Complementation between urokinase-producing and receptor-producing cells in extracellular matrix degradation*

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The respective roles of urokinase plasminogen activator (u-PA) and the u-PA receptor in extracellular matrix degradation was investigated. Human pro-u-PA and the human u-PA receptor were expressed independently by two different mouse LB6 cell lines. The matrix degradation capacity of these cell lines individually or in coculture was studied. Although pro-u-PA-producing cells alone degrade the matrix in the presence of plasminogen, u-PA-receptor producing cells do not. Cocultivation of a small fraction of pro-u-PA-producing cells with the receptor-producing cells increases the rate of matrix degradation at least threefold. By immunoprecipitation it was shown that cocultivation of the two cell lines increases the conversion of the inactive pro-u-PA to the active two chain u-PA. The enhancement of matrix degradation and of pro-u-PA activation requires actual binding of pro-u-PA to its receptor because it is inhibited by u-PA-receptor antagonists. The u-PA receptor must be cell associated, as binding of pro-u-PA to a receptor solubilized from the cell surface with phosphatidylinositol specific phospholipase C did not enhance the activation of pro-u-PA in the presence of plasminogen. The finding that activity of u-PA is enhanced when it is bound to its receptor, even when

the receptor is produced by a different cell, might have important implications for the mechanisms of u-PA-induced extracellular proteolysis in vivo.

Introduction

Plasminogen activation is a key process in the regulation of extracellular proteolysis related to tissue destruction and migration of cells in both normal and pathological conditions (for reviews see Danø *et al.*, 1985; Pöllänen *et al.*, 1991). The product of plasminogen activation, the broad-spectrum serine protease plasmin, can directly or indirectly degrade protein components of the extracellular matrix and of the basement membrane. The regulation of the conversion of plasminogen to plasmin mediated by plasminogen activators is very complex and probably not all components have been identified yet, nor has the precise function of each of the known components been well established.

Two distinct plasminogen activators have been described: tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Although it is possible that both t-PA and u-PA are involved in cell migration processes, more knowledge has been accumulated to date for u-PA (Hearing *et al.*, 1988; Axelrod *et al.*, 1989; Quax *et al.*, 1990). u-PA is synthesized and secreted as a single-chain pro-enzyme that needs activation to the two-chain active u-PA (Nielsen *et al.*, 1982; Wun *et al.*, 1982; Petersen *et al.*, 1988). The activity of u-PA on the other hand is controlled by specific inhibitors (Sprengers and Kluft, 1987; Andreasen *et al.*, 1990). Finally, u-PA activity can partition between solution and cell surface through the binding to a specific cell-surface receptor (Vassalli *et al.*, 1985; Stoppelli *et al.*, 1985; reviewed in Blasi, 1988). On some cell types receptor-bound u-PA is localized at specific sites on the cell surface, i.e., the cell to cell and the (focal) cell to substratum contacts (Pöllänen *et al.*, 1987, 1988; Hébert and Baker, 1988).

The involvement of the plasminogen activator system in cell migration and tissue remodeling processes is well established (Ossowski and

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Figure 1. Characterization of u-PA and u-PA-receptor production by mouse LB6 cells and derivatives. (Panel A) SDS-PAGE and fibrin zymography of conditioned medium from control LB6 cells, u-PA-producing CL-F cells, and u-PA receptor-producing CL-19 cells as indicated above each lane. The leftmost lane shows the zymography of a commercial preparation of human u-PA with activity at both the high molecular weight (HMW) and low molecular weight (LMW) u-PA bands. (Panel B) SDS-PAGE analysis of extracts of LB6, CL-F, and CL-19 cells after binding and chemical cross-linking to ^{125}I -DFP-treated u-PA.

Reich, 1983; Ossowski, 1988; Mignatti *et al.*, 1986), but the exact mechanism and physiological role of each individual component still needs clarification. The phenotypic characteristics related to plasminogen activation, i.e., extracellular matrix degradation and basement membrane degradation (Bergman *et al.*, 1986; Cajot *et al.*, 1989), invasion of natural and reconstituted membranes (Mignatti *et al.*, 1986; Reich *et al.*, 1988), tumor invasion (Skriver *et al.*, 1984; Kristensen *et al.*, 1990; Grøndahl-Hansen *et al.*, 1991), and metastasis (Ossowski and Reich, 1983; Hearing *et al.*, 1988) are extremely complex on their own, possibly requiring in addition to the plasminogen activation system other proteolytic activities like collagenase, which may in turn be controlled by inhibitors and presumably receptors (Tryggvason *et al.*, 1987). Moreover, it is becoming increasingly clear that the aggressive behavior of tumor cells may be influenced by interactions with nearby stromal cells, which may

in fact participate in the selection of an aggressive subpopulation (Kerbel, 1990; Basset *et al.*, 1990; Rand Pritchett *et al.*, 1989).

A deeper understanding of the role of individual components of the plasminogen activation system in the various biological functions in which it participates can be obtained through a genetic dissection of the system. To this goal we have set up an *in vitro* complementation system as an initial step in this direction. Cells unable to carry out plasminogen activation can be modified by transfection with individual genes of the plasminogen system. The resulting cells would not be able to carry out the whole reaction by themselves but should be able to complement each other. We have previously cloned the genes for several components of the human u-PA system: u-PA (Riccio *et al.*, 1985), the plasminogen activator inhibitor PAI-1 (Andreasen *et al.*, 1986; Bosma *et al.*, 1987), and the u-PA receptor (Roldan *et al.*, 1990). We now have constructed mouse cells that

Figure 2. Effect of plasmin and u-PA inhibitors on extracellular matrix degradation by mouse LB6 cells and derivatives. The capacity of control LB6 cells, u-PA producing CL-F cells, and u-PA receptor-producing CL-19 cells to degrade ^3H -labeled extracellular matrix produced by bovine smooth muscle cells was determined on a total of 10^5 cells per experiment. Cells were cultured in 10% fetal calf serum supplemented with $0.14 \mu\text{M}$ human plasminogen. No addition, filled bars; anti-human u-PA IgG ($30 \mu\text{g/ml}$), hatched bars; Trasylol (100 U/ml), dotted bars. Matrix degradation was determined after 24 h incubation as described under Materials and methods. The data represent the average of three determinations with the standard deviation.

express either human u-PA or the human u-PA receptor and have tested their respective roles in plasminogen-dependent extracellular matrix degradation.

Results

Characterization of u-PA and u-PA receptor production by transfected LB6 clones

The mouse LB6 cell line (Corsaro and Pearson, 1981) was chosen as a suitable host cell line to express the different components of the human plasminogen system, as they produce little or no mouse u-PA and t-PA nor interstitial collagenases (L. Ossowski, personal communication) and have a very poor capacity to degrade the extracellular matrix. In addition, LB6 cells can be considered to lack binding capacity for human u-PA because binding of u-PA to its receptor is largely species-specific (Vassalli and Belin, personal communication; Appella *et al.*, 1987; Estreicher *et al.*, 1989).

The synthesis of the human pro-u-PA gene product by CL-F cells, but not by parental LB6 nor CL-19 cells, was demonstrated by zymography (Figure 1A) and was further verified by immunoprecipitation and measurement of enzymatic activity with synthetic S2444 substrate (not shown). The expression of the human u-PA receptor in CL-19 cells, and not by LB6 nor CL-F cells, was verified using a binding and cross-linking assay with iodinated human u-PA (Figure 1B) or its amino terminal fragment as previously shown by Roldan *et al.*, 1990.

Extracellular matrix degradation by u-PA-producing cells

The cell lines were tested for their capacity to degrade an extracellular matrix (produced by

Figure 3. Stimulation of extracellular matrix degradation of u-PA-producing CL-F cells by cocultivation with u-PA receptor-producing CL-19 cells. Different percentages of CL-F cells (0, 5, 7.5, or 10% of total cells) were cocultured with either control LB6 cells (●—●) or CL-19 cells (○—○) (the total number of cells being in each case 10^5). Matrix degradation was determined after 24, 48, and 72 h of incubation as described under Materials and methods. The data represent the average of three determinations with the standard deviation.

smooth muscle cells) in vitro in serum-containing medium in the presence of human plasminogen. In Figure 2 the matrix degradation after 24 h is shown for pro-u-PA-producing cells (CL-F), u-PA receptor-producing cells (CL-19), and control cells (LB6). Untransfected LB6 cells or cells expressing the human u-PA receptor (CL-19) were not able to degrade the extracellular matrix. CL-F cells, producing pro-u-PA, were able to degrade the matrix. Addition of polyclonal anti-human u-PA IgGs strongly inhibited the degradation process. Trasylol, a potent inhibitor of trypsin-like proteases including plasmin, completely inhibited the degradation, whereas 1,10-phenanthroline, an inhibitor of metalloproteinases, had no effect on matrix degradation (data not shown). These results point to an involvement of u-PA and plasminogen activation in the matrix degradation process.

Role of u-PA receptor in extracellular matrix degradation

The role of the cell surface u-PA receptor in extracellular matrix degradation was studied using coculturing experiments. CL-F expressing human u-PA, CL-19 expressing the human u-PA-receptor, and control LB6 cells were cocultured in various combinations and ratios on ³H-labeled extracellular matrices. Subsequently, the rate of matrix solubilization was determined. In Figure 3 the matrix degradation is shown at various time points of cocultures containing 5%, 7.5%,

or 10% CL-F in combination with either CL-19 or control LB6 cells. Control LB6 cells in combination with CL-19 cells were used as a negative control and had a negligible matrix degrading activity, which was subtracted. Matrix degradation in cocultures of control LB6 with CL-F cells was found to be dependent on the incubation time and on the number of CL-F cells (Figure 3). In all cases, matrix degradation by CL-F cells was clearly enhanced by the cocultivation with the receptor-producing CL-19 cells. Although the CL-19 cells have no capacity to degrade extracellular matrix (see also Figure 2), they are able to considerably stimulate matrix degradation when cocultivated with CL-F cells. Also in this case, matrix degradation is totally blocked by Trasylol and anti-u-PA antibodies but not by phenanthroline (not shown). These results suggest that the presence of the receptor increases the ability of u-PA to mediate degradation of the matrix proteins.

The specificity of the effect of u-PA receptor-producing CL-19 cells has been tested with a synthetic peptide representing the receptor-binding sequence of human u-PA, u-PA[12-32(ala19)], which is able to compete with u-PA for binding to the receptor (Appella *et al.*, 1987). As shown in Figure 4, this peptide nearly completely inhibited the enhancement of matrix degradation in the cocultivation of 7.5% CL-F cells with CL-19 cells. On the contrary, the synthetic peptide u-PA[13-33(ala20)], representing the receptor-binding sequence of the mouse

Figure 4. Inhibition of matrix degradation by u-PA receptor-binding peptides. Extracellular matrix degradation by cocultures of u-PA-producing CL-F cells with u-PA receptor-producing CL-19 was determined in the presence and absence of mouse and human u-PA-receptor binding peptides. 10^5 cells were seeded on the matrix, 7.5% CL-F and 92.5% CL-19 (CL-F/CL-19), or 7.5% CL-F and 92.5% control LB6 (CL-F/LB6). The CL-F/CL-19 were also incubated in the presence of $10 \mu\text{M}$ mouse u-PA[13-33(ala20)] or $10 \mu\text{M}$ human u-PA[12-32(ala19)] peptides. Matrix degradation was determined after 72 h as described in Materials and methods. The data represent the average of three determinations with standard deviation.

enzyme, which does not bind to the human receptor (Appella *et al.*, 1987; Estreicher *et al.*, 1989), had little or no effect.

The synthetic peptides, while specific, have a relatively low affinity for the receptor (Appella *et al.*, 1987) and possibly may have a short half-life in culture conditions. Therefore we have performed competition studies with a novel recombinant u-PA derivative, ΔF -u-PA, that contains the 164 amino terminal amino acids of u-PA (and a 30 amino acids carboxy-terminal u-PA-unrelated extension). ΔF -u-PA contains the receptor binding domain of u-PA but lacks the protease domain; thus it cannot convert plasminogen to active plasmin but competes for binding to the receptor (Pedersen *et al.*, 1991). As a source of ΔF -u-PA, conditioned medium of LB6 cells stably transfected with the variant u-PA gene and secreting the truncated recombinant ΔF -u-PA (CL- ΔF) was used. As shown in Figure 5A, the presence of 10% CL- ΔF conditioned medium strongly inhibits the matrix degradation by cocultures of CL-F and CL-19 cells (in a 7.5%/92.5% ratio), whereas conditioned medium of control cells (i.e., LB6 cells transfected only with a RSV-neo plasmid) had very little effect. Conditioned medium decreases the degradation of extracellular matrix to a level even lower than that attained by the combination CL-F/LB6 cells, i.e., in the absence of receptor (Figure 5B), suggesting the presence of an inhibitor of nonreceptor-bound u-PA.

Presence of the u-PA receptor increases the conversion of single-chain u-PA to two-chain u-PA

The product of the u-PA gene is the single-chain pro-enzyme pro-u-PA (Petersen *et al.*, 1988). In vitro conversion of the soluble or receptor-bound pro-u-PA to the two-chain form by plasmin results in the formation of the fully active enzyme (Cubellis *et al.*, 1986). CL-F cells produce pro-u-PA and degrade the extracellular matrix in an u-PA-dependent manner; therefore some pro-u-PA must have been converted to the active two-chain u-PA. However, the presence of the receptor may have increased the rate of conversion of single-chain to two-chain u-PA.

To clarify this issue we have cocultivated the CL-F cells (10% of total cells) with control LB6 or receptor-producing CL-19 cells for 12 h in serum-containing medium, labeled the cells with ^{35}S for 9 h, and determined the levels of single- versus two-chain u-PA in the medium and cell surface bound by immuno-precipitation with an anti-u-PA monoclonal antibody (5B4). As shown

in Figure 6, the conditioned medium of cocultured CL-F/LB6 cells contains mainly single-chain pro-u-PA and only limited amounts of two-chain u-PA. On the other hand, in the medium from cocultured CL-F/CL-19 cells the amount of two-chain u-PA is increased. To analyze the state of receptor-bound pro-u-PA, cells were acid washed, and the acid wash was immunoprecipitated. As expected, the surface of the cocultured CL-F/LB6 cells has no cell-bound u-PA, whereas both single-chain and, predominantly, two-chain u-PA can be dissociated from cocultured CL-F/CL-19 cells.

This result can be reproduced by the addition of limiting amounts of ^{35}S -labeled pro-u-PA (in the form of ^{35}S -labeled CL-F-conditioned medium) to the receptor-producing CL-19 or control LB6 cells and determination of the ratio of single- to two-chain u-PA in the medium and on the cells surface (Figure 7A). In the presence of CL-19 cells, the labeled u-PA was scarcely detectable by the immunoprecipitation technique in the medium after 3.5 h incubation, but it could be found bound to the receptor (under these conditions most is found in the two-chain form). In contrast, most of the u-PA added to the LB6 cells remained in the single-chain form. To verify that inhibition of the matrix degradation by the ΔF -u-PA was caused by blocking the binding of the pro-u-PA to its receptor, ^{35}S -labeled u-PA (^{35}S -labeled CL-F medium) was added to CL-19 cells in the presence of conditioned medium from CL- ΔF or LB6 cells (Figure 7B). Immunoprecipitation of unbound and cell-bound u-PA shows, as before, that in the presence of the control-conditioned medium u-PA will bind to the CL-19 cells and the bound u-PA is in the two-chain form. In contrast, in the presence of the ΔF -u-PA (conditioned medium from CL- ΔF cells) there is no binding of the added ^{35}S -labeled u-PA to the cells.

We have further tested whether, in the presence of plasminogen, it is the binding to the receptor per se that induces the conversion of single-chain to two-chain u-PA by analyzing the fate of pro-u-PA when it is in the presence of a soluble form of the receptor rather than on the cell surface. It has been recently established that the u-PA receptor is attached to the cell surface via a glycolipid anchor and can be solubilized by treatment with *B. cereus* phosphatidyl-inositol specific phospholipase C (PI-PLC) (Ploug *et al.*, 1991). Therefore we prepared soluble u-PA receptor from CL-19 cells by treating them with PI-PLC. Medium with or without the solubilized receptor was incubated with labeled ^{35}S -labeled u-PA (conditioned medium of CL-F cells) for 18 h in the presence or absence of

Figure 5. Inhibition of matrix degradation by a receptor-binding u-PA antagonist. (A) Coculture of CL-F (7.5%)/CL-19 (92.5%) cells. (B) Coculture of CL-F (7.5%)/control LB6 cells (92.5%). No additions, (▲—▲); addition of 10% conditioned medium from LB6-pRSVneo cells, (○—○); addition of 10% conditioned medium from CL-ΔF cells (producing recombinant human ATF), (△—△). Matrix degradation in the medium of cocultured cells (total 10^5 cells) was determined as described in Materials and methods. The data represent the average of three determinations with standard deviation.

exogenously added plasmin (100 ng/ml). The reaction mixtures were analyzed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 8A). The presence of a solubilized u-PA receptor did not change the ratio between single- and two-chain u-PA. On the other hand, exogenously added plasmin quantitatively converted the single chain into the two-chain form, independent of the presence of the solubilized receptor. The presence and the binding activity of the u-PA receptor in the supernatant of the PI-PLC-treated cells was tested by cross-linking to 125 I-amino terminal fragment (ATF) (Figure 8B).

Discussion

In the model system presented in this paper, the role of u-PA and the u-PA receptor in extracellular matrix degradation could be studied separately or in combination. We demonstrate that the components produced by the cell lines are biologically active and that the u-PA receptor binds u-PA produced by other cells. We show that the inability of mouse LB6 cells to

Figure 6. Receptor-bound pro-u-PA is preferentially converted to two-chain (active) u-PA during cocultivation. Complementations of pro-u-PA and u-PA receptor-producing cells. 10% CL-F cells and 90% CL-19 or control LB6 cells were plated 12 h before labeling with 35 S-methionine and 35 S-cysteine for 9 h. The media (unbound) and acid-released (cell bound) material was immunoprecipitated with the anti-human u-PA monoclonal antibody 5B4 and analyzed by SDS-PAGE under reducing conditions and fluorography.

Figure 7. Preferential conversion of exogenously added pro-u-PA to the two-chain (active) form in the presence of cell-bound u-PA receptor. (Panel A) 200 μ l 35 S-labeled CL-F medium and 2.8 ml unconditioned medium was added to a semiconfluent 25-cm² flask of either CL-19 or LB6 cells. After incubation for 3.5 h, the medium (unbound) and acid released (cell bound) u-PA was immunoprecipitated with the anti-human u-PA monoclonal antibody 5B4 and analyzed by SDS-PAGE under reducing conditions and fluorography. (Panel B) Binding of 35 S-labeled u-PA (35 S-labeled CL-F medium) to CL-19 cells for 3 h in the presence of 30% conditioned medium from CL- Δ F cells or LB6 cells. Analysis of unbound and cell-bound u-PA as above.

degrade the extracellular matrix can be circumvented by transfection with the human u-PA gene, indicating that u-PA is the only component required for extracellular matrix degradation that is missing in the LB6 cells. The key role of u-PA was indicated by the strong inhibition of matrix degradation by anti-u-PA antibodies (Figure 2). The complete inhibition by Trasylol further suggested that the matrix degradation is plasmin dependent, a phenomenon also observed by Cajot *et al.* (1989). The extracellular matrix produced by smooth muscle cells appears therefore to be a suitable model system to study the physiological effects of plasmin activity. The lack of effect of o-phenanthroline, suggesting an irrelevant role of metalloproteinases, is also expected because smooth muscle cells are thought to produce collagen-poor matrices under normal cell culturing conditions unless ascorbic acid is added to the culture medium (Jones and DeClerck, 1980).

When matrix degradation was assayed using cocultures of u-PA and u-PA-receptor producing cells, an enhancement of the extracellular matrix degradation was observed. This enhancement effect is due to the binding of pro-u-PA, produced by the CL-F cells, to the u-PA-receptor of the CL-19 cells, (which in themselves have

no or only a very low capacity to degrade the matrix). This is demonstrated by the specific inhibition of the enhancement by the receptor antagonist human peptide u-PA[12-32(ala19)] (Figure 4), whereas the mouse peptide u-PA[13-33(ala20)] had no effect, as expected from the species specificity of the binding. Competition experiments were also performed using Δ F-u-PA (i.e., a recombinant ATF of human u-PA) as a competitor for receptor binding (in the form of conditioned medium from CL- Δ F). This recombinant ATF (Δ F-u-PA) behaves similarly to the natural ATF (Pedersen *et al.*, 1991) in having a much higher affinity for the receptor than the synthetic peptides. Δ F-u-PA totally inhibits the enhancement of the matrix degradation (Figure 5) and inhibits the binding of u-PA to the receptor (Figure 7B), demonstrating that the enhancing effect on matrix degradation caused by the CL-19 cells is due to binding of u-PA to its receptor. The control-conditioned medium also has a low inhibitory effect on matrix degradation by the u-PA-producing cells in the absence (Figure 5B) and in the presence (Figure 5A) of the receptor, suggesting that conditioned medium contains low levels of an inhibitor of the soluble (nonbound) u-PA activity. However, the inhibition of the several-fold enhancement of the

Figure 8. Binding of pro-u-PA to a solubilized form of the u-PA receptor is not sufficient for conversion to the two-chain (active) u-PA. Solubilized u-PA receptor was prepared by incubating a confluent 75-cm² flask of CL-19 cells with 500 μ l DMEM containing 1 U/ml PI-PLC. (Panel A) 200 μ l of the solubilized u-PA-receptor (+PI-PLC) or 200 μ l DMEM (-PI-PLC) were incubated with 100 μ l of ³⁵S-labeled pro-u-PA containing medium (CL-F medium) and 1 ml of CL-19 conditioned medium for 18 h with or without addition of 100 ng/ml plasmin. The u-PA was immunoprecipitated with the anti-human u-PA monoclonal antibody 5B4 and analyzed by SDS-PAGE and fluorography. (Panel B) Cross-linking of the medium of the PI-PLC treated cells with ¹²⁵I-ATF with (+) or without (-) unlabeled 100 nM ATF as competitor.

matrix degradation is due to blocking the receptor with Δ F-u-PA.

The secreted pro-u-PA alone mediates matrix degradation, but as the cells were cultured in medium containing plasminogen, plasmin may be present as a contaminant either as a product of the low intrinsic pro-u-PA activity or maybe produced by the effect of low levels of t-PA that might be present in the extracellular matrix itself, as suggested by its presence in reconstituted basement membrane matrix (McGuire and Seeds, 1989). However, the activity of the pro-u-PA is enhanced by the presence of the u-PA-receptor. It has been shown that plasmin generation by pro-u-PA is strongly enhanced when the activity is measured in the presence of human U937 cells due to the binding of pro-u-PA to the surface receptors. Kinetic data show that concomitant binding of u-PA (to its receptor) and plasminogen to cell surfaces increases plasmin generation primarily by increasing the rate of pro-u-PA activation (Ellis *et al.*, 1989). Because CL-

F cells express u-PA in its proenzyme form, the enhancement of extracellular matrix degradation by the u-PA receptor-producing cells is also likely to be due to the acceleration of pro-u-PA activation. And indeed, under the experimental conditions used, the presence of u-PA receptor-producing cells results in an increased ratio of two-chain versus single-chain u-PA in the medium. Noteworthy, most of the pro-u-PA bound to the receptor is largely in the activated form (Figures 6 and 7). Finally, we have shown (Figure 8) that receptor binding per se is not sufficient to activate pro-u-PA; in the presence of a solubilized form of the receptor, no pro-u-PA activation is observed. Probably the cell surface is required because it provides not only the u-PA-receptor but also binding sites for plasminogen. Moreover, binding of both components to the cell surface might cause the appropriate steric relationship (proximity) for pro-u-PA/plasminogen activation. After addition of plasmin, pro-u-PA is activated both in the presence and ab-

sence of a solubilized receptor. It is therefore possible that a crucial factor(s) introduced by the cell surface binding is a mechanism for the initiation of the plasminogen activation system.

These data agree with and provide physiological significance to the previous findings indicating an important role for the u-PA receptor in cellular invasiveness (Ossowski, 1988) and showing that surface bound pro-u-PA can catalyze plasmin formation from surface-bound plasminogen (Ellis *et al.*, 1989; Stephens *et al.*, 1989). Many cultured tumor cells have their surface receptors saturated with endogenous u-PA, and this has been suggested to be an important feature in the establishment of the invasive phenotype (Stoppelli *et al.*, 1986; see Blasi, 1988, for further references). The results reported in this paper, while identifying a physiological and biochemical basis for such property, also show that not only endogenous u-PA and pro-u-PA but also u-PA produced by other cells can bind the u-PA receptor with the same results. This phenomenon may not only occur under experimental conditions. Recent immunohistochemical and in situ hybridization studies have shown that u-PA and its receptor are produced by different cell populations in human colon adenocarcinomas: u-PA being produced by fibroblast-like cells in the tumor stroma and u-PA-receptor by cancer cells located at invasive foci, indicating a cooperation between the stromal and the cancer cells in generating cell surface proteolysis (Grøndahl-Hansen *et al.*, 1991; Pyke *et al.*, 1991).

Materials and methods

Materials

Affigel-bound monoclonal antibody 5B4 (Nolli *et al.*, 1986) was a generous gift of Dr. M.L. Nolli (Lepetit SpA, Italy). Anticatalytic polyclonal antibodies against u-PA have been previously described and characterized (Binnema *et al.*, 1986). Synthetic peptides human u-PA[12-32(ala19)] and mouse u-PA[13-33(ala20)] have been previously described (Appella *et al.*, 1987). The human peptide was dissolved in dimethyl sulfoxide and then diluted 100-fold in medium. The mouse peptide was dissolved directly in water and diluted in medium. Phosphatidylinositol-specific phospholipase C from *Bacillus cereus* was obtained from Boehringer Mannheim (Mannheim, Germany).

Cell culture

Mouse LB6 cells transfected with the human u-PA-receptor cDNA under the control of the SV40 promoter (CL-19) and expressing the surface u-PA receptor have been described previously (Roldan *et al.*, 1990). LB6 cells expressing the human u-PA gene (CL-F) have been obtained following the same procedure as described before (Nolli *et al.*, 1989). CL-ΔF cells (Pedersen *et al.*, 1991) were used to produce a conditioned medium rich in ΔF-u-PA (see Results) that was withdrawn from confluent cultures. Untransfected mouse

LB6 cells or LB6 cells transfected with the RSV-neo gene (Gorman *et al.*, 1982) have been used as control. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Between the experiments the cells were propagated in medium containing 0.4 mg/ml G418 to keep the established cell lines under mild selective pressure.

Fibrin zymography

Fibrin zymography was performed essentially as described previously (Granelli-Piperno and Reich, 1978). u-PA (British standard urokinase) was used as a standard, because it contains both the high and the low molecular weight forms of u-PA.

Extracellular matrix preparation and degradation

Extracellular matrices were produced in vitro by bovine smooth muscle cells. Bovine smooth muscle cells (kindly provided by Dr. G. Sperti) were grown to confluence in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in 2-cm² culture dishes. After cells had reached confluency and formation extracellular matrix was started, the cells were incubated for 4 d with DMEM containing a ³H-amino acid mixture (1 μCi/ml; Amersham, United Kingdom). Cells were then lysed using 0.5% Triton X-100 in phosphate-buffered saline (PBS) and the cytoskeleton was removed by 25 mM ammonium hydroxide treatment. Unincorporated ³H-amino acids were washed from the remaining extracellular matrix using two water washes and 75% ethanol. Matrices were dried and stored at -20°C until use. To assay for matrix degradation activity, cells (10⁵/dish) were seeded onto ³H-labeled matrices and presoaked with complete medium for 1 h. Cells were seeded onto the matrix in full medium, in medium supplemented with specific inhibitors (as 0.1 TIU/ml Trasylol or inhibiting antibodies against u-PA, 30 μg/ml), or supplemented with conditioned medium. In all experiments, plasminogen was added to a final concentration of 0.14 μM. After 24, 48, and 72 h of incubation, the conditioned medium was removed and the remaining matrix was degraded with 0.25% trypsin (1 h at 37°C). ³H-amino acid release by the mouse LB6 cells was determined and expressed as percentage of the total amount of ³H released by the cells plus that solubilized by trypsin treatment.

Determination and immunoprecipitation of u-PA and pro-u-PA

Production of u-PA by cultured cells was measured by a previously published enzyme-linked immunosorbent assay (Binnema *et al.*, 1986).

For biosynthetic labeling, 4 × 10⁶ cells were plated for 12 h in a 75-cm² flask, starved for 45 min in serum-free, methionine-, and cysteine-free medium, and labeled overnight with ³⁵S-L-methionine and ³⁵S-L-cysteine (120 μCi/ml each) in methionine- and cysteine-free medium supplemented with 5% FCS. For immunoprecipitation, medium was collected and Trasylol and phenylmethyl sulfonyl fluoride (PMSF) added (to 0.3 TIU and 2 mM, respectively), supplemented with 1/3 of the volume of 0.2 M potassium phosphate buffer, pH 7.0, containing 2.0 M NaCl and 0.4% Triton X-100, cleared by centrifugation, and stored at -20°C. Immunoprecipitation was carried out using anti-u-PA monoclonal antibody 5B4, directed toward the kringle domain of the A chain (Nolli *et al.*, 1986), as previously described (Stoppelli *et al.*, 1986). Immunoprecipitates were analysed by SDS-PAGE (13%) under reducing conditions. In coculture ex-

periments the cells were labeled as described above except for 9 h, and medium or acid wash (see below) collected and immunoprecipitated. In this case 3 ml of labeled medium was added to 35 μ l 5B4-agarose beads and incubated 1 h at room temperature, then overnight at 4°C by gentle rocking. After washing with the potassium phosphate-NaCl-Triton buffer, the agarose beads were eluted with 0.1 M acetic acid, pH 3.0, in 1 M NaCl.

To dissociate any ligand from the cell surface, the cells were washed three times with PBS and treated for 5 min at room temperature with 50 mM glycine, pH 3.0, in 0.1 M NaCl (Stoppelli *et al.*, 1986). The acid washes were neutralized (final volume 3.0 ml); cleared by centrifugation; supplemented with Trasylol, PMSF, and the potassium phosphate-NaCl-Triton X-100 buffer; and immuno-precipitated as above.

In the experiments in which the state of free versus receptor-bound exogenously added pro-u-PA was analyzed, 200 μ l of ³⁵S-labeled CL-F medium and 2.8 ml unconditioned medium were added to a semiconfluent 25-cm² flask containing either LB6 or CL-19 cells and incubated for 3.5 h at 37°C in 10% CO₂ atmosphere. The whole 3.0 ml were immunoprecipitated as described above.

PI-PLC release of u-PA receptor from CL-19 cells

Approximately 6×10^6 cells were washed once in DMEM containing 25 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (HEPES) and incubated at 37°C for 1 h in 0.5 ml of DMEM-HEPES containing 10 μ l PI-PLC from *Bacillus cereus* (final concentration 0.4 μ g/ml). At the end of the incubation, the medium was collected, cleared by centrifugation, and stored at -20°C. Conditioned medium (1 ml) of CL-19 cells was added to 200 μ l of fresh medium or of supernatant of the PI-PLC-treated cells and incubated with 200 μ l of ³⁵S-labeled CL-F medium, with or without plasmin (100 ng/ml) at room temperature for 18 h. At the end of the incubation, the whole mixture was immunoprecipitated as described above.

Cross-linking of iodinated ATF or u-PA to the u-PA receptor

Diisopropylfluoro-phosphate-treated human u-PA or the amino terminal fragment of u-PA, ATF (Stoppelli *et al.*, 1985), were iodinated and cross-linked to the u-PA receptor as previously described (Behrendt *et al.*, 1990; Ploug *et al.*, 1991). Specific activity was about 1500 cpm/fmole.

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