Distinct Localizations of Urokinase-type Plasminogen Activator and Its Type 1 Inhibitor under Cultured Human Fibroblasts and Sarcoma Cells

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Abstract. We studied the immunocytochemical localization of urokinase-type plasminogen activator (u-PA) and the type 1 plasminogen activator inhibitor (PAI-1) in human fibroblasts and sarcoma cells, using both polyclonal and monoclonal antibodies. The u-PA was found to be located at discrete cell-substratum contact sites, and also at areas of cell–cell contacts, whereas PAI-1 was distributed as a homogenous carpet excluding strialike areas on the substrate under the cells. To confirm the extracellular localization of u-PA and PAI-1, we stained the cells live at 0°C before fixation. A double-labeling experiment showed different distribution of u-PA and PAI-1 under the cells, and especially their peripheral parts. The staining pattern of u-PA and PAI-1 resisted treatment with 0.2% saponin followed by mechanical removal of cells, a method previously reported to isolate focal contact membranes of fibroblasts. We further demonstrated the deposition of u-PA to the contact areas of cells obtained by saponin treatment by zymography, and that of PAI-1 by metabolic labeling, reverse zymography, immunoblotting, and immunoprecipitation. Fibronectin was also present in the preparations. The deposition of both PAI-1 and fibronectin by the sarcoma cells was enhanced, after treating the cells with 10⁻⁶ M dexamethasone. The confinement of u-PA to discrete contact sites and the more uniform distribution of PAI-1 on the cell substratum may explain how cells producing large amounts of enzyme inhibitors can produce PA-mediated focal proteolysis.

Plasminogen activators (PAs)¹ are serine proteinases, which by limited proteolysis convert the abundant extracellular proenzyme plasminogen into the active proteinase plasmin. Two different types of PAs have been recognized in mammalian tissues: the urokinase type (u-PA) and the tissue type (t-PA), with approximate Mr 50,000 and 70,000, respectively. Evidence has accumulated indicating that u-PA plays a role in tissue degradation both in the normal organism and in cancer, whereas t-PA is supposed to be a key enzyme in thrombolysis (for reviews see references 12, 13, 16, 46, 58, 59).

Several of the plasma proteinase inhibitors interact with PAs. Specific protein inhibitors of PA have also been identified in a number of tissues, body fluids, and cell cultures (10, 14, 18, 19, 22, 26, 30, 35, 42, 44, 56, 66, 70, 73, 78, 80, 81, 83), though the mutual relationship of these inhibitors is not yet fully clarified. An M₇, 54,000 plasminogen activator inhibitor (PAI) was recently purified from the human fibrosarcoma cell line HT-1080 (4), and immunologic evidence suggested that it is of the same type as the inhibitors from the rat hepatoma cell line HTC (66), bovine endothelial cells (18, 42, 44, 56, 70), and human blood platelets (19). This inhibitor has been designated plasminogen activator type 1 (PAI-1).² Other types of inhibitors acting on plasminogen activators include the protease nexin I (33, 64, 65) and that present in human placenta (PAI-2) (26, 30, 83).

Plasmin has a relatively broad specificity, being able to degrade known extracellular matrix glycoproteins and to activate latent collagenase (52, 54, 60). The affinity of plasminogen and plasminogen activators for laminin (61), fibronectin (62), and fibrin (45) further suggests the importance of these proteinases in the turnover of matrix components. Clearly, proteolysis taking place at cellular contact sites is of considerable importance for the interaction between the membrane structures and the surrounding matrix components. Proteolytic targets of the pericellular matrices of cells in culture have been reported to include fibronectin (74) and an M₇, 66,000 matrix-associated protein (31), which seems to be a plasminogen-independent cellular target for u-PA. In

¹ Abbreviations used in this paper: HES, human embryonic skin; PA, plasminogen activator; PAI, PAI-1, or PAI-2, plasminogen activator inhibitor, or specifically, PAI type 1 or type 2; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; TNP, trinitrophenyl.

² Nomenclature recommended by the Subcommittee on Fibrinolysis of the International Committee on Thrombosis and Hemostasis, 8 June 1986; Kruijthof, E. K. O., personal communication.
addition, a metallo-endoprotease seems to be involved in the focal degradation of fibronectin substrate by Rous sarcoma virus-transformed chicken and rat cells (11).

We now report that u-PA is located at cell-substratum and cell–cell contact sites of cultured human fibroblasts and sarcoma cells, whereas a specific $M_0 54,000$ PAI is homogeneously distributed on the substrate under the cells.

**Materials and Methods**

**Cell Cultures**

The human fibrosarcoma cell line HT-1080 was obtained from the American Type Culture Collection (CCL 121, Rockville, MD). The human embryonic skin fibroblast cultures (2-HES) were established locally by conventional methods and were used at the 10–20 passage level. The cells were grown either in plastic petri dishes (53 cm$^2$) or in plastic Linbro wells (2 cm$^2$; 0.2% saponin (white; BDH Chemicals Ltd., Poole, England) and shaken slowly. In the morning the cultures were pipetted and washed in PBS and then incubated with the primary antibodies for 30 min. Staining of live cells was carried out at 0°C and consisted of incubation with the primary antibody prior to fixation with –20°C methanol and incubation with the fluorochrome-conjugated secondary antibody. For double-immunofluorescence studies of u-PA or PAI-1, the cells were incubated with antibodies in the following sequence: rabbit anti-u-PA IgG, sheep anti-rabbit immunoglobulins (FITC), mouse anti-PAI-1 clone 1, goat anti-mouse immunoglobulins (TRITC). Immunocytochemical controls included incubation with polyclonal antibodies preabsorbed with either highly purified u-PA (34) or PAI-1, and with preimmune rabbit IgG or mouse monoclonal anti–TNP-IgG, and omission of one of the steps in the staining procedure. After washing with PBS the coverslips were mounted in a solution consisting of glycerol and PBS in a one-to-one ratio.

**Contact Area Preparations**

Two procedures for removal of cells were used to prepare samples for immunofluorescence microscopic observations. Method I consisted of washing the cell layers at 0°C three times with PBS (10 mM NaPO$_4$, 145 mM NaCl, pH 7.4). The cultures were then placed in PBS supplemented with 0.2% saponin (white; BDH Chemicals Ltd., Poole, England) and shaken for 20 min at 0°C on a rotary shaker at 100 rpm. Then the coverslips were intensively pipetted in PBS and the material remaining bound to coverslips was fixed for 20 min in 3% (wt/vol) paraformaldehyde, prepared in buffered saline. In method II, instead of saponin, the cultures were incubated overnight in cold PBS, thereby letting the cells detach from the coverslips more slowly. In the morning the cultures were pipetted and washed in PBS and fixed as in method I.

Methods I and II were applied when the proteins remaining associated with the coverslip were to be analyzed. Cells were seeded onto Linbro wells, each well containing one coverslip. After a 24-h incubation at 37°C the medium was changed into serum-free MEM lacking methionine, and the cultures were further incubated for 2 h. The cultures were then metabolically labeled for 2 h with 50 pCi/ml [35S]methionine (Amersham International, Buckinghamshire, England). The extraction procedure was carried out as detailed above (methods I and II). After excess buffer had been drained and removed by aspiration, the material remaining attached to the coverslips was dissolved at 0°C in Laemmli's sample buffer. The supernatants were precipitated in 35% (v/v) trichloroacetic acid, redissolved in sample buffer, and the samples were heated at 100°C for 2 min. Methods I and II gave quantitatively similar results.

**Dexamethasone Treatment**

Dexamethasone (Sigma Chemical Co., St. Louis, MO) was diluted from ethanol-based stock solution. The final dexamethasone concentration in the culture medium was 10$^{-6}$ M.

**Immunofluorescence**

Polyclonal purified rabbit IgG and monoclonal (clone 12) mouse antibodies against u-PA, and mouse monoclonal (clone 1) and affinity-purified polyclonal rabbit IgG antibodies against PAI-1 from the HT-1080 cell line were prepared as described elsewhere (4, 34, 49, 50). Monoclonal antibody against the trinitrophenyl group, anti-TNP-IgG, was from the hybridoma clone designated Hy 2.15 (63). Rabbit antisera to fibronectin (25) and antivinculin sera (76, 77) and monoclonal antibody fpg-9 to fibronectin (25) and antivinculin sera (76, 77) were raised and its specificity against the trinitrophenyl group, anti-TNP-IgG, was from the hybridoma clone 12 anti-u-PA (D), polyclonal anti-u-PA absorbed with purified u-PA (F) and monoclonal fpg-9 anti-fibronectin (G). Indirect immunofluorescence and anti-rabbit IgG FITC (A–C, E, and F), and anti-mouse IgG TRITC (D and G) were used. Bars, 10 µm.

![Figure 1. Immunofluorescence localization of u-PA and fibronectin in cultured human HT-1080 sarcoma cells and 2-HES fibroblasts.](image-url)
pore Corp., Bedford, MA) was performed using immunoperoxidase staining according to Towbin et al. (23) as modified (75). Commercially available low molecular weight markers (Pharmacia Uppsala, Sweden) and 3H-labeled markers (Amersham International) were used.

Zymography

The contact area preparations were analyzed for PA activity as described previously (23). Briefly, after electrophoresis in an 8% polyacrylamide slab gel under nonreducing conditions and without heating the specimen, SDS was removed from the gels with extensive washing on a gyratory shaker for 6 h in PBS with 2.5% Triton X-100, and an indicator agarose gel containing casein and plasminogen was placed in contact with the polyacrylamide gel. An agarose gel lacking plasminogen was used as a control. To determine the type of the PA activity, contact area preparations were dissolved in 0.5% Triton-X-100 and run through a column of Sepharose-coupled monoclonal antibody against PAI-I before running the specimens in SDS PAGE. PAIs in polyacrylamide gels were detected by washing the gel first in the same buffer and then containing 5 IU of u-PA or 100 IU of plasminogen was placed in contact with the polyacrylamide gel. An agarose gel containing plasminogen was used as a control. To determine the type of the PA activity, contact area preparations were dissolved in 0.5% Triton-X-100 and run through a column of Sepharose-coupled monoclonal antibody against PAI-I before running the specimens in SDS PAGE. PAIs in polyacrylamide gels were detected by washing the gel first in the same solution as above, but containing 5 IU of u-PA in 100 ml of washing solution (Calbiochem Behring Corp., La Jolla, CA). When layered over an agarose gel containing casein and plasminogen, a general lysis of the casein took place except where inhibitors were present in the polyacrylamide gels. These positions were revealed by opaque lysis-resistant zones: reverse zymography (20).

u-PA and PAI-1 Preparations

u-PA was from a commercial preparation (M, 54,000, 60,000 IU/mg, Calbiochem). PAI-1 was purified from conditioned culture medium of dexamethasone-treated HT-1080 cells as described in detail elsewhere (4). Indication of u-PA and PAI-1 was carried out with the Iodo-Gen method (Pierce Chemical Co., Rockford, IL).

Immunoprecipitation

For the immunoprecipitation experiment, metabolically labeled cells were extracted using method 1 (see above), the contact area material being dissolved in 500 μl of 0.1% (wt/vol) SDS. 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl. 70 μl of 10% (wt/vol) solution of washed Protein A-Sepharose CL-4B beads (Pharmacia) in 50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl was added, and after 15 min beads were separated by centrifugation at 9,000 g for 1 min. 5 μl of the monoclonal antibody against PAI-1 was added to the supernatant, and the mixture was incubated overnight at 4°C. The samples were further incubated with Protein A-Sepharose beads as above for 60 min at 37°C in an end-over-mixer. The precipitate was washed four times with the dissolving buffer and once with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, before resolubilization by SDS PAGE on 8% acrylamide gels. The dried gels were exposed on to Kodak XAR-5 film for autoradiography.

Conversion of Added[3H]-u-PA to a High Molecular Mass Form in Contact Area Preparations

[3H]-u-PA prepared as described above and diluted in PBS (106 cpm/ml), was added to freshly prepared contact area material of 2-HES fibroblasts obtained by saponin treatment as described. After incubation for 60 min at 0°C the preparations were washed with PBS, and the bound proteins were dissolved in Laemmli's sample buffer under nonreducing conditions, and analyzed in SDS PAGE followed by autoradiography.

Results

Immunocytochemical Localization of u-PA and PAI-1

HT-1080 and 2-HES cells were grown on glass coverslips overnight. After rinsing with ice-cold PBS, the cells were fixed or removed either by treating them with 0.2% saponin or by letting them shed overnight in PBS at 4°C as described in Materials and Methods. The fixed cells and the material remaining on the coverslips were examined by immunofluorescence microscopy.

Using both a polyclonal and a monoclonal antibody we found u-PA to be located at cell substratum contact sites of HT-1080 fibrosarcoma cells. Staining was also detectable in areas of cell–cell contacts (Fig. 1, A and C). The label associated with the substratum resisted the saponin extraction procedure, making the footprint contact pattern of immunofluorescence even more evident (Fig. 1, B and D). When live HT-1080 cells were stained, we detected patches of staining at cell–cell contacts and a weak diffuse pattern of immunofluorescence over the dorsal surfaces of cells (see Fig. 4 A), in addition to that in the contact sites on the substratum (see Fig. 4 B). These results demonstrate the extracellular location of the u-PA antigen. Intracellular anti-u-PA staining was also seen, showing highest intensity in the perinuclear region (Fig. 1, A and C). This was particularly the case, when cells were fixed with paraformaldehyde followed by various permeabilization procedures (not shown). At the electron microscopic level we could observe patches of polyclonal anti-u-PA staining extracellularly in association with the plasma membrane. Occasionally, a patch of stain formed a contact between two neighboring HT-1080 cells (Fig. 2 A). Human fibroblasts showed much lower staining intensity for u-PA than sarcoma cells. Only rarely were we able to detect a contact site staining pattern similar to that visualized in Fig. 1 E.

Staining with a monoclonal antibody to PAI-1 gave a similar pattern in fibrosarcoma cells and in fibroblasts. The immunofluorescence consisted of a homogeneously layer on the substratum. There was also intracellular perinuclear staining with very intensive labeling, presumably indicating PAI-1 synthesis (Fig. 3, A and B). After saponin extraction of cells, the immunofluorescence consisted of a homogenous carpet in the cell peripheral area interrupted by striae of negative staining (Fig. 3 C and D), which were also very faintly present in cells fixed with -20°C methanol (see Fig. 5 A). Affinity-purified polyclonal rabbit anti-PAI-1 IgG gave similar staining patterns as the monoclonal antibody. In cells stained live at 0°C with anti-PAI-1 antibodies, the immunofluorescence was restricted to the peripheral parts of the cells whereas the central areas appeared negative (Fig. 4 C). We interpret this to be the result of antigen in accessibility on the substrate under live cells in that the antigen was seen in the saponin-treated preparation on the substratum (Fig. 3, C and D). Double-stained HT-1080 cells, featured in Fig. 5, A and B, show that u-PA and PAI-1 have distinct staining patterns especially on the peripheral parts of the cells.

Fibronectin was detected with a polyclonal and the fpg-9 monoclonal antibody in both cell lines. The saponin contact preparations of fibroblasts displayed a typical matrix pattern, whereas in HT-1080 preparations the corresponding pattern was dominated by dots and striae. Fig. 1 G shows the fpg-9 staining pattern in a contact area preparation of dexamethasone-treated HT-1080 cells. Neither HT-1080 nor fibroblast contact preparations displayed vinculin or laminin, as investigated by immunofluorescence using polyclonal antibodies. Specificity controls including the use of up to 50 μg/ml preimmune IgG corresponding to the anti-u-PA or anti-PAI-1 and monoclonal anti-TPN-IgG were negative, as were experiments in which one of the steps in the staining protocol was omitted (Fig. 2 B). Absorption of the antibodies with either highly purified u-PA (Fig. 1 F) or PAI-1 (not shown) completely abolished the staining described above.

[3H]Methionine Labeling and Immunoprecipitation

To characterize the macromolecular components of the con-
tact area preparations obtained by method I, we labeled the cells metabolically with $[^{35}S]$methionine in the presence or absence of $10^{-6}$ M dexamethasone in the culture medium. The material attached to the glass coverslip was analyzed in SDS-PAGE followed by autoradiography. One major band run at $M_r$, 54,000 and coelectrophoresed with purified radio-labeled PAI-1 (Fig. 6, lane 5) in HT-1080 as well as fibroblast contact preparations. The monoclonal anti-PAI-1 antibody also immunoprecipitated a similar band from the preparation (see Fig. 10). However, the precipitation of PAI-1 did not result in a quantitative depletion of the $M_r$, 54,000 band from the immune supernatant. Thus, it cannot be excluded that there are at least two $M_r$, 54,000 proteins comigrating in the metabolically labeled contact area preparation. Enriched in the contact area preparations were also bands at $M_r$, 60,000, 75,000, and 250,000. Dexamethasone in the culture medium increased the intensity of the $M_r$, 54,000 and 250,000 bands on the HT-1080 contact preparations (Fig. 6, lanes 1-2 and 8-9), as expected of the PAI-1 (2, 4, 14, 66) and fibronectin subunits (53). The $M_r$, 54,000 band also appeared increased...
Figure 3. Immunofluorescence localization of PAI-1 in (A and C) cultured HT-1080 sarcoma cells and (B and D) 2-HES fibroblasts. Fixation was with methanol in A and B. Cells were removed using method I in C and D. Indirect immunofluorescence, monoclonal clone 1 anti-PAI-1 antibodies and TRITC-conjugated anti-mouse IgG were used. Note the lack of staining in strialike areas in the otherwise uniform staining under the cells in A and especially C and D. Bars, 10 μm.
in the contact area preparations from dexamethasone-treated fibroblasts with SDS-PAGE under reducing conditions, but not with SDS PAGE under nonreducing conditions. This apparent discrepancy is due to the fact that less total protein was undeliberately applied to the gel lane corresponding to fibroblasts without dexamethasone electrophoresed under reducing conditions.

**Zymographic and Reverse Zymographic Analysis of the Contact Area Preparations**

Molecular forms of the plasminogen activators present in the contact area preparations were investigated by SDS-PAGE under nonreduced conditions followed by casein-agarose zymography (Fig. 7). In the contact area preparations of HT-1080 cells (lanes 1 and 2), PA activity migrated as a well-defined band at Mr 50,000, as expected of u-PA. Additionally, higher molecular mass forms of PAs were detected as a smear of activity between Mr 70,000 and 100,000, with a distinctive band at the latter end. Dexamethasone slightly diminished the PA activity in the preparations. All the PA activity in the preparations is u-PA, in that preparations run through a column of Sepharose coupled monoclonal anti-u-PA antibodies lost all their PA activity, while treatment in a similar anti-t-PA column had no effect on the PA activity pattern of the preparations (data not shown). This indicates that PAI-1 and u-PA form a complex in the preparation which dissociates partly during electrophoresis, in agreement with previous findings (4). The reason that the lysis zone is not continuous between positions corresponding to Mr 50,000 and 100,000 can be ascribed to the fact that excess inhibitor migrates slightly behind uncomplexed u-PA, preventing lysis in that position. Virtually no PA activity was detected in fibroblast contact preparations using this method (lanes 3 and 4); only after several days' exposure did a weak lysis appear at Mr 50,000. Control gels lacking plasminogen showed no caseinolytic activity.

Reverse casein agarose zymography was carried out to test for PA inhibiting activities (Fig. 8). Analysis of the contact area preparations of HT-1080 cells as well as fibroblasts revealed a major inhibitor zone at Mr 54,000. Dexamethasone markedly increased the intensity of the inhibitor zone of the HT-1080 contact area preparations (lanes 1 and 2), but this effect was virtually non-existent in the fibroblast preparations (lanes 3 and 4).

**Immunoblotting**

Contact area preparations of dexamethasone-treated HT-1080 cells were subjected to immunoblotting analysis under reducing conditions. The monoclonal PAI-1 antibody detected a single band at Mr 54,000 (Fig. 9, lane 1). Lane 2 is a specificity control track immunoblotted with anti-TNP-IgG.

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**Figure 4.** Immunofluorescence localization of (A and B) u-PA and (C) PAI-1 in HT-1080 cells. The cells were stained with the primary antibodies, (A and B) polyclonal rabbit anti-u-PA IgG, (C) mouse monoclonal anti-PAI-1, before fixation with methanol and incubation with the secondary antibodies. The entire experiment was performed at 0°C. In A the focus is above the growth substratum at the cell–cell contacts and in B and C the microscope was focussed on the substratum. Bars, 10 μm.
Complexing of $^{125}$I-u-PA in Contact Area Preparations of Fibroblasts

When $^{125}$I-u-PA ($M_r$ 50,000, Fig. 11, lane a) was added to preparations obtained from cultures of 2-HES fibroblasts using method I, and incubated for 60 min in the cold, a large proportion of the bound label was converted to a high molecular mass form: $M_r$ 90,000 (Fig. 11, lane b). A similar high molecular mass complex was obtained after incubation of $^{125}$I-u-PA with excess amount of PAI-1 purified from the HT-1080 cell line (lane c).

Discussion

In the present study we have described the distribution of u-PA and an $M_r$ 54,000 PAI in cultured HT-1080 human fibrosarcoma cells and in human fibroblasts using both polyclonal and specific monoclonal antibodies. In our experiments, u-PA was found to be located at discrete cellular contact sites under the fibrosarcoma cells, whereas its inhibitor was distributed as a homogeneously thick carpet on the sub-
Figure 8. Reverse zymography after 8% SDS PAGE of the contact area preparations. 70-μl samples of the contact area preparations of dexamethasone-treated (lane 1) and untreated (lane 2) HT-1080 cells, and dexamethasone-treated (lane 3) and untreated (lane 4) 2-HES cells were analyzed. Development at 37°C for 24 h. The positions of the Mr markers are indicated to the right in kilodaltons.

Figure 9. Immunoblotting of PA inhibitor under reduced conditions in a contact area preparation of dexamethasone-treated HT-1080 contact area preparation, using 50 μg/ml monoclonal anti-PAI, clone 1 (lane 1) or monoclonal anti-TNP-IgG (lane 2). The positions of the Mr markers are indicated in kilodaltons.

stratum, but lacking in strialike areas. When live cells were stained, PAI-1 could not be detected under the central parts of the cells. Thus, we cannot exclude the possibility that the negative strialike areas in saponin-treated cell preparations would be due to inaccessibility of the PAI-1 antigen to the antibodies used. At the electron microscopic level we detected patches of anti-u-PA staining associated with the plasma membrane. This result, obtained by staining live cells in the cold with the primary antibody, indicated that u-PA on the cell surface is externally located. The deposition of fibronectin was also detected using a monoclonal antibody. Normal human fibroblasts showed low intensity of anti-u-PA immunoreactivity, while producing PA inhibitor quite abundantly.

Additional lines of evidence were obtained, suggesting that u-PA and its inhibitor are indeed deposited to the contact areas of cells. First, the contact areas could be isolated with 0.2% saponin assisted mechanically with a stream of buffer. When such cell-free substrate-attached material, prepared from [35S]methionine-labeled cells, was studied by fluorography, we found that an Mr 54,000 band was the major one in the preparations. This band includes PAI-1, as judged by immunoprecipitation experiments, although the presence of other proteins in the same position cannot be excluded. Still, its amount in the preparations was responsive to dexamethasone treatment of the HT-1080 cell cultures in agreement with the previous findings (2, 4, 14, 53, 66). PAI-1 in the contact area preparations could be immunoblotted with a specific monoclonal antibody. Apparently, u-PA is relatively minor component in the contact area preparations; we were unable to detect u-PA in these preparations using immunoblotting. The PA activity of the contact area preparations was, however, confirmed with zymography which showed a major band at M, 50,000 as well as a minor band at M, 100,000. Reverse zymography showed a single Mr, 54,000 inhibitor in the preparations. Furthermore, when 125I-u-PA was incubated with the preparations, much of it was converted into a high molecular mass form (M, 90,000). This observation is consistent with the presence of PAI-1 in contact area preparations, but further experiments are needed to determine whether the complexing of 125I-u-PA is specifically with PAI-1.

The accessibility of u-PA for antibodies at the contact sites differed under various fixation and permeabilization conditions, methanol fixation at −20°C giving the clearest results. Even then, the monoclonal antibodies gave much less staining than the polyclonal anti-u-PA antibodies we used. After removal of cells to obtain the contact area preparations both types of antibodies gave a similar pattern of staining, suggesting that the determinant of the monoclonal antibody is restrictedly exposed.

The subcellular localization of u-PA has been previously explored by cell fractionation studies and by the immunocytochemical approach. Despite conflicting results, there appears to be a consensus among the various cell fractionation reports that PA activity is often localized in membrane-rich fractions. In Rous sarcoma virus-transformed chicken embryonal fibroblasts, u-PA has been localized to fractions containing the Golgi apparatus and the plasma membrane (57), whereas in growing and virus-transformed 3T3 cells exclusively to the plasma membrane-enriched fraction (28, 29). However, in a study of HT-1080 cells PA of non-determined type was found associated solely with a cellular fraction representing either the Golgi apparatus or secretory granules rather than plasma membrane (39). Interestingly, either u-PA or PA of nondetermined type from several cell lines includ-
Figure 10. Autoradiography of 8% SDS PAGE of an immunoprecipitate of PAI-1 from the contact area preparation of dexamethasone-treated HT-1080 cells. The cells were labeled for 2 h in medium containing 50 μCi/ml [35S]methionine. After extraction of cells, the labeled substrate-attached proteins were immunoprecipitated using monoclonal anti-PAI-1 (clone 1). Lane 1: HT-1080 preparation; lane 2: the immunoprecipitate.

HT-1080, has been found resistant to various solubilization procedures that would be expected to solubilize non-membrane integrated proteins (28, 39, 41, 43, 57, 69). As for the light microscopic studies the subcellular distribution of u-PA differs greatly in different cell types (38, 67). Only one study at the electron microscopic level PA (apparently u-PA) immunoreactivity has been reported to be associated with the cell membranes of porcine kidney cells (55). Furthermore, in two recent studies of human monocyte-like U937 cells and peripheral blood monocytes (71, 79), u-PA was shown to bind with its noncatalytic aminoterminal domain to specific plasma membrane receptors, whereas the catalytic, carboxy-terminal domain remains exposed on the cell surface. Additionally, Fibbi et al. (21) have demonstrated the binding of a urokinase-ferritin conjugate to cell surface sites in A431 human epidermoid carcinoma cells and proposed that these represented single and clustered receptor. Our data fit well with the findings above. If indeed u-PA is bound to a receptor also in HT-1080 cells, the anti-u-PA immunoreactivity at the contact sites and on the plasma membrane (Fig. 2 A) could be based on local clustering of such receptors.

Several different procedures have been used to isolate material designated either as cellular contact sites, cell substrate-attached material, foot pads, or focal contact membranes (5, 7, 9, 15, 37, 40, 47). These procedures include use of chelating agents, low concentrations of nonionic or zwitter-ionic detergents, or mechanical extraction. All these procedures to isolate the material from the growth substratum should be considered operational only, because the material obtained is expected to contain both extracellular matrix components and material binding from the culture medium to the glass or plastic surface, besides the actual cellular contact site components. Saponin, which we used as a cholesterol-removing detergent, has also been used to isolate acetylcholine receptor clusters from cultured rat myotubes (8). In addition, using saponin and mechanical extraction Neyfakh et al. (47) found Mf 51,000 and 47,000 proteins in isolated focal contact membranes of mouse and chick fibroblasts, respectively. Interestingly, Bayley and Rees (7) detected a major new protein component with an Mf 50,000-55,000 in fibroblast focal adhesion preparations that remains attached to a glass substratum after cells are mechanically removed. Our results indicated that a contact area preparation obtained by saponin treatment contains PAI-1. However, the inhibitor was not confined to discrete focal contacts, nor was it found to have a matrix-type distribution, instead it was found to be deposited under the ventral surface of the cells excluding strialike areas.

Extracellular PA activity may be regulated at the level of cellular production and release of the activators themselves, and by modulators of the enzyme activity of the activators (for reviews, see references 16 and 59). Furthermore, the fact that PAs act as a part of a cascade reaction implies a more complicated set of control mechanisms for the final proteolytic effect. Recent demonstration of proenzymes with little or no activity (3, 17, 27, 32, 51, 67, 68, 82) suggests that mechanisms, although at present unknown, exist that control the conversion of zymogens to active enzymes in the extracellular space. Moreover, u-PA is inhibited by the abundant protease inhibitors of the human plasma, by the protease nexin, and by the recently reported more specific inhibitors (see our introductory text for references). Binding by pro-
tease nexin may initiate cellular uptake and degradation of u-PA.

Our results demonstrating u-PA and PAI-1 in the contact area preparations add a novel dimension to the complex regulatory scenario of pericellular plasminogen activation. The protein concentration being too low in our preparations, we were not able to decide by immunoblotting, whether u-PA at the sarcoma cell contact sites is present as the one-chain inactive proenzyme. The results obtained by zymography, demonstrating a PAI-1/u-PA complex that is not formed by pro-u-PA (4), do not settle that question because pro-u-PA may have been activated during the zymography procedure. The difference in distribution between u-PA, being enriched into discrete contact sites, and PAI-1, being distributed as an extracellular carpet on the growth substratum, could conceivably permit focal u-PA-mediated proteolysis to occur.

The large amounts of the PAI on the growth substratum was a rather unexpected finding. This could be an indication of the need to secure the inhibition of PA in the pericellular area, to maintain cell surface integrity. Proteins bound in a similar way on to glass substrata include serum spreading factor (vitronectin), and the M₈₀₀₀,₀₀₀ protein of serum-containing media (48). Vitronectin is an adhesive glycoprotein that promotes spreading of a variety of cell types on culture dishes (6, 24). The deposition of PAI-1 suggests that it may have functions other than enzyme inhibition. We are currently exploring this possibility.

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