., IMM13, La Jolla, CA 92037. Tel.: (619) 554-8124. Fax: (619) 554-6705.

1. *Abbreviations used in this paper:* DTSSP, [3,3'-dithiobis (sulfosuccinimidylpropionate)]; GPI, glycosylphosphatidylinositol; OGP, *n*-octyl β -D-glucopyranoside; uPA, urokinase-type plasminogen activator; uPAR, uPAR receptor.

coat containing the marker protein caveolin (Rothberg et al., 1992). The folate receptor (Rothberg et al., 1990) and other GPI-linked proteins (Ying et al., 1992; Keller et al., 1992) were demonstrated by immunocytochemistry to be localized in caveolae. Cholesterol and glycolipid rich low density Triton X-100 insoluble complexes isolated from cell lysates (Brown and Rose, 1992; Sargiacomo et al., 1993) contain a membrane fraction highly enriched for caveolae. Both caveolin and GPI-linked proteins have been identified biochemically in such detergent insoluble complexes isolated from different cell types, including MDCK cells (Sargiacomo et al., 1993), endothelial cells from human lung (Lisanti et al., 1994), and smooth muscle cells from chicken gizzard (Chang et al., 1994).

We reasoned that the subcellular distribution of uPAR may be directed by its GPI-anchor resulting in a clustering of uPAR in caveolae. To study the localization of uPAR, we used a highly metastatic human melanoma cell line that expresses very high levels of uPAR (Stahl and Mueller, 1994). Here, we present three lines of evidence demonstrating that the subcellular localization of uPAR in these melanoma cells is indeed in caveolae: (a) uPAR, uPA, and caveolin are exclusively found in the low density detergent insoluble fraction of cell lysates; (b) uPAR colocalizes with caveolin when analyzed by indirect immunofluorescence; and (c) the ultrastructural localization of uPAR is largely confined to small, nonclathrin-coated invaginations of the plasma membrane. The finding that uPAR is localized in caveolae suggests that caveolae may serve as a specialized compartment for pericellular plasminogen activation. In support of this idea, we could demonstrate that the pretreatment of melanoma cells with drugs that interfere with the structural integrity of caveolae markedly reduced cell surface plasminogen activation.

Materials and Methods

Antibodies

The monoclonal mouse anti-human uPAR antibody mAb 3936 (IgG2a) and polyclonal rabbit anti-human uPAR antibodies, 399R, and polyclonal rabbit anti-human uPA antibodies were kindly provided by Dr. Richard Hart, American Diagnostica (Greenwich, CT). An inhibiting anti-uPA mouse monoclonal antibody was purchased from York Biologicals International (Stony Brook, NY). Normal mouse and rabbit sera were purchased from Dako (Carpenteria, CA). A monoclonal mouse anti-22 kD RSV-SRC substrate (caveolin) antibody was purchased from ICN Biomedicals, Inc. (Costa Mesa, CA). Mouse monoclonal anti-annexin heavy and light chain antibodies were purchased from Transduction Laboratories (Lexington, KY).

Tumor Cell Line

The human melanoma cell line M24met has been described previously (Mueller et al., 1991). M24met cells were routinely cultured in RPMI 1640 supplemented with 10% FBS and 2 mM glutamine (Whittaker Bioproducts, Walkersville, MD). Cells were passaged using versene, 0.5 mM EDTA, 0.15 M NaCl, and 0.02 M HEPES.

Immunofluorescence Staining and Laser Scanning Confocal Microscopy

Cells were grown on gelatin-coated coverslips to the desired cell density; washed once with HBSS (with 1 mM Ca^{2+} and Mg^{2+}) and fixed with 4% fresh paraformaldehyde for 30 min at room temperature (RT). Results of preliminary experiments indicated that cells fixed in 4% paraformaldehyde, 0.5% glutaraldehyde showed no difference in their staining patterns when compared with cells fixed in 4% paraformaldehyde only. Cells were per-

meabilized for 5 min on ice with 0.05% saponin in HBSS and blocked with 5% BSA in HBSS for 30 min on ice. These permeabilized cells were incubated with primary antibodies (rabbit anti-uPAR and mouse anti-caveolin) 1 h on ice, washed three times with HBSS and incubated for 40–60 min on ice with the secondary antibody (rhodamine-conjugated goat-anti-mouse IgG and FITC-conjugated goat-anti-rabbit IgG; TAGO, Burlingame, CA). For the staining of F-actin, 5 μl of a 6.6- μM rhodamine phalloidin (Molecular Probes, Eugene, OR) stock solution were used. Cells were washed three times with HBSS and once in distilled water. Slides were mounted with Slow Fade (Molecular Probes, Eugene, OR) and sealed with nail polish. The double-labeled specimen were examined with a (MRC 600) laser scanning confocal microscope equipped with an argon/krypton laser ($\lambda_1 = 488 \text{ nm}$, blue line for FITC; $\lambda_2 = 568 \text{ nm}$, yellow line for rhodamine) (BioRad Labs, Hercules, CA). The recorded images were merged to illustrate the colocalization of uPAR and caveolin to the membrane. The gain and black levels were set to ensure that the collected images reflected the full range of grey level values from black (0 pixel intensity) to maximal white (255 pixel intensity). Optical sections of immunolabeled samples were examined at high magnification (1,000, Planapo 63 \times , NA 1.4, with oil). A section plane was collected at true focus using Kalman averaging at an optical zoom setting of one or more.

Electron Microscopy

M24met cells were seeded in 24-well plates and incubated on ice with the polyclonal anti-uPAR antibody. After three PBS washes, bound antibody was detected with goat-anti-rabbit IgG 5-nm gold conjugates. After washing the cells, they were fixed in modified Karnovsky's fixative (2% paraformaldehyde, 1.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4) for 2 h at 4°C and postfixed in 1% OsO_4 in the same buffer for 1 h at RT. The fixed cells were stained in 50% saturated aqueous uranyl acetate solution for 1 h, dehydrated in graded ethanol and flat embedded in 812 epoxy resin (TAAB Laboratories Equipment, Reading, England). Thin sections were cut on an LKB Ultratome III, mounted on a copper grid, stained in uranyl acetate and lead citrate, and viewed with a Hitachi HU 12A electron microscope at 75 kV acceleration.

Isolation of Triton-Insoluble Complexes by Sucrose Gradient Centrifugation

Low density, Triton-insoluble complexes were prepared, as described (Sargiacomo et al., 1993). Briefly, between $6\text{--}10 \times 10^7$ M24met cells were harvested with versene and lysed in 2 ml Mes-buffered saline (MBS; 25 mM Mes, pH 6.5, 0.15 M NaCl) 1% Triton X-100 with 10 strokes of a Dounce homogenizer. The lysates were adjusted to 40% sucrose and overlaid with 10 ml of a 30–5% linear sucrose gradient. The lysates were centrifuged overnight at 40,000 rpm (190,000 g) in a SW41 rotor (Beckman Instrs., Palo Alto, CA). Gradients were harvested and fractionated with fraction 1–6 spanning 5–30% sucrose and fraction 7 containing 40% sucrose. Fraction 8 was the pellet solubilized by boiling in 200 μl 10% SDS for 5 min. The protein content in each fraction was measured using the BCA assay (Pierce, Rockford, IL). Equal amounts of protein ($\sim 10 \mu\text{g}$ per lane) were separated on 5–15% SDS gradient gels and specific proteins were detected by Western blotting. The distribution of the blotted protein over the gradient fractions was quantified by laser densitometry on a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) using local background correction.

Sequential Extraction and Cross-linking

Sequential extraction and cross-linking were performed essentially, as described (Lisanti et al., 1993). A monolayer of $\sim 10^6$ cells was washed briefly with PBS, and then extracted for 30 min on ice with 10 ml of extraction buffer A (MBS, pH 6.5, 1% Triton X-100; 1 mM PMSF; 10 $\mu\text{g}/\text{ml}$ aprotinin; 1 mM sodium ortho vanadate). The extraction buffer was removed and 10 ml of a 1 mg/ml solution of [3,3'-dithiobis(sulfosuccinimidylpropionate)] (DTSSP; Pierce, Rockford, IL) in extraction buffer A was added to the cells. Cross-linking of the Triton X-100 insoluble complexes was carried out for 30 min on ice without agitation. After removing the cross-linking solution a second round of extraction was performed in 10 ml of extraction buffer B (TBS, pH 7.8; 60 mM *n*-octyl β -D-glucopyranoside (OGP); 1 mM PMSF; 10 $\mu\text{g}/\text{ml}$ aprotinin; 1 mM sodium ortho vanadate) or extraction buffer C (10 mM Tris, pH 7.8, 0.15 M NaCl, 3% Triton X-114; 1 mM PMSF; 10 $\mu\text{g}/\text{ml}$ aprotinin; 1 mM sodium ortho vanadate). For this second extraction the cells were agitated on an orbital shaker for 30 min

at 4°C. OGP extracts were directly used for immunoprecipitation, while Triton X-114 extracts were subjected to temperature induced phase separation, as described (Behrendt et al., 1993).

Surface Biotinylation

Surface biotinylation with NHS-LC-biotin (Pierce) was done according to the manufacturer's suggestions. Briefly, 5×10^6 M24met cells were harvested with versene and washed twice in PBS. The cells were then resuspended in 5 ml PBS containing 100 μ l of a NHS-LC-biotin stock solution (10 mg/ml) and incubated on a rotator for 60 min at room temperature. Cells were washed twice in PBS and resuspended in 1 ml NP-40 lysis buffer (10 mM Tris/HCl, pH 7.4; 150 mM NaCl; 1% NP-40; 1 mM EDTA; 1 mM PMSF; 10 μ g/ml Aprotinin). Lysates were precleared and subjected to immunoprecipitation.

Immunoprecipitation and Western Blotting

Immunoprecipitation of extracts from sequential extractions was preceded by preclearing of lysates with Pansorbin (Calbiochem, La Jolla, CA) and incubated for 1 h with 10 μ g of the appropriate antibody that was previously bound to 50 μ l of a slurry of protein A-Sepharose. Immunoprecipitates were washed four times with the buffer used for the second extraction (see Sequential Extraction) and three times with PBS, 0.1% Tween 20 (PBS-T) and finally boiled for 3 min in 20 μ l of reducing Laemmli buffer. Proteins were separated on a 5–15% denaturing polyacrylamide (Protogel, National Diagnostics, Atlanta, GA) gradient gel and transferred to a nitrocellulose membrane (BioBlot-NC, Costar, Cambridge, MA) using a semidry blotting apparatus (0.8 mA/cm² membrane; transfer buffer containing 20 mM Tris, 150 mM glycine, 0.1% SDS, 20% methanol). Membranes were blocked overnight in PBS-T containing 5% nonfat dry milk powder. The primary antibodies used for Western blotting were diluted in PBS-T containing 1% nonfat dry milk powder to a concentration of 2 μ g/ml and incubated with the membranes for 1 h. Excess antibody was removed by three washes with PBS-T. The specifically bound primary antibody was detected by species-specific goat antibodies conjugated to horseradish peroxidase (Bio-Rad Labs). The membranes were incubated with the secondary antibody for 30 min and washed five times in PBS-T. Secondary antibodies were visualized with an ECL kit (Amersham Corp., Arlington Heights, IL) following the manufacturer's instructions.

Plasminogen Activation Assays

M24met cells, 5×10^4 per well, were seeded into 96-well plates and incubated overnight at 37°C. The cells were rinsed with RPMI without phenol red and groups of eight wells were either treated with nystatin, filipin, PMA (all from Sigma Chem. Co., St. Louis, MO), or with inhibiting anti-uPAR antibodies for 1 h. Cells were rinsed again and 100 μ l of a reaction mix, 2 μ M plasminogen (Sigma), and 2.2 mM of H-D-Val-Leu-Lys-p-nitroanilide (S-2251) (Chromogenix, Franklin, OH) in RPMI without phenol red, were added to the cells. Plasmin generation was measured every 5 min as Δ OD₄₀₅ in a ThermoMax plate reader (Molecular Devices, Menlo Park, CA).

Results

uPAR Has a Punctated Localization Pattern on the Cell Surface and Colocalizes with Caveolin

The cellular distribution of uPAR on M24met human melanoma cells was analyzed by indirect immunofluorescence with a rabbit polyclonal anti-uPAR antibody. Paraformaldehyde fixed, saponin permeabilized cells that were stained with anti-uPAR showed a staining pattern characteristic for a caveolar localization, i.e., a punctated pattern with clusters of uPAR localizing to defined regions of the plasma membrane (Fig. 1, A, D, and G). Cells fixed with paraformaldehyde and glutaraldehyde demonstrated an identical staining pattern (data not shown). To further support our contention that uPAR is localized in caveolae, we used immunofluorescence double labeling to demonstrate colocalization of uPAR with a caveolar marker protein, caveolin. Antibodies against

a 22-kD v-src substrate from Rous sarcoma virus-infected chicken fibroblasts have been shown to react specifically with human and chicken caveolin (Glenney and Zokas, 1989). Thus, such a mouse monoclonal anti-caveolin antibody was used together with a polyclonal rabbit serum against uPAR for immunofluorescence double labeling of paraformaldehyde fixed and saponin permeabilized M24met cells. Laser scanning confocal microscopy indicated that both antigens are localized mainly in discrete spots on the plasma membrane. The anti-caveolin antibody also stained, in some cases, a cytoplasmic compartment that likely corresponds to the Golgi network (Fig. 1 C); however, uPAR was strictly confined to the punctated membrane regions and showed a high degree of colocalization with caveolin (Fig. 1, B, E, and H). The colocalization of uPAR and caveolin was confirmed by three dimensional analysis of the cellular localization (data not shown) and by high resolution scanning of the corresponding membrane areas (Fig. 1 J). The punctated pattern of membrane regions accumulated into stretches along cell-cell contact sites and membrane ruffles (Fig. 1 K). Scans along the Z-axis of cells revealed that both uPAR and caveolin were mainly localized in the upper layers of the cell while hardly any receptor was found at the substrate contact sites. In addition, the receptor could not be colocalized with the actin cytoskeleton except for sites of membrane ruffles (Fig. 1 L). The nearly exclusive localization of uPAR in membrane regions clearly above the cell substrate contact sites and the distinct localization of actin stress fibers and uPAR make a localization of the receptor in focal contact sites, as described for other cells, rather unlikely. Taken together, these data suggest that uPAR is localized in caveolae where it colocalizes with caveolin.

The subcellular localization of uPAR was confirmed by immunogold electron microscopy on intact M24met cells. Cells were incubated on ice with anti-uPAR antibodies followed by secondary antibodies conjugated to 5-nm gold particles. Anti-uPAR antibodies decorated the plasma membrane with most of the gold particles being localized in plasmalemmal caveolae (Fig. 2). Caveolae were frequently observed in rows along the plasma membrane. Vertical sections showed no preference for a basal or apical labeling with the anti-uPAR antibody.

uPA and uPAR Are Enriched in Low Density Triton-Insoluble Complexes

To further determine whether uPAR is localized in caveolae, we took advantage of some distinct properties of this cell compartment. Caveolae are insoluble in certain detergents, such as 1% Triton X-100, and form glycolipid rich low density complexes that are enriched in the 22-kD caveolar marker protein caveolin (Sargiacomo et al., 1993). Insoluble, low density complexes from M24met cells were prepared in sucrose density gradients, as described. The gradients were divided into fractions with fraction 1–6 spanning the 5–30% linear sucrose gradient, fraction 7, the 40% sucrose fraction, and fraction 8, the pellet. The protein concentrations of the fractions were determined and equal amounts of total protein from each fraction were separated by SDS-PAGE and transferred to nitrocellulose membranes. The distribution of uPAR over the gradient was visualized using a polyclonal rabbit anti-uPAR antibody. The receptor was detected as a \sim 55-kD signal with 69% of the total uPAR signal

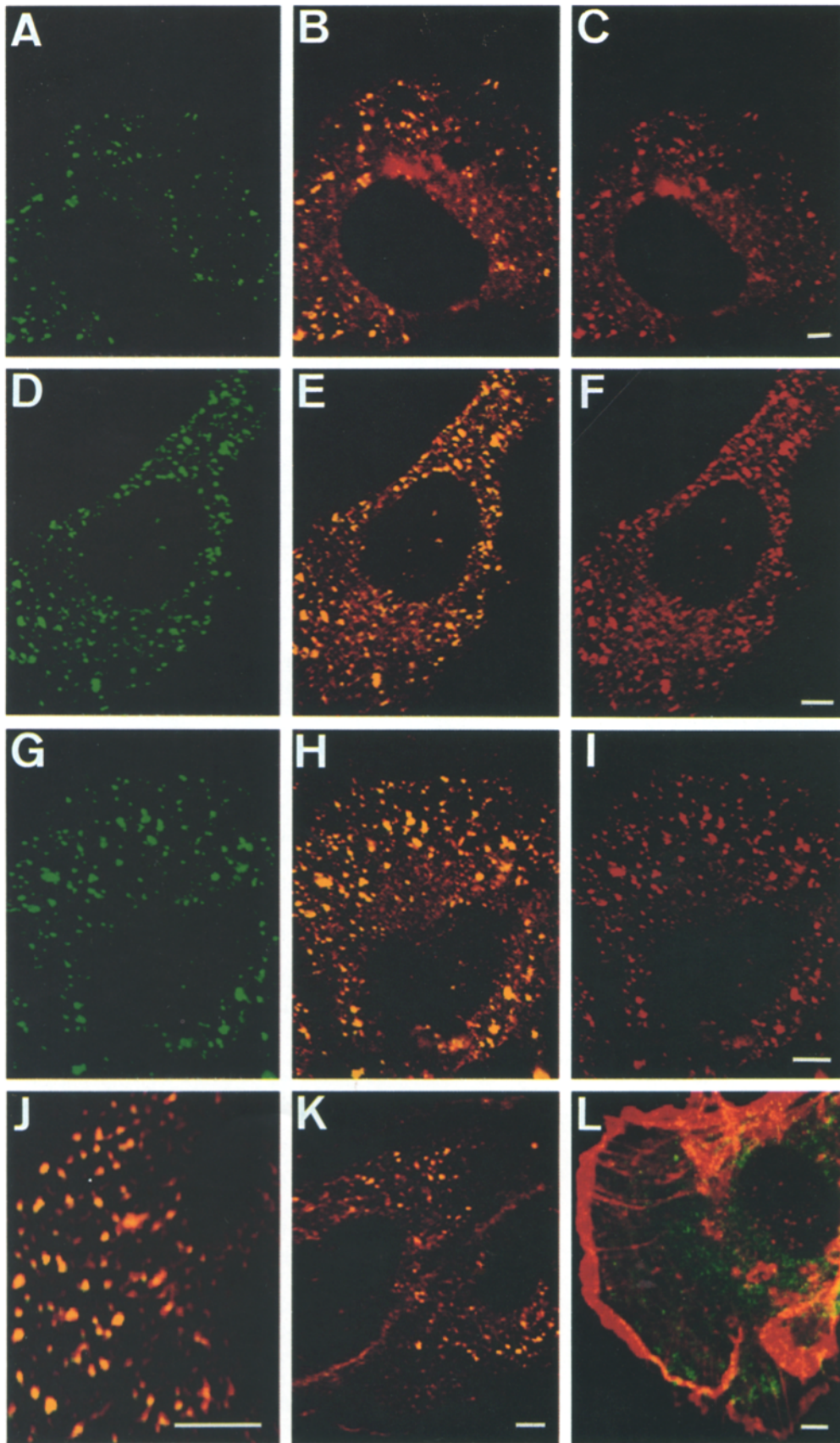


Figure 1. Double immunofluorescence labeling of M24met melanoma cells with antisera specific for uPAR (A, D, and G) and caveolin (C, F, and I). Cells were fixed and stained, as described in the Materials and Methods section. To demonstrate the colocalization of uPAR and caveolin images from both fluorescence, channels were superimposed (B, E, H, J, and K), colocalization is indicated by yellow color. Staining of the cells with normal mouse and rabbit sera as a control was negligible. Localization of F-actin was visualized by staining with rhodamine-phalloidin (1 U/slide) and compared with the cellular distribution of uPAR (L). Bars: 5 μ m.

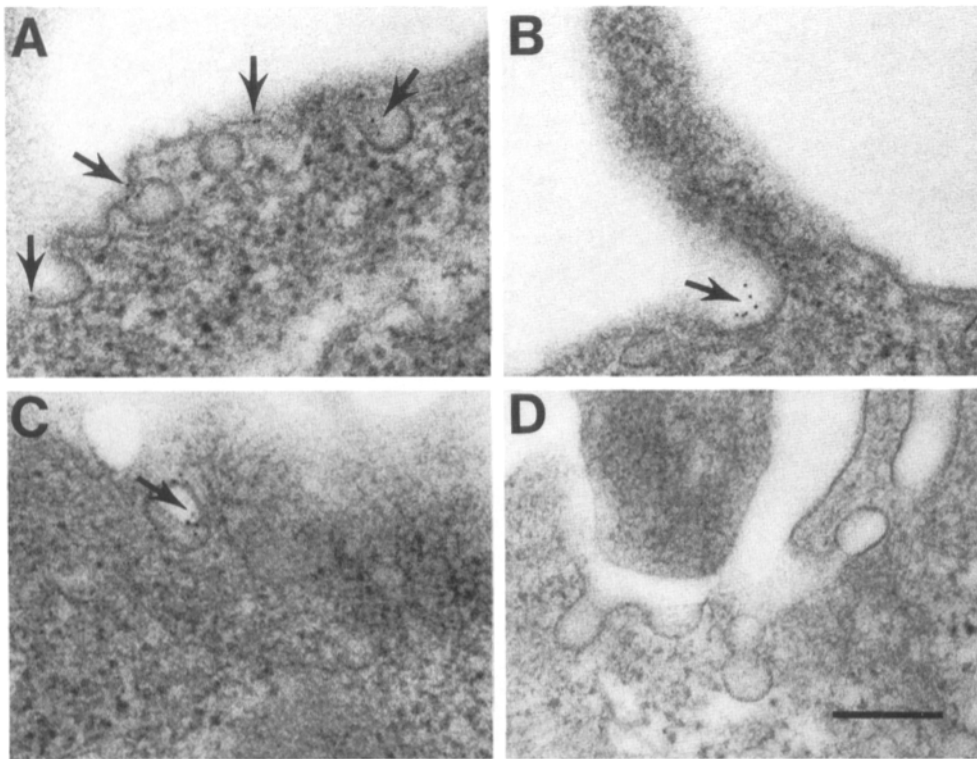


Figure 2. In vivo electron microscopy of M24met melanoma cells. Living cells were incubated with anti-uPAR specific rabbit polyclonal antibody (A–C) or preimmune rabbit serum (D), as described in the Materials and Methods section. The receptor was localized using a goat-anti-rabbit IgG 5-nm gold conjugate, arrows indicate the location of anti-uPAR antibodies. Bar, 200 nm.

in fractions 2 and 3, corresponding to a density range from 9.3 to 17.5% sucrose (Fig. 3). An additional 24% of the receptor specific signal was detected in fraction 4 (17.5–21.7% sucrose). Western blotting of the endogenously produced, cell surface-bound uPA with a polyclonal rabbit anti-uPA antibody showed a distribution similar to uPAR with 91% of the total signal in fractions 2 and 3 (Fig. 3). We compared the distribution of uPAR and its ligand in the density gradient with that of known markers for caveolae, namely caveolin and annexin II heavy and light chain. Western blotting of caveolin with a mouse monoclonal anti-caveolin antibody showed that 99% of the total signal was in fractions 2 and 3 (Fig. 3). This finding is in accordance with previous reports that low density Triton X-100 insoluble complexes representing caveolae are enriched in the 15–20% sucrose range of density gradients (Lisanti et al., 1993). Immunoblotting with mouse monoclonal anti-annexin II heavy and light chain antibodies showed that 16 and 20%, respectively, of the annexin II signals were in fractions 2 and 3 (Fig. 3).

uPAR Associates with Caveolin

GPI-linked proteins were reported to form stable complexes with cell surface molecules of distinct molecular sizes (14, 22–29, 40, 50, and 80 kD), caveolin (22–29 kD) being one of the major components (Lisanti et al., 1993). We reasoned that similar proteins may be associated with uPAR. M24met melanoma cells were surface biotinylated, lysed in NP-40 lysis buffer and immunoprecipitated with anti-uPAR antibodies. Both the mouse monoclonal and the polyclonal rabbit anti-uPAR antibodies coprecipitated uPAR (Fig. 4, *asterisks*) with other molecules in the approximate molecular mass ranges of 22, 40, 65, and 80 kD (Fig. 4), suggesting that uPAR is contained in complexes similar to those de-

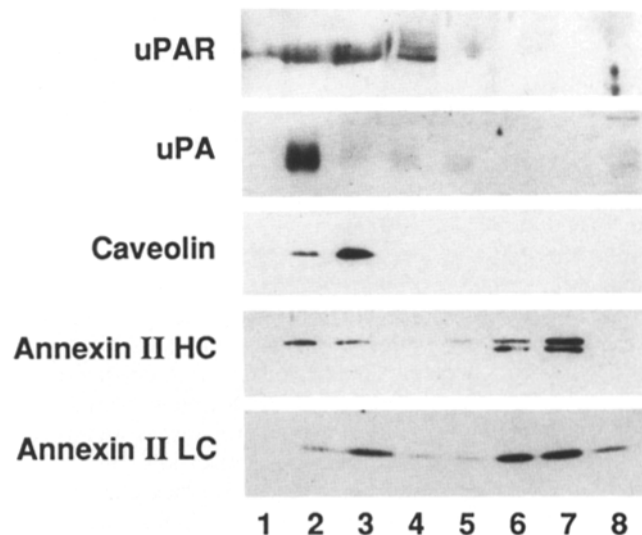


Figure 3. Western blotting of low-density Triton X-100 insoluble complexes from M24met cells. Cells were lysed and subjected to sucrose gradient centrifugation, as described in the Materials and Methods section. The sucrose gradients were divided into fractions with fraction 1–6 containing the 5–30% linear sucrose gradient, fraction 7 containing 40% sucrose, and fraction 8 the pellet. Equal amounts of protein from each fraction were separated by SDS-PAGE using a 5–15% acrylamide gradient gel and transferred to nitrocellulose. The indicated proteins were detected in their appropriate molecular mass range using specific antibodies against uPAR (55–60 kD), uPA (55 kD), caveolin (22 kD), annexin II heavy chain (HC, 39 kD), and annexin II light chain (LC, 11 kD).

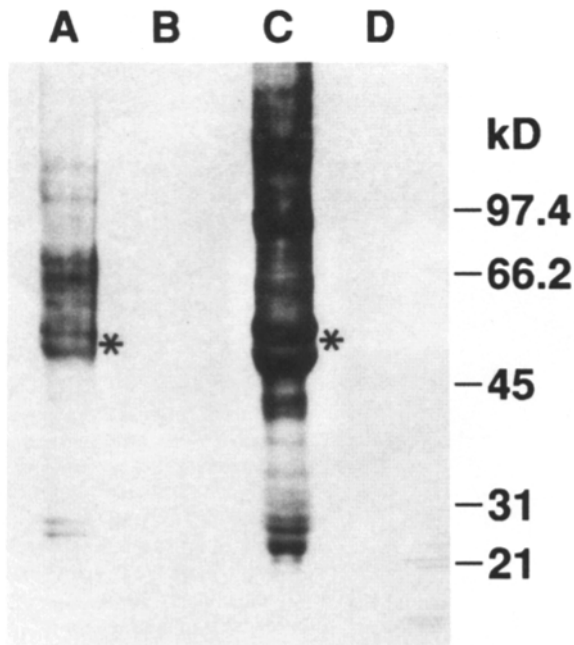


Figure 4. Immunoprecipitation of uPAR from surface biotinylated M24met cells. Immunoprecipitation of uPAR with either a uPAR specific mouse monoclonal antibody (A) or rabbit polyclonal serum (C) resulted in the specific coprecipitation of surface proteins in the molecular size range of 22, 40, 65, and 80 kD. The position of uPAR is indicated by asterisks. Immunoprecipitations were specific, since preimmune mouse serum (B) or rabbit serum (D) showed no signals.

scribed for other GPI-linked proteins (Lisanti et al., 1993). The finding of multiple bands in the 22–29-kD range implied the uPAR associates with caveolin. To further test this hypothesis, we performed cross-linking experiments with Triton X-100 insoluble proteins. To this end, M24met cells were extracted with 1% Triton X-100 for 30 min on ice. Under these conditions, the caveolae remained in the insoluble fraction attached to the cytoskeleton (Lisanti et al., 1993). The remaining insoluble proteins were cross-linked with DTSSP, a water-soluble homobifunctional NHS-ester cross-linker, that can be cleaved under reducing conditions. A second round of extraction was then performed with either 60 mM OGP or 3% Triton X-114. Both detergents have been reported to solubilize GPI-anchored proteins, as well as caveolin (Kurzchalia et al., 1992; Lisanti et al., 1993). The cross-linked complexes from the second extraction were then subjected to immunoprecipitation with a polyclonal rabbit antibody against uPAR. The specifically bound proteins were boiled in reducing sample buffer, separated by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with the monoclonal antibody against caveolin. Under both detergent conditions, anti-uPAR antibodies coprecipitated caveolin (Fig. 5), although extraction with OGP seemed to be more efficient. These results demonstrate that in M24met cells, uPAR and caveolin exist in detergent insoluble complexes in close spatial association, since a distance of 12Å or less between the two molecules can be deduced from the length of the cross-linker's spacer arm.

Disruption of Caveolae by Nystatin Reduces Cell-Surface Bound uPA Activity

The clustering of uPAR in caveolae suggested caveolae as a compartment for cell surface plasminogen activation. To study whether the structural integrity of caveolae is required for efficient plasminogen activation, we used the cholesterol-binding drug nystatin. Nystatin selectively disrupts caveolae, but does not effect clathrin-coated pits, actin cables, or other submembranous structures (Rothberg et al., 1992; Lisanti et al., 1993). Treatment of M24met cells with 50 µg/ml nystatin under serum-free conditions for up to 16 h had no effect on the morphology or viability of the cells (data not shown). In a cell free system, nystatin had no effect on the rate of plasmin generation by uPA or plasmin's ability to cleave the substrate peptide H-D-Val-Leu-Lys-p-nitroanilide. Furthermore, pretreatment of the cells with nystatin did not alter the amount of endogenous cell surface-bound uPA, as tested by indirect immunofluorescence, indicating that nystatin does not interfere with the binding of uPA to uPAR.

To test the effect of nystatin on cell surface plasmin generation by uPA/uPAR, M24met cells in 96-well plates were treated with nystatin at 50 µg/ml for 1 h. Plasminogen and a chromogenic substrate for plasmin, H-D-Val-Leu-Lys-p-nitroanilide, were added and the initial increase in reaction velocity was measured as ΔOD_{405} nm with measurements every 5 min. The endogenously produced uPA on the cell surface of the M24met cells converted plasminogen into plasmin leading to a rapid and linear increase in the cleavage of the plasmin substrate over the first 30 min of the reaction (Fig. 6). A polyclonal anti-uPA antibody, as well as an inhibiting monoclonal antibody against uPA, could block the reaction (Fig. 6 and Table I) demonstrating that uPA was the only significant plasminogen activator in this system. Nystatin treatment of the cells resulted in a 50% reduction of the rate of plasmin generation, measured as the increase in ΔOD_{405} nm (Fig. 6), indicating that the structural integrity of the caveolae plays an important role for the efficiency of plasminogen activation by surface-bound uPA. Our contention that the observed reduction of the rate of plasmin generation upon nystatin treatment was caused by the disruption of caveolae was supported by the effect of two other drugs that had also been reported to interfere with the morphology of caveolae. We used filipin, another cholesterol-binding antibiotic, that causes, like nystatin, an unclustering of GPI-linked molecules (Rothberg et al., 1990) and the protein kinase C-activating phorbol ester PMA, which had been shown to markedly reduce the number of caveolae and to inhibit potocytosis (Smart et al., 1994). Table I summarizes several experiments in which M24met cells were pretreated with nystatin, filipin, PMA, or anti-uPA antibodies. Both filipin and PMA treatment resulted in a dose-dependent reduction in the rate of plasmin generation.

Discussion

The aim of our present study was to determine the subcellular distribution of uPAR in a highly metastatic human melanoma cell line. This cell line was previously shown to use uPA/uPAR for the degradation of interstitial matrix and basement membrane (Montgomery et al., 1993), as well as for

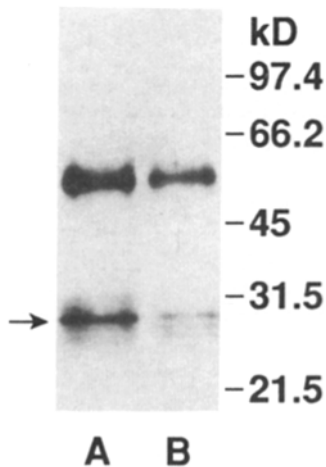


Figure 5. Western blot detection of caveolin coimmunoprecipitated with uPAR. Adherent M24met cells were first extracted with 1% Triton X-100, insoluble proteins were cross-linked with DTSSP, and then extracted with either OGP or Triton X-114. Extracts were subjected to immunoprecipitation with anti-uPAR serum, separated by SDS-PAGE under reducing conditions and caveolin was detected by Western blotting with an anti-caveolin mAb. OGP lysates are shown in lane A and Triton X-114 lysates in lane B. Note that the signal ~60-kD corresponds to the heavy chain of the antibody used for immunoprecipitation.

invasion through reconstituted basement membrane and for haptotactic migration (Stahl and Mueller, 1994). Here, we demonstrate that uPAR on M24met human melanoma cells is localized in caveolae and that caveolae provide an environment allowing for enhanced cell surface plasminogen activation.

Immunoelectron microscopy indicated that anti-uPAR antibodies localized the receptor on the surface of M24met cells in membrane invaginations which were observed in clusters along the plasma membrane and appear to be caveolae. When visualized by indirect immunofluorescence, anti-uPAR antibodies stained these cells in a punctated pattern, which completely colocalized with the signal from anti-

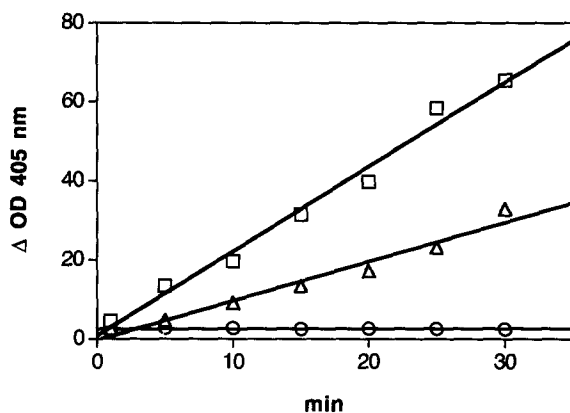


Figure 6. Inhibition of plasminogen activation by nystatin. Plasmin generation by endogenously produced cell surface-bound uPA on untreated M24met human melanoma cells (\square) was compared to cells treated with either 50 $\mu\text{g/ml}$ nystatin (Δ) or 0.2 mg/ml of an inhibiting mouse anti-uPA mAb (\circ). Plasmin generation was measured as ΔOD_{405} after the addition of plasminogen (2 μM) and H-D-Val-Leu-Lys-p-nitroanilide (2.2 mM) to the cells. Data points represent the mean of 8 wells. The experiment was performed three times with similar results.

Table I. Inhibition of Cell Surface Plasmin Generation by Drugs Interfering with the Morphology of Caveolae

Pretreatment	Concentration	Inhibition %
Nystatin	25 $\mu\text{g/ml}$	42.6
	50 $\mu\text{g/ml}$	53.5
Filipin	5 $\mu\text{g/ml}$	41.5
	20 $\mu\text{g/ml}$	42.8
PMA	0.1 μM	10.4
	1 μM	42.1
Rabbit polyclonal anti-uPA	0.2 mg/ml	86.2
Mouse monoclonal anti-uPA	0.2 mg/ml	99.0

Chromogenic plasmin assays were performed, as described in the Materials and Methods section. The rate of plasmin generation was calculated for each treatment using linear regression, as shown in Fig. 6.

caveolin antibodies. Based on these observations of native, as well as fixed and saponin permeabilized cells, we conclude that M24met human melanoma cells possess abundant plasmalemmal caveolae and that uPAR is localized in these caveolae. This is, to our knowledge, the first description of caveolae on neuro-ectodermal cells suggesting a general importance of these organelles on different types of cells, including tumor cells of neuro-ectodermal origin. The localization of uPAR in such plasmalemmal caveolae is in agreement with the previously described subcellular distribution of other GPI-linked proteins on cultured cells. In this regard, endogenous GPI-linked proteins (Rothberg et al., 1990; Ying et al., 1992), as well as GPI-linked proteins expressed after cDNA transfection (Ying et al., 1992; Keller et al., 1992) were demonstrated to be preferentially localized in caveolae.

In addition to the localization of uPAR in caveolae, immunogold staining for uPAR also labeled sporadically, but consistently, coated pits and cellular processes of M24met cells (data not shown). The localization of some uPAR in coated pits is consistent with previous work (Nykjaer et al., 1992; Li et al., 1994) that demonstrated that the α_2 macroglobulin receptor/low density lipoprotein receptor-related protein mediates the internalization of uPA-plasminogen activator inhibitor complexes bound to uPAR, a process occurring most likely in clathrin-coated pits.

Occasionally, immunofluorescence demonstrated uPAR and caveolin on M24met cells concentrated in areas of cell-cell contacts or membrane ruffles. Similarly, uPAR was described previously in areas of cell-cell contact for HT 1080 cells (Pöllänen et al., 1987) and rhabdomyosarcoma cells (Myöhänen et al., 1993). In contrast, we found no evidence for a preferential localization of uPAR at the interface between cell and substrate or for the presence of the receptor in sites of focal contacts. In this regard our observations differ from the distribution of uPAR on HT 1080 fibrosarcoma cells and cultured fibroblasts on which uPAR was described to colocalize with vinculin in focal contacts (Pöllänen et al., 1988; Hebert and Baker, 1988). Generally, the distribution of uPAR on M24met melanoma cells resembles

more closely the punctated pattern of uPAR staining found on amnion WISH cells (Busso et al., 1994) and the granular pattern that was described for MCF7 breast carcinoma cells (Zhang et al., 1993).

The localization of uPAR in caveolae was further supported by our biochemical analysis of low density, detergent insoluble complexes that were previously shown to contain membrane fractions highly enriched for caveolae (Sargiacomo et al., 1993). We demonstrated uPAR, its ligand uPA, and caveolin by immunoblotting almost exclusively in low density, Triton X-100 insoluble fractions of M24met lysates. uPAR exhibited a somewhat broader density distribution than uPA and caveolin, with 93% of the uPAR signal in the ranges of 9–22% sucrose, and 91% of the uPA and 99% of the caveolin signal, respectively, in the ranges of 9–17.5 sucrose. Thus, ~70% of uPAR is colocalized with caveolin, while a subpopulation of uPAR appears to be associated with neither uPA nor caveolin. This distribution might suggest that under certain thus far undefined conditions, GPI-linked molecules exist in Triton X-100 insoluble, low density complexes that differ from caveolin-coated caveolae.

After surface biotinylation of intact living cells, anti-uPAR antibodies coprecipitated a molecule with a similar molecular weight as caveolin. A close spatial association between uPAR and caveolin was further suggested by their coprecipitation from the Triton X-100 insoluble fraction of M24met cells after cross-linking. The presence of uPAR in low density, detergent insoluble complexes and the spatial association between uPAR and caveolin indicate that uPAR is localized in caveolae in the absence of cross-linking antibodies. It was suggested recently (Mayor et al., 1994) that GPI-linked proteins cluster in caveolae only as a result of physiologically or experimentally induced cross-linking. Accordingly, the presence of uPAR in caveolae on our metastatic melanoma cell line may imply that the receptor is cross-linked as a consequence of uPA binding and/or interaction with vitronectin (Wei et al., 1994) or other components of the extracellular matrix.

GPI-linked proteins have been demonstrated to interact with nonreceptor tyrosine kinases of the src family (Stefanova et al., 1991; Thomas and Samelson, 1992; Shenoy-Scaria et al., 1992) suggesting a role for GPI-linked proteins in signal transduction processes. Based on the analysis of low density detergent insoluble complexes, it has been proposed that the src substrate caveolin may function as a transmembrane adaptor protein that couples GPI-anchored proteins with cytoplasmic signaling molecules (Sargiacomo et al., 1993). Catalytically inactive uPA can induce cellular responses, such as proliferation and migration (Kirchheimer et al., 1989; Odekon et al., 1992; Gyetko et al., 1994; Stahl and Mueller, 1994) implicating uPAR as a molecule involved in signal transduction. The mechanism by which GPI-linked proteins, such as uPAR, could activate cells is presently unknown. The close spatial association between uPAR and caveolin described here, suggests that caveolin could be involved in signal transduction mediated by uPAR.

Both uPAR and its ligand, uPA, were detected exclusively in the low density, Triton X-100 insoluble fraction of melanoma cell lysates, which suggests that caveolae may serve as a specialized compartment for pericellular plasminogen activation. The structural integrity of caveolae depends on cholesterol (Rothberg et al., 1992). Cholesterol-binding drugs,

such as nystatin, have been shown to disperse both the striated coat of caveolae and clustered GPI-linked folate receptors (Rothberg et al., 1992) without affecting submembranous structures. Treatment of M24met cells with nystatin and other drugs interfering with the structure of caveolae reduced the rate of cell surface plasmin generation up to 50% indicating that the clustering of uPAR and uPA in caveolae enhances cell surface plasminogen activation. This inhibition was not due to a direct effect of the drugs on the catalytic activity of uPA or plasmin or on the binding of uPA to uPAR. The effect was also not restricted to cholesterol-binding drugs, since PMA, which reduces the number of caveolae probably by activating protein kinase C, caused also a reduction in the rate of plasmin generation. The contention that uPAR enhances uPA's catalytic activity at least partly by localizing it in caveolae is strengthened by the recent finding that recombinant uPA linked with a GPI-anchor to the cell membrane, and thus likely clustered in caveolae, shows almost the same rate of enhanced catalytic activity as uPA bound to uPAR (Lee et al., 1994).

Caveolae may allow for enhanced cell surface plasminogen activation by clustering uPAR/uPA and other components of the fibrinolytic system, thus providing a favorable environment for protease cooperation. In this regard, it may be significant that we find annexin II, which has been described as a receptor for plasminogen and tissue type plasminogen activator (Hajjar et al., 1994; Cesarman et al., 1994), also in the low density Triton X-100 insoluble fraction of M24met melanoma cells. The ganglioside GM1, which is another plasminogen-binding molecule (Miles et al., 1989), is also localized in caveolae (Parton, 1994). Furthermore, a close association of uPA- and plasminogen-receptors has been suggested (Zhang et al., 1993) by colocalization of anti-uPAR antibodies and biotinylated plasminogen on MCF7 cells.

In summary, we demonstrate here that the GPI-anchored protease receptor uPAR on a metastatic human melanoma cell line is localized in caveolae and that the structural integrity of caveolae is a prerequisite for efficient cell surface plasminogen activation, thus implicating caveolae as a cell membrane specialization involved in the regulation of cell activation and pericellular proteolysis.

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