A Cellular Binding Site for the Mr 55,000 Form of the Human Plasminogen Activator, Urokinase

JEAN-DOMINIQUE VASSALLI, DANIEL BACCINO, and DOMINIQUE BELIN*
Institute of Histology and Embryology and *Department of Pathology, University of Geneva, Geneva 4, Switzerland

ABSTRACT The secretion of plasminogen activators has been implicated in the controlled extracellular proteolysis that accompanies cell migration and tissue remodeling. We found that the human plasminogen activator urokinase (Uk) (Mr 55,000 form) binds rapidly, specifically, and with high affinity to fresh human blood monocytes and to cells of the monocyte line U937. Upon binding, Mr 55,000 Uk was observed to confer high plasminogen activator activity to the cells. Binding of the enzyme did not require a functional catalytic site (located on the B chain of the protein) but did require the noncatalytic A chain of Mr 55,000 Uk, since Mr 33,000 Uk did not bind. These results demonstrate the presence of a membrane receptor for Uk on monocytes and show a hitherto unknown function for the A chain of Uk: binding of secreted enzyme to its receptor results in Uk acting as a membrane protease. This localizes plasminogen activation near the cell surface, an optimal site to facilitate cell migration.

Various physiological and pathological processes, e.g., organogenesis during embryonic development, invasive and metastatic spreading of malignant tumors, and inflammatory reactions, require that certain cell types transgress the normal anatomical boundaries of tissues and migrate in and out of different body compartments. To allow such cellular migrations, mechanisms that provide for the focal degradation of components of the extracellular matrix must be available. Although the enzymatic basis for such degradation is not completely understood, several lines of evidence have suggested that extracellular proteolysis catalyzed by the secretion of plasminogen activators may play an important part in the degradative events necessary for migration of cells in tissues (24).

The plasminogen activator urokinase (Uk),1 originally identified in human urine as an activator of the fibrinolytic system (27), is a serine protease of trypsin specificity; it converts plasminogen, a zymogen, into plasmin, a neutral protease of broad specificity. Active human Uk consists of two polypeptide chains linked by a disulfide bond, and exists in a high molecular weight (Mr 55,000) and a low molecular weight (Mr 33,000) form; the latter is a proteolytic product of Mr 55,000 Uk lacking most of the A chain but retaining the active site-containing B chain (10). It is now well established that a variety of cell types synthesize and secrete Uk-type plasminogen activators (9); these include cells endowed with migratory and invasive properties, such as monocytes-macrophages (34), polymorphonuclear leukocytes (37), and implanting trophoblast (30), and cells derived from malignant tumors (19).

We describe here the specific binding of Mr 55,000 Uk to the surface of U937 cells, a monocyte-like human cell line (31), and to freshly prepared human peripheral blood monocytes. This interaction provides a means by which Uk-producing cells can express both a secreted and a membrane-associated form of the enzyme. Enzymatic activity of membrane-bound Uk is localized to the vicinity of the cell surface, where it can catalyze the focal lysis of extracellular substrates, and may thereby facilitate monocyte migration.

MATERIALS AND METHODS

Cell Culture: U937 cells were grown in RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Gibco Laboratories), and cultures were split 1/3 every 4 d.

Monocyte-enriched cultures were prepared from Ficoll-Hypaque-purified human peripheral blood mononuclear cells (3). Mononuclear cells were plated in fibrinogen-coated (10 µg/cm²) Nunc (Bayer AG, Basel, Switzerland) 24-well tissue culture plates (3 x 10⁶ cells in 1 ml RPMI 1640 with 10% FBS that had been heated for 30 min at 56°C [HI-FBS]). 5 h later the wells were washed twice vigorously with RPMI 1640, and the cultures were incubated further for 16 h in RPMI 1640 with 10% HI-FBS.

1 Abbreviations used in this paper: AT-BSA, acid-treated BSA; DFP, diisopropylfluorophosphate; EGF, epidermal growth factor; FBS, fetal bovine serum; TA, tissue activator; Uk, urokinase.
Binding Studies: U937 cells were collected by centrifugation, washed twice in RPMI 1640, and resuspended at 1 x 10^6 cells/ml in RPMI 1640 containing 1 mg/ml acid-treated (17) BSA (A-7638, Sigma Chemical Co, St Louis, MO) (AT-BSA). Aliquots of 0.3 ml were distributed to Falcon No. 2058 tubes (Falcon Labware, Div., of Becton, Dickinson & Co., Oxnard, CA) and incubated at 37°C in the presence of rabbit anti-human Uk IgG or irrelevant IgG (0.1 mg/ml) for 1 h at 8°C using DuPont Cronex Par-Speed intensifying screens (E.I. Du Pont de Nemours & Co., Newton, CT). Molecular weights were calculated from the position of markers (Pharmacia, Uppsala, Sweden; low molecular weight calibration kit) in the stained gels.

Immunofluorescence: Cultured monocytes-macrophages were washed twice with PBS + 1 mg/ml AT-BSA, incubated for 30 min at 24°C, washed three times with PBS + BSA, incubated for 15 min at 4°C in the presence of fluorescein-labeled sheep immunoglobulins anti-rabbit IgG (heavy and light chains) (Institut Pasteur Production, Paris, France) (10 µg/ml), washed twice with PBS and PBS, incubated for 15 min at 24°C in the presence of rabbit anti-human Uk IgG or irrelevant IgG (0.1 mg/ml) in PBS + BSA, washed three times with PBS + BSA, incubated for 15 min at 24°C in the presence of fluorescein-labeled sheep immunoglobulins anti-rabbit IgG (heavy and light chains) (Institut Pasteur Production, Paris, France) (10 µg/ml) in PBS, and finally washed four times with PBS + BSA. Photographs were taken on a Zeiss Photomicroscope 2 with RS3 epifluorescence optics onto Kodak Ektachrome 400 film.

Plasminogen Activator Plaque Assay: U937 cells were incubated with Uk, washed by centrifugation through sucrose as described above under binding studies, resuspended in casein-agar-plasminogen medium (36), and plated in Falcon 35-mm tissue culture dishes. Ficoll-Hypaque-purified mononuclear cells were plated in Falcon 35-mm tissue culture dishes (1.3 x 10^6 cells in 1.5 ml RPMI 1640 with 10% HI-FBS). After 24 h of incubation, the cultures were washed four times with PBS, kept for 30 min at 37°C in RPMI 1640 + 1 mg/ml AT-BSA with or without added Uk, washed again three times with PBS, and overlaid with the casein-agar-plasminogen medium.

Radioactivity remaining in the medium was quantitatively recovered as two major bands (lane 1): an Mr 55,000 band, corresponding to the added 125I-Uk, and an Mr 94,000 band, corresponding to a covalent complex formed between Uk and a plasminogen activator-specific inhibitor secreted by U937 cells (34); this complex was not observed in the presence of excess unlabeled Uk (lane 3), the secreted inhibitor being saturated under these conditions. Cellular binding of 125I-Uk showed only little temperature dependence: after 60 min of

RESULTS

The plasminogen activator secreted by human monocytes-macrophages and U937 cells is electrophoretically and immunologically identical to M, 55,000 Uk (34). To investigate the fate of Uk after secretion, we added 125I-labeled M, 55,000 enzyme to U937 cells in culture. Cells and media were collected after 30 min of incubation at 37°C, and the cell-associated radioactivity was determined; the samples were also subjected to SDS PAGE under nonreducing conditions, and the gel was analyzed by autoradiography (Fig. 1). A substantial fraction (13.2 ± 0.1%) of the total radioactivity added to the cultures was associated with the cells and migrated with an apparent Mr of 55,000 (lane 2); in the presence of a 60-fold excess of unlabeled M, 55,000 Uk, only 1.1 ± 0.2% of the added radioactivity was cell associated (lane 4). Radioactivity remaining in the medium was quantitatively recovered as two major bands (lane 1): an Mr 55,000 band, corresponding to the added 125I-Uk, and an Mr 94,000 band, corresponding to a covalent complex formed between Uk and a plasminogen activator-specific inhibitor secreted by U937 cells (34); this complex was not observed in the presence of excess unlabeled Uk (lane 3), the secreted inhibitor being saturated under these conditions. Cellular binding of 125I-Uk showed only little temperature dependence: after 60 min of

![Figure 1 Binding of M, 55,000 125I-Uk to U937 cells. Lanes 1 and 3, media; lanes 2 and 4, cells. Incubation (30 min, 37°C) of M, 55,000 125I-Uk (3 x 10^-10 M) with U937 cells was performed in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of unlabeled M, 55,000 Uk (1.8 x 10^-8 M). Culture media and cell lysates were prepared and subjected to SDS PAGE followed by autoradiography.](image-url)
incubation, 15.5 ± 0.6% of the added 125I-Uk was cell associated at 37°C, and 12.3 ± 0.3% at 0°C, indicating that endocytosis of the enzyme was probably not involved. These results suggested the existence of a saturable binding site for free 125I-Uk on the surface of U937 cells, and further experiments were carried out to characterize this interaction.

To investigate the contribution of the enzyme's catalytic site on the cellular binding of Uk, we reacted M, 55,000 125I-Uk with DFP, an inhibitor of serine esterases that irreversibly phosphorylates their active site. DFP-inactivated M, 55,000 125I-Uk bound to U937 cells as effectively as the active enzyme (Fig. 2, lanes in A and C), and this binding was also completely prevented in the presence of an excess of unlabeled M, 55,000 Uk (Fig. 2, lanes in B and D), or M, 55,000 DFP-Uk (Table I). We concluded that enzymatic activity was not necessary for cellular binding of Uk, and that the catalytic site was probably not involved in this binding. Since DFP-125I-Uk does not form a covalent complex with the U937-produced plasminogen activator-specific inhibitor, 125I-Uk binding to U937 cells did not appear to be a consequence of covalent complex formation with this ligand. Nevertheless, to avoid the influence of possible secondary interactions between bound Uk and this ligand (such interactions being presumably responsible for the M, 94,000 radioactivity associated with the cells after longer incubations with M, 55,000 125I-Uk [compare Fig. 1, lane 2, and the lanes in Fig. 2A]), we chose to carry out the following experiments using DFP-inactivated 125I-Uk.

Cellular binding of M, 55,000 DFP-125I-Uk was fast, half-maximal specific binding being achieved in <7 min at 20°C (Fig. 3A). We also determined the amount of enzyme bound in the presence of increasing concentrations of M, 55,000 DFP-125I-Uk (Fig. 3B). By Scatchard analysis of these data (Fig. 3C), we estimated the number of binding sites per U937 cell at approximately 60,000; the Kd of the interaction was calculated to be on the order of 4 x 10^-11 M.

### Table I

| No competitor | DFP-Uk55 | DFP-Uk33 | TA |
|---------------|----------|----------|----|
| DFP-125I-Uk55 | 5.2 ± 0.2 | 0.4 ± 0.1 | 4.8 ± 0.7 |
| DFP-125I-Uk33 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.1 |
| 125I-TA | 0.9 ± 0.1 | 0.8 ± 0.1 | NT |

U937 cells were cultured for 90 min at 4°C in the presence of the ligands and competitors as indicated. After incubation the cells were washed and the cell-associated radioactivity (percent of total) was determined (results are expressed as the mean ± SEM of two determinations). Ligands were added at the following concentrations: M, 55,000 DFP-125I-Uk, 5.4 x 10^-10 M; M, 33,000 DFP-125I-Uk, 1.5 x 10^-9 M; M, 33,000 DFP-Uk, 1.5 x 10^-9 M; M, 33,000 DFP-Uk, 2.3 x 10^-9 M. Competitors: M, 55,000 DFP-Uk, 2 x 10^-9 M; M, 33,000 DFP-Uk, 4.3 x 10^-9 M; TA, 1 x 10^-9 M; NT, not tested. (Uk55 and Uk33, M, 55,000 and 33,000 Uk, respectively, here and in the following tables.)

UK is synthesized and secreted as a one-chain, M, 55,000, zymogen (21, 38). Limited proteolysis by plasmin can convert this proUk into the two-chain, M, 55,000, active enzyme (21, 38), composed of a 158 amino acid A chain and a 253 amino acid B chain, linked by a disulfide bond (11). This Uk form predominates in urine (8). An M, 33,000 form is also found in urine and cell culture media; it is composed of an intact B chain and a "mini" A chain consisting of the C-terminal 21 amino acids of the A chain (10, 29). We have analyzed the binding of M, 33,000 DFP-Uk to U937 cells (Table I); we did not detect any specific binding of the 125I-labeled protein; in addition, using a nearly 100-fold molar excess of M, 33,000 DFP-Uk, we found no inhibition of binding of M, 55,000 DFP-125I-Uk. These results indicate that Uk binding to U937 cells is a property of the M, 55,000 form of the enzyme. Since M, 33,000 Uk differs from M, 55,000 Uk only by the absence of most of the enzyme's A chain (10), the cellular binding of Uk requires the presence of determinants of the intact A chain.
The amino acid sequence of human Uk has recently been elucidated (11, 29). Two regions of the A chain of Uk show homologies with other proteins: an N-terminal region (amino acids 13 to 43) presents clusters of sequences that are also elucidated (1 l, 29). Two regions of the A chain of Uk show residues 50 to 132 show 42% of identity with the fifth kringle found in murine EGF and in bovine blood factor X, whereas homologies with other proteins: an N-terminal region (amino
tional catalytic site, the bound enzyme may be active, and it is therefore not the property of a subpopulation or a contaminant in our cultures.

Since the cellular binding of Uk does not require a functional catalytic site, the bound enzyme may be active, and Uk secretion followed by binding to a high-affinity cellular site could result in Uk functioning in part as a cell-bound enzyme. To examine the validity of this hypothesis, we incubated U937 cells and monocytes with M, 55,000 or 33,000 Uk, washed the cells, and analyzed their plasminogen activator activity by the casein-agar overlay procedure (Fig. 5). After incubation, the cells were washed, and cell-associated radioactivity was determined. Specifically bound Uk was calculated by subtracting the counts per minute bound in the presence of 1.6 × 10⁻⁶ M unlabeled M, 55,000 DFP-Uk. (Nonspecific binding never exceeded 1% of total counts per minute in the cultures, and 8% of total counts per minute bound to the cells.) (C) Scatchard analysis of the data presented in B.

![Figure 3](image-url) Binding of M, 55,000 DFP-¹²⁵I-Uk to U937 cells. (A) U937 cells were cultured at 20°C for various periods of time in the presence of M, 55,000 DFP-¹²⁵I-Uk (6 × 10⁻¹⁰ M), with (O) or without (x) the addition of unlabeled M, 55,000 DFP-Uk (1.4 × 10⁻⁸ M). After incubation the cells were washed, and cell-associated radioactivity was determined. (B) U937 cells (2 × 10⁶/ml, 0.3 ml/culture) were incubated for 90 min at 20°C, in the presence of twofold increasing concentrations of M, 55,000 DFP-¹²⁵I-Uk (2.2 × 10⁻¹¹ M to 1.4 × 10⁻⁸ M). After incubation the cells were washed, and cell-associated radioactivity was determined. Specifically bound Uk was calculated by subtracting the counts per minute bound in the presence of 1.6 × 10⁻⁶ M unlabeled M, 55,000 DFP-Uk. (Nonspecific binding never exceeded 1% of total counts per minute in the cultures, and 8% of total counts per minute bound to the cells.) (C) Scatchard analysis of the data presented in B.

### Table II

| Added component | Final concentration | Inhibition of binding M % |
|-----------------|---------------------|--------------------------|
| Uk              | 2.5 × 10⁻⁸          | 93 ± 1                   |
| DFP-Uk          | 2.5 × 10⁻⁸          | 92 ± 2                   |
| Plasminogen     | 1 × 10⁻⁷            | -6 ± 5                   |
| DFP-plasmin     | 1 × 10⁻⁷            | -8 ± 3                   |
| Factor Xa       | 4 × 10⁻⁹            | 11 ± 1                   |
| Thrombin        | 4 × 10⁻⁶            | 8 ± 1                    |
| EGF             | 1.7 × 10⁻⁶          | -25 ± 13                 |
| Insulin         | 1.7 × 10⁻⁶          | 82 ± 2                   |

The experimental protocol is the same as for Table I. Percent inhibition was calculated relative to controls containing M, 55,000 DFP-¹²⁵I-Uk (3 × 10⁻¹⁰ M) added to the cells in the absence of competitors; under these conditions bound counts per minute represented 11.9 ± 0.8% of total counts per minute added to the cultures.

### Table III

| Binding of M, 55,000 Uk to Human Monocytes | No competitor | DFP-UkSs | DFP-UkSs |
|------------------------------------------|---------------|----------|----------|
| Monocytes                                | 5.2 ± 0.5     | 0.5 ± 0  | 5.2 ± 0  |
| Control plates                           | 0.4 ± 0.1     | 0.5 ± 0  | 0.4 ± 0  |

Monocytes were cultured for 30 min at 20°C in presence of the ligand M, 55,000 DFP-¹²⁵I-Uk, 5.8 × 10⁻¹⁰ M) and competitors (M, 55,000 DFP-Uk, 1.7 × 10⁻⁸ M; M, 33,000 DFP-Uk, 2.8 × 10⁻⁸ M) as indicated. After incubation the cells were washed, and the cell-associated radioactivity (percent of total) was determined. Control plates were prepared and treated in parallel, except that mononuclear cells were not added.
FIGURE 4  Immunofluorescence detection of Mr 55,000 Uk bound to cultured monocytes-macrophages. Cells were incubated in the absence (A) or presence (B) of Mr 55,000 Uk (1 x 10^{-8} M), and bound Uk was visualized on live, unfixed cells by indirect immunofluorescence using rabbit antibodies directed against human Uk. Controls included cells incubated with Mr 33,000 Uk and cells incubated with Mr 55,000 Uk followed by irrelevant IgG; fluorescence of control cultures was comparable to that of A. × 600.

FIGURE 5  Plasminogen activator plaque assay of U937 cells and monocytes. Effect of exogenous M, 55,000 Uk. U937 cells were incubated for 60 min at 4°C, without (A) or with (B) Mr 55,000 Uk (1.1 x 10^{-9} M) or Mr 33,000 Uk (C) (1.8 x 10^{-9} M). The cells were washed, resuspended in casein-agar-plasminogen medium, and plated in tissue culture dishes. Photographs were taken after 3 h of incubation at 37°C. Monocytes were incubated for 30 min at 37°C, without (A) or with (B) Mr 55,000 Uk (1.2 x 10^{-9} M) or (C) Mr 33,000 Uk (2.4 x 10^{-9} M). The cells were washed and overlayed with casein-agar-plasminogen medium. Photographs were taken after 40 min of incubation at 37°C. × 1.6.
many more plaques (Fig. 5B). These were due to the enzymatic activity of the exogenously added Uk, and not to increased Uk synthesis by treated cells, since cycloheximide (5 μg/ml) did not prevent the increase in plasminogen activator activity after exposure to Mr 55,000 Uk, and since cells that had bound Mr 55,000 DFP-inactivated Uk did not produce more plaques than did control cells (data not shown). Finally, addition of Mr 33,000 Uk did not increase the proteolytic activity of U937 cells (Fig. 5C). Similar results were obtained with human monocytes: no lysis was observed in control cultures (Fig. 5A) or in cultures that had been exposed to Mr 33,000 Uk (Fig. 5C), whereas Mr 55,000 Uk-treated cultures produced many proteolytic plaques (Fig. 5B); phase-contrast microscopy confirmed that the zones of lysis surrounded monocytes-macrophages. We concluded that cell-bound Mr 55,000 Uk remains at least in part enzymatically active, and can thereby catalyze plasmin-mediated proteolysis around monocytes and U937 cells.

DISCUSSION

The cellular binding of the plasminogen activator Uk (Mr 55,000 form) provides a mechanism by which the enzyme can function not only as a secreted protease, diffusing away from its site of synthesis, but also in a membrane-associated form, catalyzing focalized plasmin formation and thereby high proteolytic activity in the close environment of the cell. Although the role of plasminogen activator in the biology of mononuclear phagocytes remains a matter of conjecture, enzyme production is thought to be related to the migratory properties of these cells (35). In this context, a membrane-bound plasminogen activator is optimally located to generate proteolytic activity along the path of monocyte migration. In addition, membrane-bound Uk may be less susceptible to the protease inhibitors present in the extracellular fluid (4, 5). Plasminogen activation has been postulated to be essential for the migratory and invasive properties of other Uk-producing cells, such as implanting murine trophoblasts (30) or malignant cells (19, 22); similar Uk-binding sites may thus exist on the surface of these cells as well.

Binding of Mr 55,000 Uk to human monocytes and U937 cells may be related to the fact that these cells themselves produce Uk (34). We propose that during or following secretion, at least some of the (pro?)enzyme binds to the cell surface. In this respect, it is of interest to note that murine macrophages, which synthesize a Uk-type enzyme (in preparation), express a membrane-associated form of plasminogen activator (5, 16, 28). The interaction described here suggests that this membrane-associated form of the enzyme is due to the binding of secreted Uk to a high-affinity cell membrane receptor, rather than to the synthesis and membrane insertion of a distinct species of plasminogen activator. It should also be recalled that Uk and proUk are present in human plasma (32, 39), at a concentration (1–2 × 10^{-10}M) which is on the order of the \( K_d \) value determined in this paper for Mr 55,000 Uk binding to U937 cells. One may thus speculate that cells can bind circulating enzyme, or enzyme produced by neighboring cells in the environment of a tissue.

The Mr 33,000 form of Uk did not bind to the cells studied; it differs from Mr 55,000 Uk by the absence of the first 135 amino acids of the 158 amino-acid A chain. This indicates that determinants of the A chain are necessary for binding to occur, although at this time it cannot be taken to imply that the A chain itself does bind to the cellular receptor. To further characterize the cellular binding of Mr 55,000 Uk, it will be of interest to evaluate the binding of proUk, and of various fragments derived from the A chain of the enzyme. In any event, the requirement for the presence of an intact A chain for cellular binding of Uk provides a novel function for this domain of the molecule.

We did not detect any significant competition for binding of Mr 55,000 Uk by other proteins with homologies to the A chain of the enzyme, nor by thrombin, suggesting that the binding site is specific for Uk. Binding of both thrombin and a functional prothrombinase complex (consisting of factors Va and Xa) to human monocytes have also recently been described (7, 33). Fibrin formation as well as fibrinolysis can thus be catalyzed at or near the surface of monocytes-macrophages; membrane binding sites for these seemingly antagonistic reactions appear to be different, and regulation of their expression may control the overall deposition and removal of fibrin around these cells, in particular within inflammatory lesions.

The cellular binding of Mr 55,000 Uk contrasts with the absence of interaction between the other human plasminogen activator, namely TA, and the cells studied in this work. We suggest that Mr 55,000 Uk is a form of plasminogen activator with an affinity for the cell surface, where it catalyzes plasmin formation in the close environment of the cell, in the same way that TA represents a form of plasminogen activator with an affinity for fibrin clots where it catalyzes a high plasmin-mediated thrombolytic activity (20). The determinants that direct these specific extracellular localizations appear to reside in the A chains of both enzymes. The recently described adsorption of TA to cultured fibroblasts (13) suggests that this form of plasminogen activator also can become cell associated. However, in contrast with the cellular binding of Uk described here, binding of TA was due to the formation of an SDS-resistant complex between the enzyme and a cellular ligand; furthermore, TA adsorption to fibroblasts was slow as compared with that of Uk to U937 cells, and appeared to be involved in the clearance of the enzyme.

Finally, the interaction of Uk with U937 cells and monocytes could be considered as that of a hormone with its membrane receptor. The region within the A chain of Mr 55,000 Uk which is homologous to murine EGF has been described as a "growth factor domain" (11). Our studies show that EGF does not compete for the Uk binding site; nevertheless, we intend to search for possible "trophic" effects of Mr 55,000 Uk on monocytes and other cells. Increased levels of Uk have been observed in malignant tumors (19), and autocrine effects (14) may be responsible for some aspects of the malignant phenotype.

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VASSALLI ET AL. Cellular Binding of M, 55,000 Urokinase 91
