

# Ultrastructural Localization of Plasma Membrane-associated Urokinase-type Plasminogen Activator at Focal Contacts

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**Abstract.** We have recently shown that urokinase-type plasminogen activator (u-PA) and plasminogen activator inhibitor type 1 are both found extracellularly beneath cultured human skin fibroblasts and HT-1080 sarcoma cells, but in distinct localizations. Here, the ultrastructural distribution of u-PA was studied using immunoferritin electron microscopy. In HT-1080 cells, u-PA on the extracellular aspect of the plasma membrane was detected at sites of direct contact of the cell with the growth substratum beneath all parts of the ventral cell surface. The ferritin-labeled adhesion plaques, which were enriched in submembraneous microfilaments, were frequently seen at the leading lamellae of the cells as well as in lamellipodia and microspikes. Besides the cell-substratum adhesion plaques, ferritin

label was detected at cell-cell contact sites.

Double-label immunofluorescence showed a striking colocalization of u-PA and vinculin in both HT-1080 cells and WI-38 lung fibroblasts, which is consistent with u-PA being a focal contact component. The u-PA-containing focal contacts of WI-38 cells had no direct codistribution with fibronectin fibrils. In WI-38 cells made stationary by cultivation in a medium containing 0.5% FCS, vinculin plaques became highly elongated and more centrally located, whereas u-PA immunolabel disappeared from such focal adhesions. These findings show that plasma membrane-associated u-PA is an intrinsic component of focal contacts, where, we propose, it enables directional proteolysis for cell migration and invasion.

**A**DHESIVE interactions of cells with the extracellular matrix and their reversal are critical events for morphogenetic movements during embryonic development and for cancer cell invasion during metastasis (30, 49). The molecular structures at the sites where cultured cells contact each other and the growth substratum are thus of great interest. Focal contacts, the sites of closest cell-substratum apposition, can be visualized either by electron microscopy (1) or by interference reflection microscopy (23). Focal contacts are localized at the termini of actin-containing microfilament bundles, and contain vinculin (7, 19) and talin (6) on the cytoplasmic side, but apparently do not have fibronectin on the extracellular side (9). The so-called close contacts exhibit a greater substrate separation distance, do not contain vinculin, and probably represent a more labile type of contact (23). A third kind of substrate adhesion site termed the fibronexus (42) or extracellular matrix contact site (9) is characteristic of well-spread and stationary fibroblasts. It features a codistribution of fibronectin, its plasma membrane receptor, actin, vinculin, and alpha-actinin throughout the entire contact site and is located at central regions of the cell rather than at its margins (11, 15). Fibronexuses show very close transmembrane associations of actin microfilaments and fibronectin fibrils (42, 43).

Urokinase-type plasminogen activator (u-PA)<sup>1</sup> is one of the two well-characterized types of serine proteinases that convert the extracellular proenzyme plasminogen into the active proteinase plasmin (for reviews see references 4, 16). It is synthesized as an inactive single-chain proactivator (pro-u-PA) by many types of cells (17, 32, 44, 45, 52), and most of the enzyme is in the proactivator form also in vivo (28). Pro-u-PA is converted to the active two-chain form in a reaction that may be catalyzed by plasmin. Specific u-PA receptors were recently detected in human monocytes and monocyte-like cells (46, 50), and also in several other cell types (for reviews see references 3, 4). These receptors bind u-PA at its growth factor domain (2), with a high affinity,  $K_d$   $10^{-10}$  M. According to experiments with human A-431 epidermoid cells, pro-u-PA is first secreted and then bound to its specific plasma membrane receptors (47).

PAs are thought to play an important role in controlling extracellular proteolysis (37). Plasminogen and PAs have affinity for extracellular matrix components laminin (40) and fibronectin (39), and a  $M_r$  66,000 matrix-associated protein

1. *Abbreviations used in this paper:* anti-TNP-IgG, monoclonal mouse IgG antibody against trinitrophenyl group; pro-u-PA, inactive single-chain proactivator; TRITC, tetramethylrhodamine isothiocyanate, u-PA, urokinase-type plasminogen activator.

seems to be a plasminogen-independent target for u-PA (27). Furthermore, plasmin is able to activate latent collagenases, proteinases involved in the degradation of interstitial matrix and basement membranes (33, 34, 38). Extracellular matrix degradation by Rous sarcoma virus-transformed fibroblasts probably consists of both plasminogen-dependent and -independent pathways (18), and a direct catalytic role for PA in the process has been suggested (48). External proteolysis taking place at cellular contact sites would be advantageous to cells actively migrating or invading surrounding tissues. Focal proteolysis of fibronectin substrate occurs in Rous sarcoma virus-transformed fibroblasts (10, 12), involving a  $M_r$  150,000 metalloendoproteinase and a  $M_r$  120,000 serine proteinase (8).

We have previously shown that u-PA is extracellularly located beneath cultured cells, and that the distributions of u-PA and plasminogen activator inhibitor type 1 under the cells are mostly distinct (35). In this report, we have identified the u-PA-containing structures. To do this, immunoferritin bridge electron microscopy was chosen as a method to examine the ultrastructural distribution of extracellular u-PA in HT-1080 sarcoma cells. In addition, double-label immunofluorescence staining of u-PA and vinculin was performed.

## Materials and Methods

### Cell Cultures

The human fibrosarcoma cell line HT-1080 and WI-38 human embryonic lung fibroblasts were obtained from American Type Culture Collection, Rockville, MD (ATCC, CCL 121, and CCL 75, respectively). The cells were grown in plastic dishes (53 cm<sup>2</sup> or 19.7 cm<sup>2</sup>) in Eagle's minimal essential medium (MEM) supplemented with 10% FCS (Gibco, Paisley, UK), 100 IU/ml penicillin and 50 µg/ml streptomycin. Cells were plated on glass coverslips and fixed and processed after 24 h of subculture at subconfluent density or were processed as specified in the text.

### Immunofluorescence

Polyclonal purified rabbit IgG against u-PA was prepared as described elsewhere (29) and detected both active u-PA and its proenzyme (31). Monoclonal anti-vinculin mouse IgG<sub>1</sub> was from Bio-Yeda (Kiryat Weizmann, Rehovot, Israel). Monoclonal mouse IgG<sub>1</sub> fpg-9 to fibronectin was described previously (35). Monoclonal mouse IgG antibody against the trinitrophenyl group (anti-TNP-IgG) was from the hybridoma clone designated Hy 2.15 (41). FITC-conjugated sheep anti-mouse IgG was from Cappel Laboratories, Cochranville, PA, and tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG from Nordic Immunological Laboratories, Tilburg, The Netherlands.

For immunofluorescent staining, rinsed coverslip cultures of cells were fixed with -20°C methanol. Cells were treated with the primary antibodies diluted in PBS at a concentration of 50 µg/ml (anti-u-PA-IgG) or hybridoma supernatants diluted 1:30 (monoclonal antivinculin or fpg-9 antifibronectin) for 30 min. This was followed by a short rinse in PBS and treatment with the fluorochrome (FITC/TRITC)-conjugated secondary antibodies for 30 min. For double immunofluorescent studies of u-PA and vinculin/fibronectin the cells were treated in the following sequence: rabbit anti-u-PA IgG, goat anti-rabbit IgG-TRITC, monoclonal anti-vinculin/fpg-9, sheep anti-mouse IgG-FITC. Immunocytochemical controls consisted of anti-u-PA-IgG preabsorbed with highly purified u-PA (29), preimmune IgG from the u-PA-immunized rabbit or mouse monoclonal anti-TNP-IgG, as well as omission of any one of the steps in the staining procedure. After rinsing with PBS the coverslips were mounted in glycerol-PBS. The specimens were viewed with an EB microscope (Leitz Dialux, Wetzlar, Germany) and photographed as previously described (35).

### Immunoferritin Electron Microscopy

To select an optimal method of processing the cells for experiments at the EM level, we tested several alternatives in immunofluorescence using FITC-

conjugated anti-ferritin-IgG (22). The cells were fixed with various concentrations of paraformaldehyde (0.1–4%) and/or glutaraldehyde (0.01–2%, EM grade; Leiras, Turku, Finland), either before or after the entire labeling sequence, or after treatment with the primary antibody.

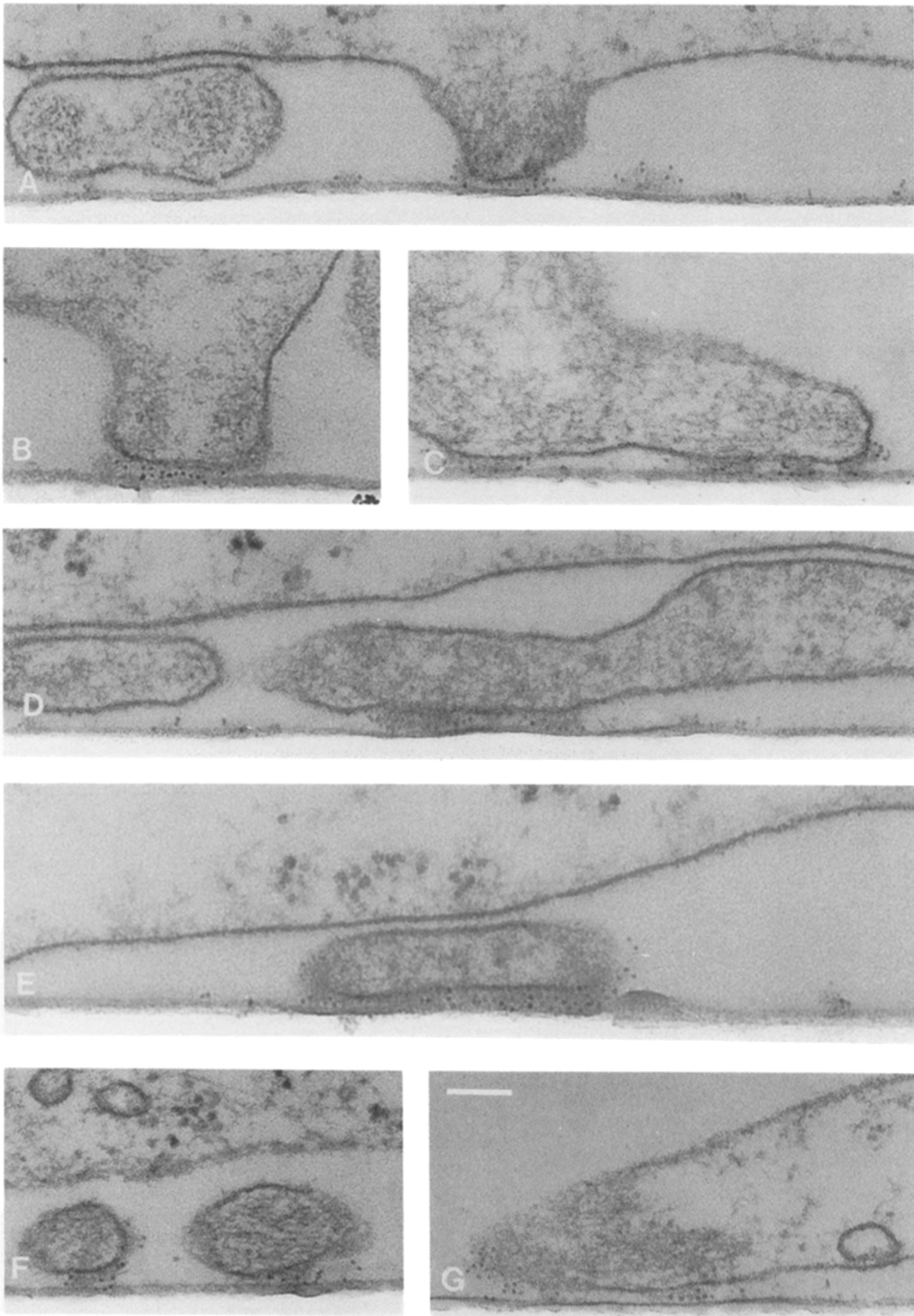
In the immunoferritin experiments, cultured HT-1080 cells were briefly rinsed three times with Dulbecco's PBS (DPBS) containing 0.2% BSA at 0°C, and then incubated with the primary antibody (anti-u-PA-IgG, 50 µg/ml). After gently rinsing three times with DPBS-BSA, labeling was performed using the ferritin bridge procedure (51) with incubations in the following sequence: affinity purified goat anti-rabbit IgG (100 µg/ml; Jackson Immuno Research Laboratories Inc., Avondale, PA), affinity-purified rabbit anti-horse ferritin (100 µg/ml; Jackson Immuno Research Laboratories Inc.), ferritin (100 µg/ml; Sigma Chemical Co., St. Louis, MO). Each step of the labeling sequence was performed at 0°C for 30 min and was followed by three 5-min rinses with DPBS-BSA. Then the cells were fixed with 2% glutaraldehyde (Leiras) in 0.1 M phosphate buffer, pH 7.2, for 30 min. As a specificity control for the immunoferritin staining, 50 µg/ml of the preimmune IgG was used. The rinsed samples were postfixed with 1.5% OsO<sub>4</sub> in 0.1 M cacodylate buffer, pH 7.2, for 1 h at 23°C and dehydrated with ethanol. The culture dishes were mounted in Epon 812. After 2 d polymerization, the Epon blocks were sectioned vertically and horizontally against the cell layer. Thin sections were post-stained with uranyl acetate and lead citrate, and were observed and photographed in a Jeol 1200 EX electron microscope at 60 kV at the Department of Electron Microscopy, University of Helsinki, Finland. The position of the u-PA antigen was estimated from photographic prints at a final magnification of 60,000. From the prints, the distance of 200 ferritin cores was measured from perpendicularly sectioned segments of the plasma membrane.

## Results

### Distribution of u-PA in Immunoferritin Electron Microscopy

To study the external distribution of u-PA at the EM level, we labeled HT-1080 sarcoma cells using the immunoferritin bridge procedure. In preliminary experiments, we tested several methods of processing the cells for immunofluorescence using FITC-conjugated anti-ferritin-IgG. The cells were fixed with paraformaldehyde and/or glutaraldehyde, either in the beginning or at the end of the entire ferritin bridge sequence, or after treatment with the primary antibody. Of these, labeling of live cells at 0°C before fixation gave the strongest signal in the areas of cell-substratum contact, and detected exclusively extracellular antigen. The resulting pattern of u-PA staining was qualitatively identical to that obtained using methanol prefixation in the ferritin bridge sequence, as well as in conventional immunofluorescence (35; figures not shown). Thus, the pattern obtained by the antibody treatment of live cells in the cold reflected the situation at physiological temperature examined under prefixation conditions.

At the EM level, the u-PA immunoreactivity was detected as discrete patches of ferritin cores associated with the plasma membrane. The ferritin was predominantly found at sites where the cells made contact with the growth substratum beneath all parts of the ventral cell surface. Such contacts were best observed in sections cut vertically against the cell layer. These ferritin-labeled plaques had a close substratum separation distance of 15–50 nm (Fig. 1, A and B). In rare instances, e.g., Fig. 1 A, some ferritin was on the growth substratum without any apparent relationship to the plasma membrane. The mean distance of the ferritin cores from the plasma membrane at these locations, such as illustrated in Fig. 1, was  $21 \pm 5$  nm. These plaques were enriched in a thick submembraneous patch of microfilaments and were frequently seen at the leading lamellae of the cell (Fig. 1, C, D, and G).



**Figure 1.** Immunoferritin EM labeling for u-PA in cultures of HT-1080 cells using polyclonal rabbit IgG. The cells were stained live with the immunoferritin bridge procedure at  $\pm 0^{\circ}\text{C}$  before fixation with 2% glutaraldehyde. Note the plasma membrane-associated ferritin labeling at focal cell adhesion sites (A-G). A and B are from the central regions of the cell; C, D, and G from the leading lamellae; E and F from cross-sectioned cell extensions (lamellipodia). Vertical thin sections with uranyl acetate-lead citrate post-staining. Bar, 100 nm.

Ferritin was often detected between cross-sectioned cellular extensions (lamellipodia and microspikes) and the substratum (Fig. 1, *E* and *F*). Such microspikes could be well visualized in horizontal sections made along the cell-substratum interface (Fig. 2). These extensions were also often enriched in a submembraneous patch of microfilaments, the linear orientation of which is clearly seen inside the microspikes in Fig. 2, *A* and *B*.

In addition to the cell-substratum adhesion plaques, ferritin had a preferential localization at sites of cell-cell contact (Fig. 3, *A* and *B*). Control cultures in which the primary antibody was substituted with 50 µg/ml preimmune u-PA IgG, gave virtually no binding of ferritin cores to the plasma membrane (Fig. 4).

#### **Characterization of u-PA-containing Sites as Focal Contacts**

To further characterize the sites with u-PA immunoreactivity seen at the EM level, we performed double immunofluorescent labeling of cultured HT-1080 and WI-38 cells with polyclonal anti-u-PA IgG and monoclonal anti-vinculin IgG. Focalized u-PA immunoreactivity was found in all parts of the ventral cell surface, including the trailing edge, and in considerable amounts at the leading edge. Additionally, diffuse granular intracellular localization of u-PA was detected (Figs. 5 *B* and 6 *B*). u-PA and vinculin displayed a striking colocalization at the focal contact sites on the ventral cell surface (Fig. 5, *A* and *B*). A similar codistribution of u-PA and vinculin was also detected in WI-38 human embryonic lung fibroblasts (Fig. 6, *A* and *B*). In these cells the

labeled sites were a little more elongated than those in the HT-1080 cells.

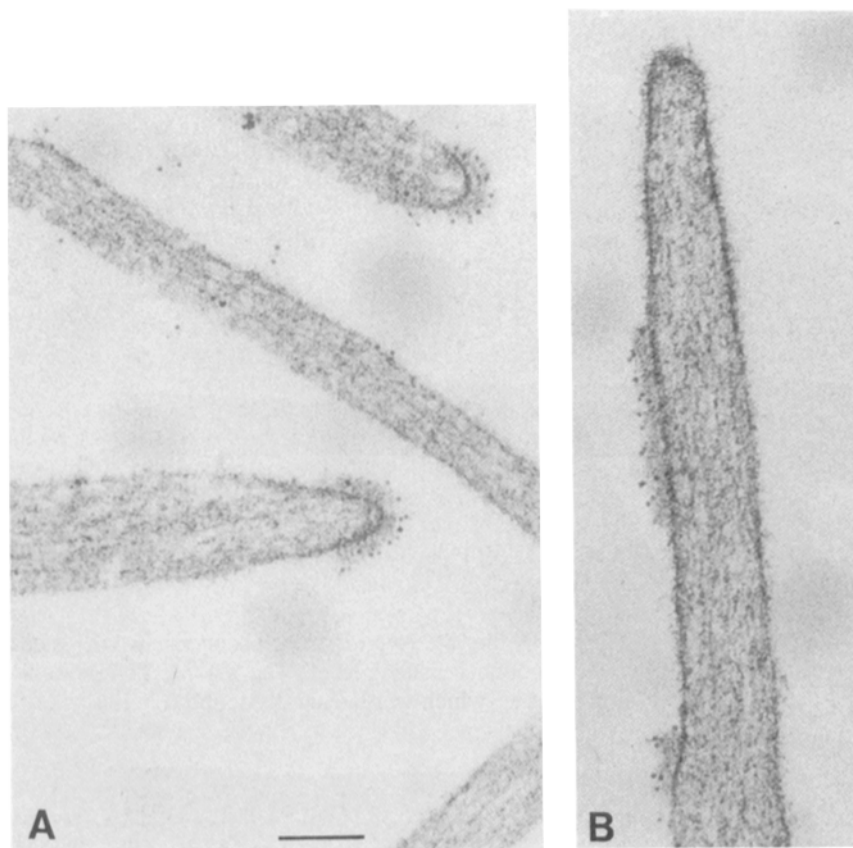
We also compared the distributions of u-PA and fibronectin in these two cell lines. HT-1080 cells deposit very little pericellular matrix fibronectin in contrast to WI-38 fibroblasts grown for 24 h in 10% serum. As judged by double immunofluorescent labeling, most of the fibronectin fibrils were not superimposed with the u-PA and vinculin containing contact sites in WI-38 cultures (Fig. 6, *C* and *D*).

In WI-38 lung fibroblasts growth arrested in 0.5% FCS, anti-u-PA label diminished markedly. Specifically, no focalized u-PA immunostaining was seen after 4 d of serum depletion. Double-label immunofluorescence of the cells at this stage detected vinculin arranged in highly elongated striae that were more centrally located (Fig. 7, *A* and *B*). The stationary WI-38 cells deposited an abundant fibronectin matrix (not shown). Confluent control cultures grown in 10% serum for 4 d showed a colocalization of u-PA and vinculin (not illustrated) similar to that in Fig. 6, *A* and *B*.

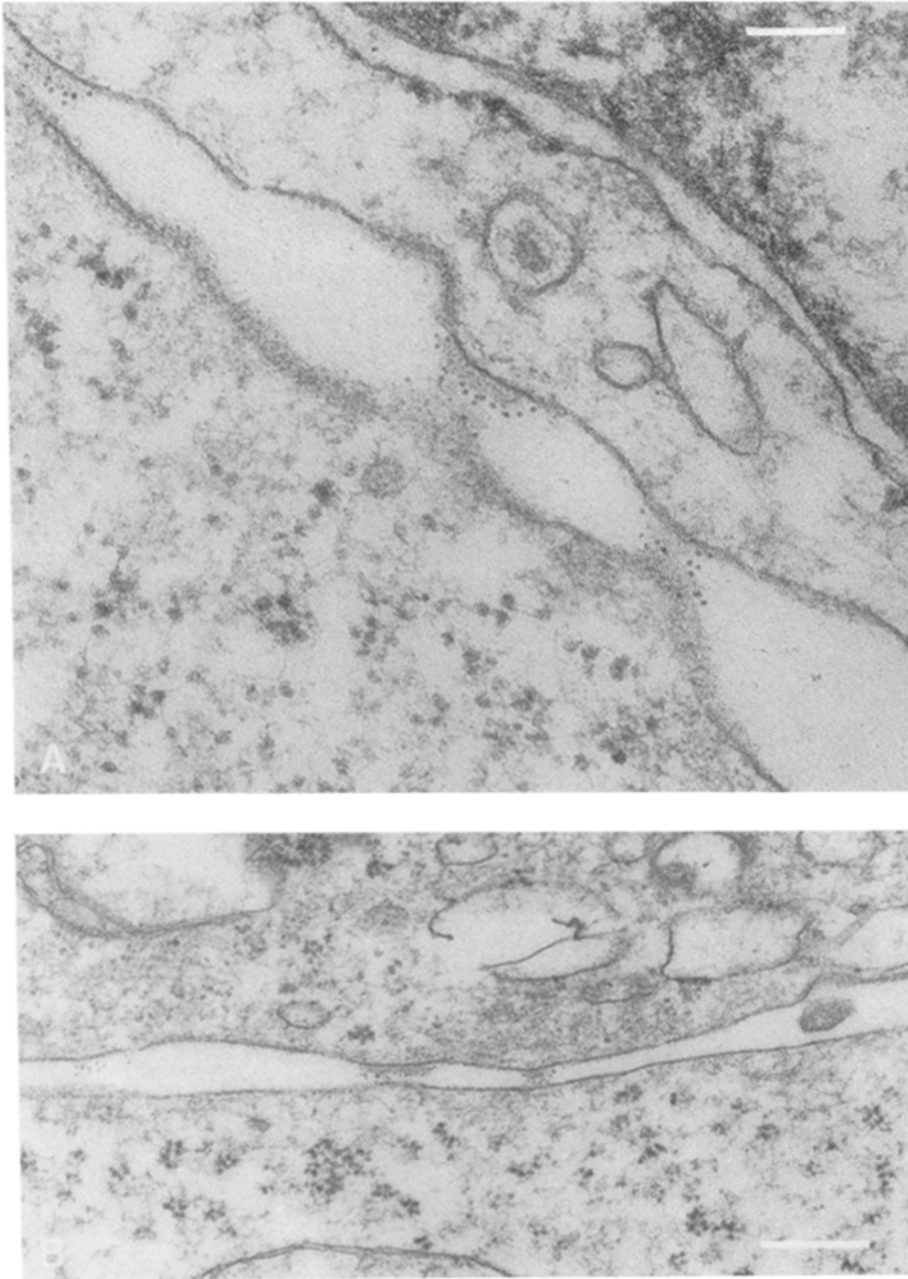
Each staining reaction was specific as tested by either u-PA-blocked or preimmune anti-u-PA IgG, as shown before (35), or using anti-TNP-IgG, and/or by omission of any step in the staining sequence (figures not shown).

#### **Discussion**

In the present study, we have shown that urokinase-type plasminogen activator is an intrinsic component of focal contacts of human lung fibroblasts and sarcoma cells, in contrast to the two other types of cell-substratum contact, i.e. close



**Figure 2.** Immunoferritin EM labeling for u-PA on HT-1080 cells: staining of live cells at  $\pm 0^{\circ}\text{C}$  by the immunoferritin bridge procedure before fixation with 2% glutaraldehyde. Thin sections were cut horizontally against the cell layer. Ferritin labeling is seen on the plasma membrane in lamellipodia and microspikes (*A* and *B*). Uranyl acetate-lead citrate post-staining. Bar, 100 nm.



*Figure 3.* Immunoferritin EM labeling for u-PA (*A* and *B*) on HT-1080 cells. Staining of the cells at  $\pm 0^{\circ}\text{C}$  before fixation with 2% glutaraldehyde. Note the ferritin patches associated with the plasma membrane at sites of cell-cell contacts. Horizontal (*A*) and vertical (*B*) thin sections with uranyl acetate-lead citrate post-staining. Bars, 100 nm in *A*, 200 nm in *B*.

contacts and fibronexuses, in which u-PA was not detected. This finding was based upon (*a*) immuno-EM, and (*b*) double-label immunofluorescence of u-PA and vinculin. In addition to focal contacts, u-PA was located at cell-cell contact sites. In previous cell fractionation studies, PA has been generally detected in membrane-rich fractions (reviewed in reference 16). These biochemical studies did not, however, exclude the possibility that u-PA became bound to the membranes after disruption of cells. The immuno-EM data presented here (Figs. 1-3) show that u-PA was located at specific sites on the extracellular aspect of the plasma membrane. This is consistent with recent reports on specific cell-surface receptors for u-PA (reviewed in reference 3, 4).

In immunoferritin bridge electron microscopy of sarcoma cells, plasma membrane-associated u-PA antigen was detected at (*a*) adhesion plaques, (*b*) cell-cell contacts, and (*c*)

on cellular extensions, lamellipodia and microspikes. These features in the ultrastructural localization of u-PA distinguish it, e.g. from the major extracellular matrix protein fibronectin, which is not located at close cell-cell contacts (22). The possibility that the ferritin patches obtained were due to antibody-induced redistribution of the u-PA antigen seems remote, because the labeling protocol used in immuno-EM gave an identical staining pattern with methanol prefixation followed by the same ferritin bridge sequence when tested at the immunofluorescence level. The u-PA containing adhesion plaques, which were generally found in all parts of the ventral cell surface, were frequently seen at the leading lamellae of the cells, bearing a close resemblance in ultrastructure to those described in motile fibroblasts (1). Earlier studies on normal locomotory fibroblasts have shown that new focal contacts are formed at the active edge by advancing

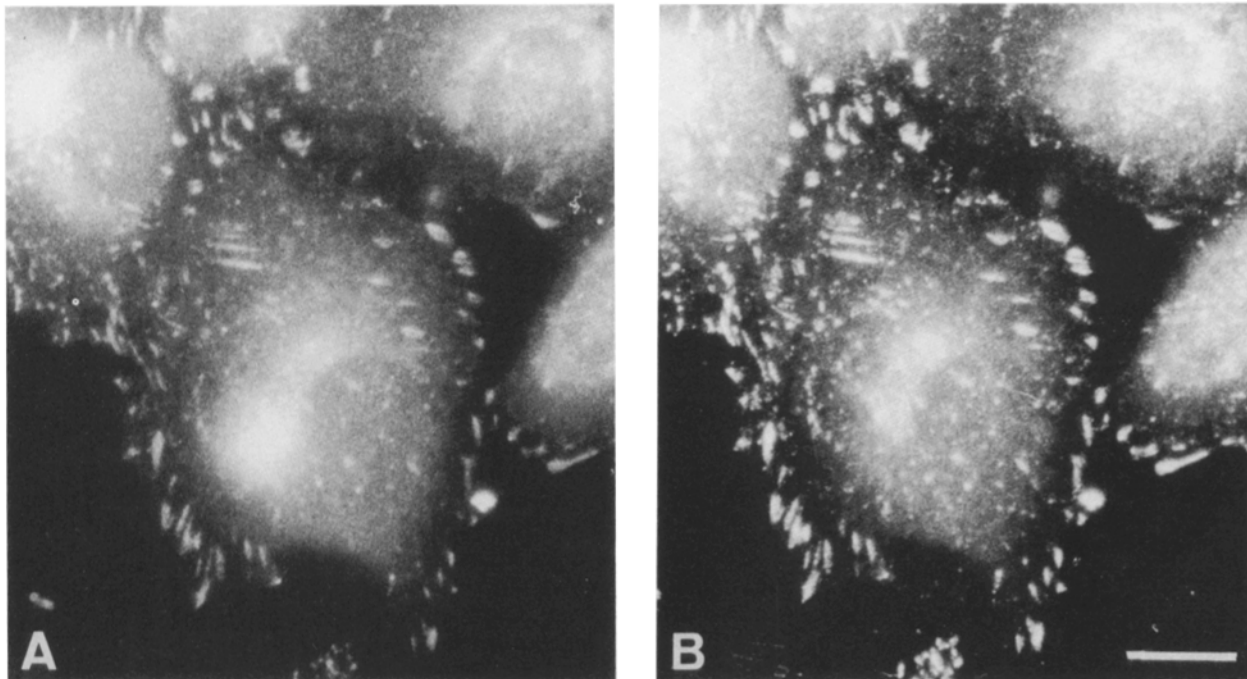


**Figure 4.** Specificity control for immunoferritin labeling. HT-1080 cultures were stained with 50  $\mu\text{g/ml}$  preimmune anti-u-PA IgG as the primary antibody. No ferritin particles are seen in the vertical thin section that features a cellular extension in contact with the substratum. Bar, 100 nm.

microspikes and lamellipodia, but later the contacts increased in size (5, 24). On the other hand, few reports exist on the focal contacts of transformed cells (36).

Double-label immunofluorescence showed a striking colocalization of u-PA and vinculin in HT-1080 cells and WI-38 fibroblasts. The u-PA plaques had no direct codistribution

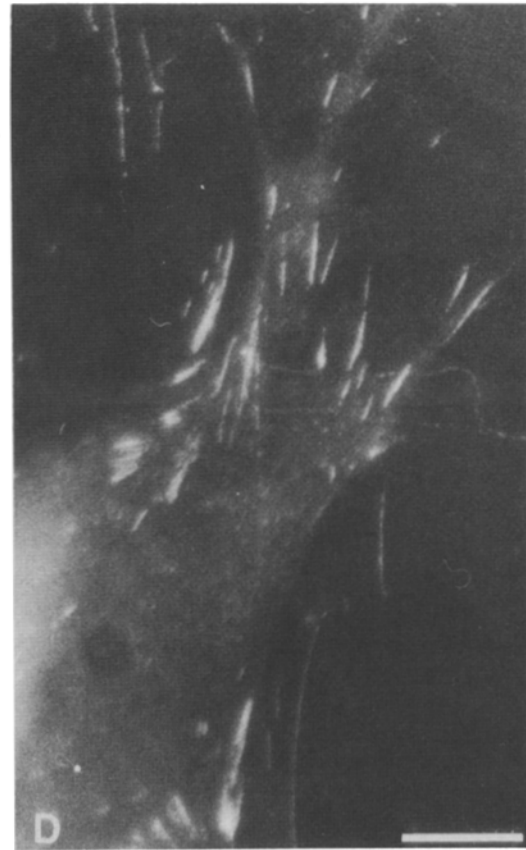
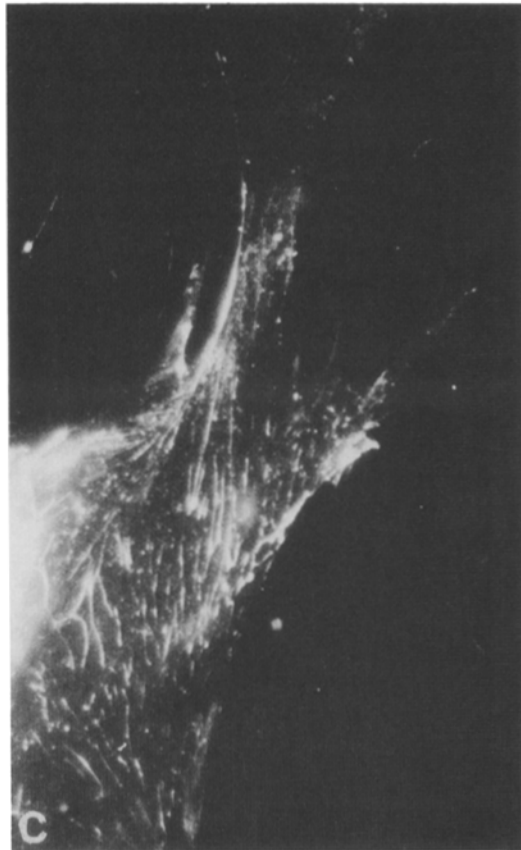
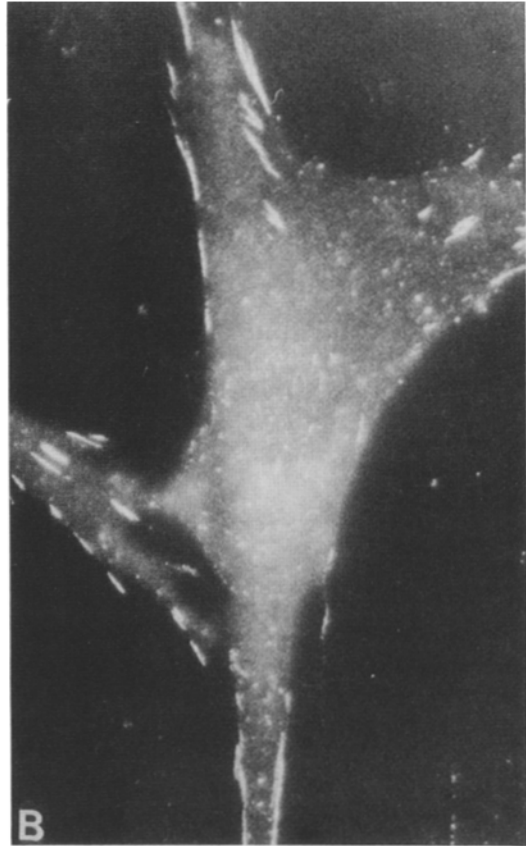
with fibronectin fibrils in WI-38 cells. Both these features, the presence of vinculin (19, 20) and the apparent absence of fibronectin (9 and references quoted therein), have been reported to be characteristic of focal contacts. However, u-PA was not found in adhesions made by stationary fibroblasts in 0.5% serum. During such conditions u-PA immunostaining

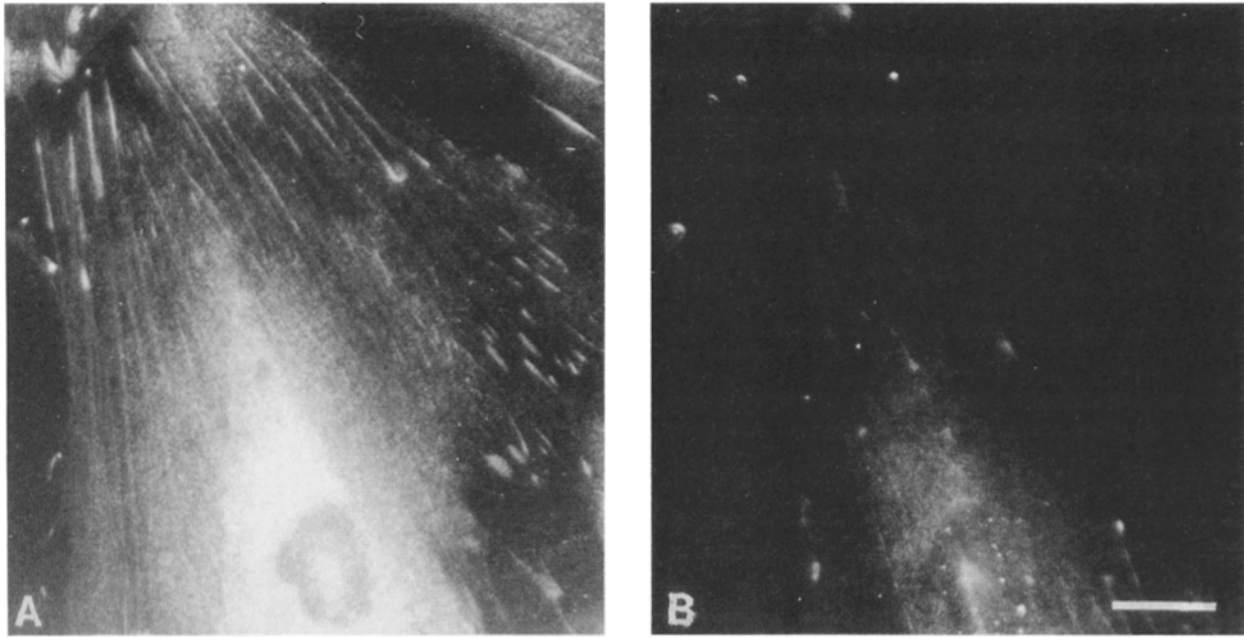


**Figure 5.** Double immunofluorescent localization of vinculin (A) and u-PA (B) in HT-1080 fibrosarcoma cells prefixed with  $-20^{\circ}\text{C}$  methanol. (A) Vinculin staining using monoclonal anti-vinculin mouse IgG. FITC fluorescence. (B) The same cells stained with polyclonal anti-u-PA rabbit IgG. TRITC fluorescence. The sequence in the staining procedure is described in Materials and Methods. Note the striking colocalization of vinculin and u-PA at discrete sites on the ventral cell surface. Bar, 15  $\mu\text{m}$ .

**Figure 6.** Double immunofluorescent localization of vinculin (A) and u-PA (B); and fibronectin (C) and u-PA (D) in WI-38 human embryonic lung fibroblasts prefixed with methanol. Primary antibodies: monoclonal anti-vinculin mouse IgG (A), polyclonal anti-u-PA IgG (B and D), monoclonal fpg-9 antifibronectin (C). Anti-u-PA labeling codistributes with vinculin plaques, but is apparently unrelated to the fibronectin fibrils. Bar, 15  $\mu\text{m}$ .







**Figure 7.** Double immunofluorescent labeling of vinculin (A) and u-PA (B) in quiescent WI-38 fibroblasts cultured in 0.5% FCS for 4 d. Prefixation, labeling conditions identical to those in Figs. 5 and 6. Note simultaneous disappearance of u-PA immunostaining from the focal contact sites and the elongation of the vinculin plaques. Bar, 15  $\mu$ m.

disappeared from the contact sites whereas vinculin plaques became highly elongated, and more centrally located. These results are in line with the earlier cell fractionation data (25, 26), which showed u-PA in the plasma membrane-enriched fraction in growing and transformed 3T3 cells, whereas in quiescent cultures PA was almost exclusively intracellular and of the tissue type. The results also agree with a recent report showing that u-PA mRNA is increased in quiescent cultured fibroblasts and keratinocytes upon growth stimulation (21). The elongation of the vinculin plaques in quiescent WI-38 fibroblasts is consistent with earlier findings (43), also describing the induction of fibronexus contact sites under similar conditions. The well-spread phenotype is thought to be maintained by tensile forces mediated by the actin-containing microfilaments (20). Moreover, the contracted state of fibroblasts has been associated with decreased motility and the beginning of growth (13, 14).

The confinement of u-PA to focal contacts of fibroblasts under serum-containing conditions when the cells are motile would suggest a role for plasma membrane-associated u-PA in the attachment-detachment sequence. At this specific localization, u-PA could then enable directional proteolysis for cell migration and invasion. It should be noted that the experiments reported here studied the distribution of u-PA antigen, and not the expression of u-PA enzyme activity. Although many types of cells secrete mainly the pro-u-PA form (17, 32, 44, 45, 52), it remains unknown whether the u-PA at the focal contacts is actually in that proenzyme form, as our polyclonal antibodies are able to detect active enzyme and enzyme-inhibitor complexes as well (31). Several levels of regulatory mechanisms are likely to operate to insure that net enzyme activity at the focal contacts would only be expressed when it is required (4, 16). These include the levels of proenzyme activation vs. inactivation, the specific fast-acting PA inhibitors, and matrix modulation of inhibition.

However, the present data establish that there is a process resulting in selective accumulation of the enzyme to the cell/matrix interface, and specifically to focal contacts.

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