

Urokinase Plasminogen Activator Receptor, β 2-Integrins, and Src-kinases within a Single Receptor Complex of Human Monocytes

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Summary

The glycosylphosphatidylinositol (GPI)-anchored membrane protein urokinase plasminogen activator-receptor (uPA-R; CD87) is one of the key molecules involved in migration of leukocytes and tumor cells. uPA bound to uPA-R provides the cell proteolytic potential used for degradation of extracellular matrix. uPA-R is also involved in induction of cell adhesion and chemotaxis. Here, we provide a molecular explanation for these uPA-R-related cellular events. By size fractionation of monocyte lysate and affinity isolation on its natural ligand uPA, we demonstrate uPA-R as a component of a receptor complex of relatively large size. Reprecipitation and immunoblotting techniques allowed us to detect the protein tyrosine kinases (PTKs) p60^{lyn}, p53/56^{lyn}, p58/64^{hck}, and p59^{src} as components of this "uPA-R complex". Activation of monocytes even with enzymatically inactivated uPA resulted in induction of tyrosine phosphorylation, suggesting modulation of uPA-R-associated PTKs upon ligand binding. In spite of their presence in large complexes, we did not find the GPI-linked proteins CD14, CD58, and CD59 in the uPA-R complex, which indicates the presence of different receptor domains containing GPI-linked proteins in monocytes. However, we identified the leukocyte integrins LFA-1 and CR3 as components of the uPA-R complex as indicated by coisolation of these molecules, as well as by cocapping and comodulation of uPA-R and leukocyte integrins on the monocyte surface. The assemblage of uPA-R, PTKs and membrane spanning β 2-integrins in one receptor complex indicates functional cooperation. In regard to the involvement of these molecules in pericellular proteolysis, signal transduction, as well as adhesion and chemotactic movement, we suggest uPA-R complex as a potential cellular device for cell migration.

Cell activation, adhesion, and extracellular proteolysis are causally connected in leukocyte migration, a process indispensable for immunity and inflammation. The complexity of this process requires concerted action and interaction of molecules underlying the mechanisms involved. Indeed, molecules mediating adhesion, cell surface proteolysis as well as signal transduction have been colocalized in regions of cell contact (1). One of these critical molecules seems to be uro-

kinase plasminogen activator-receptor (uPA-R)² (2, 3) recently assigned as CD87 antigen (4). uPA-R is a highly glycosylated 50–65-kD protein linked to the plasma membrane by glycosylphosphatidylinositol (GPI) (5). It is expressed on mononuclear phagocytes (6), neutrophils (7), activated T cells (8), endothelial cells (9), and several types of tumor cells (10). uPA-R focuses and controls the fibrinolytic system

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² Abbreviations used in this paper: DFP, diisopropyl fluorophosphate; GPI, glycosylphosphatidylinositol; MFI, mean fluorescence intensity; PTK, protein tyrosine kinase; TRITC, tetramethylrhodamine isothiocyanate; uPA-R, urokinase plasminogen activator-receptor.

at the cell surface. Its ligand, uPA, is converted into the active form upon binding to uPA-R, and thus gains the ability to enzymatically digest plasminogen to the serine protease plasmin. This delivers to cells a spatially and temporally regulated proteolytic potential necessary for remodeling of pericellular space during migration as well as for generation of active substances employed in this process. Internalization, ligand clearing, and reexpression of uPA-R are crucial devices controlling uPA activity, and therefore the plasminogen system (3, 11). Therefore, uPA-R can be considered as one of the possible targets in treatment of inflammatory disorders and metastasis (10).

In addition to its function in the fibrinolytic system, recent reports attribute a further active role to uPA-R in cell migration. It has been shown that incubation of myeloid cells with uPA results in adhesion induction (12, 13) and chemotactic movement (14, 15). Thus, there is evidence that interaction of uPA-R with its ligand participates in the activation of migrating cells, which is required for complete migration and development of the inflammatory response. These observations classify uPA-R as a potential signal-transducing molecule in cells. Indeed, changes in tyrosine phosphorylation (16) and diacylglycerol formation (17) have been observed as a result of uPA-R engagement. However, the mechanisms responsible for the signaling capacity of uPA-R and its effects on adhesion and chemotaxis regulation, have so far remained unclear.

Here, we report that uPA-R is a component of a multimeric receptor complex in human monocytes. Using its natural ligand, uPA, we coisolated four members of the Src-protein tyrosine kinase (PTK) family with uPA-R, and provide evidence for their involvement in signal transduction by induction of tyrosine phosphorylation through uPA. Furthermore, we demonstrate the presence of β 2-integrins, molecules functionally important for cell adhesion and migration, in this receptor complex.

Materials and Methods

Cells. Peripheral blood monocytes were isolated from the peripheral blood of healthy individuals by density gradient centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden), followed by removal of the E-rosette-forming fraction (18). These preparations contained at least 75% CD14-positive cells and were free of other blood cells expressing uPA-R, as analyzed by flow cytometry.

Antibodies. Mouse mAbs E4 (IgG2b), F10 (IgG1), and H2 (IgG1) against uPA-R were obtained as a result of immunization with recombinant soluble human uPA-R (19). Using an uPA-R ELISA (19), mAbs E4 and F10 were found not to compete for binding of uPA to uPA-R, whereas anti-uPA-R mAbs VIM5 (IgG1; reference 7) and no. 3936 (IgG2a; purchased from American Diagnostica, Greenwich, CT) inhibited uPA binding. mAbs MEM-57 (IgG2a) to CD3; MEM-83 (IgG1) to CD11a; MEM-95 (IgG1) to CD11a; 5E6 (IgG1) to CD11a; MEM-170 (IgG1) to CD11b; VIM12 (IgG1) to CD11b; MEM-18 (IgG1) to CD14; MEM-48 (IgG1) to CD18; 7E4 (IgG2a) to CD31; MEM-59 (IgG1) to CD43; MEM-56 (IgG2b) to CD45RA; MEM-43 (IgG2a) to CD59; 132 (IgG2b) to M6; VIAP (IgG1) to alkaline phosphatase; and B2M-01 (IgG2a)

and B2M-02 (IgG1) both against human β 2-microglobulin, were prepared and characterized in our laboratories. Specificities of these mAbs were confirmed by the 3rd, 4th, and 5th International Workshop and Conference On Human Leucocyte Differentiation Antigens, with the exception of mAbs B2M-01 and B2M-02 (20), MEM-48 (21), 7E4 (22), 5E6 (23), VIAP, 132, and MEM-170. A clone TS2/9 producing mAb to CD58 (IgG1) was obtained from the American Type Culture Collection (Rockville, MD). mAb BU-15 (IgG1) to CD11c was purchased from The Binding Site (Birmingham, UK) and mAb IV.3 (IgG1) to CD32 from Medarex Inc. (West Lebanon, NH). mAbs PEN-2 (IgG2a) to CD11b and Ki-M1 (IgG1) to CD11c were samples from the 3rd and 5th International Workshops On Human Leucocyte Differentiation Antigens. mAb KIM127 to CD18 (24) was a gift from Dr. M. K. Robinson (Stanford University, Palo Alto, CA). The mouse mAb and the rabbit polyclonal Ab to caveolin were purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal Abs against Lyn, Fgr, and Hck were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antiserum against Fyn was kindly provided by Dr. A. Veillette (McGill University, Montreal, Canada). Rabbit antiserum against the p85 subunit of phosphatidylinositol 3-kinase and anti-phosphotyrosine mAb 4G10 were from UBI (Lake Placid, NY).

Preparation of uPA-Sepharose. Inactivated uPA (Serva, Heidelberg, Germany), or BSA were coupled to CNBr-activated Sepharose 4B (Pharmacia) as recommended by the manufacturer, except that the coupling was performed in a buffer containing 50 mM H_3BO_3 and 0.5 M NaCl (pH 8.0), at room temperature for 90 min.

Immunoprecipitation and In Vitro Kinase Assay. Freshly isolated monocytes were pretreated with 5 mM diisopropyl fluorophosphate (DFP) for 6 min, and lysed in ice-cold lysis buffer containing 20 mM Tris (pH 8.2), 140 mM NaCl, 2 mM EDTA, 1% Brij-58 (Pierce, Rockford, IL), 5 mM iodoacetamide, aprotinin and leupeptin (both 10 μ g/ml), 1 mM PMSF, 0.1 mM quercetin, 0.1 mM N-tosyl-L-phenylalanine chlormethyl ketone, 0.1 mM N_α -p-tosyl-L-lysine chlormethyl ketone, 0.1 mM N-CBZ-L-phenylalanine chlormethyl ketone (all from Sigma Chemical Co., St. Louis, MO) for 30 min. The lysates were subjected to immunoprecipitation by the solid phase immunoisolation technique on 96-well plastic plates, as described (25). For isolation of uPA-R with its natural ligand, uPA (40 μ g/ml) was directly coated on plastic plates instead of mAbs. Alternatively, uPA-Sepharose (see above) was used as an adsorption matrix. After washing isolated material with lysis buffer, an in vitro kinase assay was performed by adding 25 mM Hepes (pH 7.2), 5 mM $MnCl_2$, 20 mM $MgCl_2$, and 1 μ Ci of γ -[^{32}P]ATP (3,000 Ci/mmol) (NEN, Boston, MA). ^{32}P -labeled proteins were analyzed by SDS-PAGE and autoradiography.

For analysis by reprecipitation, uPA-R was affinity-isolated on uPA-Sepharose by incubation with void volume fractions of cell lysate (see below) for 4 h at 4°C. After in vitro kinase assay, phosphorylated proteins were eluted from uPA-Sepharose by lysis buffer containing 1% SDS (instead of Brij-58) for 10 min at room temperature, and then diluted 10 \times with lysis buffer containing 1% NP-40. This material was subjected to reprecipitation with indicated Abs for 3 h on ice. Immune complexes were collected with a fixed cell suspension of *Staphylococcus aureus*-Protein A (Sigma Chem. Co.), washed and analyzed by SDS-PAGE and autoradiography. Alternatively, the uPA-Sepharose isolated material was eluted by the SDS-PAGE sample buffer and analyzed by immunoblotting.

Immunoprecipitation of integrins was performed with mAbs covalently attached on Eupergit C1Z particles (Röhm GmbH, Weiterstadt, Germany) (26).

Immunoblotting. Nitrocellulose membrane replicas were blocked for 1 h in 5% nonfat milk, and immunostained by sequential incubation with specific mouse or rabbit Abs, followed by appropriate second Abs conjugated with peroxidase (Bio-Rad Labs, Richmond, CA). The enhanced chemiluminescence detection system (Amersham, Aylesbury, UK) was used for visualization of labeled proteins.

Gel Chromatography. The minicolumns (0.8 cm × 5 cm, total volume 3 ml) of Sepharose 4B (Pharmacia) were used as described (27). Briefly, after equilibration of the column with lysis buffer, 0.3 ml of cell lysate was applied on the top. Subsequently, 0.3-ml fractions of the eluate were collected, which were used either for immunoprecipitation or immunoblotting. Calibration of this column revealed its void volume in fractions 4, 5 (the elution volume of erythrocytes), and the elution volumes of IgM, and IgG were in the fractions 7 and 9, respectively.

Tyrosine Phosphorylation Analysis. Freshly isolated monocytes (2×10^6 cells/ml in RPMI 1640 medium with 10% FCS) were incubated with 50 nM uPA (kindly provided by Technoclon, Vienna, Austria) or 50 nM DFP-inactivated uPA for 5 and 15 min at 37°C. Afterwards, the cells were briefly pelleted in a microfuge and solubilized as described above, except that 1% NP-40 and 0.1% deoxycholic acid were contained as detergents in the lysis buffer. Cell lysates were analyzed by SDS-PAGE and immunoblotting using mAb 4G10 (UBI) for detection of tyrosine-phosphorylated proteins. DFP-inactivated uPA manifested undetectable enzymatic activity, as assayed by a plasminogen-dependent system (28). The LPS concentrations of the reagents employed in the treatment of monocytes were measured as described (29) by using the turbidimetric LAL-endotoxin assay from Pyrotell (Woods Hole, MA), and found to be <10 pg/ml in each sample. This is >1 log below the reported stimulatory level of the LPS-induced protein tyrosine phosphorylation in macrophages (30).

Cocapping. Freshly isolated monocytes were treated for 3 min with acid glycine (50 mM glycine, 100 mM NaCl, pH 3.0) to remove cell-bound uPA and incubated for 30 min at 4°C with 4 mg/ml of human Ig to block nonspecific FcR-mediated binding of mAbs. Cells (5×10^5 in 50 μ l RPMI 1640 medium containing 10% FCS and 20 mM HEPES) were incubated for 30 min at 4°C with 1 μ g mAb, washed, and the FITC-conjugated sheep F(ab')₂ anti-mouse IgG+IgM Abs (Grub, Scandic, Vienna, Austria) were added. To induce capping, the samples were shifted to 37°C for 5 min. After washing with PBS containing 1% BSA and 0.1% sodium azide, uPA-R was stained by sequential incubation with biotinylated uPA (biotin-X-NHS; Calbiochem-Novabiochem, La Jolla, CA) and tetramethylrhodamine isothiocyanate (TRITC)-conjugated streptavidin (Accurate Chemical & Scientific Corp., Westbury, NY). For coclustering analysis of PTKs, capped cells were permeabilized with 0.3% saponin, immunolabeled with the respective rabbit antisera to the Src-PTKs, fixed with 1% paraformaldehyde, and stained with TRITC-conjugated goat anti-rabbit IgG Abs (Accurate Chemical & Scientific Corp.). The cells were examined by fluorescence microscopy.

Antigen Modulation. Untreated or formaldehyde fixed (1%) mononuclear cells were incubated with either 10 μ g/ml of CD18 mAb KIM127 or isotype control mAb VIAP for 15 min at 37°C. Afterwards, the cells were washed once with cold PBS and stained with PE-labeled mAbs against different cell surface molecules for 30 min on ice. After washing twice with PBS, the cells were analyzed by flow cytometry. The monocytes were identified by their light-scatter distribution. The alteration in mAb binding due to mAb KIM127 preincubation was calculated by the comparison of

the mean fluorescence intensity (MFI) of mAb KIM127 to control mAb incubated monocytes and expressed in percent change of MFI.

Results

uPA-R is Associated with Protein Kinases. To evaluate association of uPA-R with protein kinases, we solubilized human monocytes using the mild detergent Brij-58 and subjected the lysate to immunoprecipitation followed by *in vitro* kinase assay and SDS-PAGE analysis. Independent of the isotype of the anti-uPA-R mAbs used for precipitation, and the fine specificities of these antibodies, *i.e.*, competing (mAbs VIM5, no. 3936) or non-competing (mAbs E4; F10 [not shown]) with binding of the natural ligand u-PA, we obtained a similar pattern of labeled proteins with molecular masses of ~55–60, 85, 100, and 120 kD. Interestingly, a similar pattern of *in vitro* phosphorylated zones, with an additional strong 40-kD component, was obtained with mAb IV.3 directed to the Fc γ -receptor type II (Fc γ RII, CD32) (Fig. 1 A). This indicates that in monocytes also, Fc γ RII is associated with protein kinases as was described with granulocytes (31), and monocytic cells (32) previously. Therefore, to rule out the possibility that coprecipitation of the protein kinase activity by mAbs to uPA-R was due to their interaction with Fc γ Rs, we performed *in vitro* kinase assay on the uPA-R affinity isolated from monocyte detergent lysate using its natural ligand, uPA. As shown in Fig. 1 B, the same pattern of *in vitro* phosphorylated proteins was obtained. Thus, coisolation of phosphoproteins pp55-60, pp85, pp100, pp120, and protein kinase activity with uPA-R is independent of Fc γ R, indicating that uPA-R is a component of a mul-

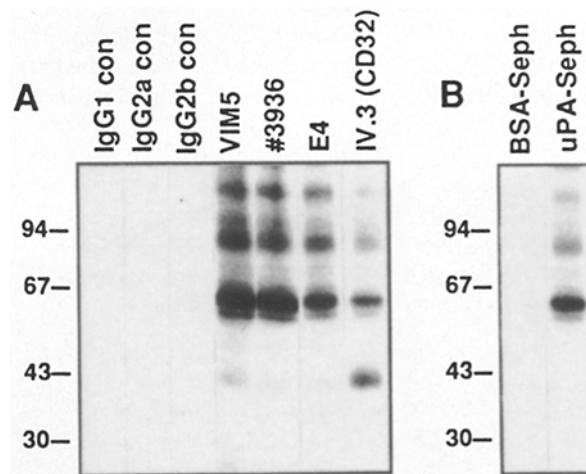


Figure 1. Coisolation of protein kinase activity with uPA-R. Monocytes were lysed and *in vitro* kinase assay on isolated uPA-R was performed. Presence of *in vitro*-labeled proteins was detected by SDS-PAGE and autoradiography. (A) uPA-R was immunoprecipitated with mAbs VIM5, no. 3936, and E4. As isotype control, mAbs B2M-01, B2M-02, and 132 were used. (B) uPA-R was affinity isolated on its ligand uPA, coupled to Sepharose 4B. BSA-coupled Sepharose 4B was used as a control matrix. Molecular mass standards are indicated in kD.

timolecular membrane complex. This material isolated by uPA is therefore designated in the text as "uPA-R complex". It should be noted, that virtually identical results were obtained when a more stringent detergent, NP-40, was used for solubilization (data not shown).

Determination of the Size of the uPA-R Complex. The existence of large detergent resistant complexes containing GPI-anchored proteins, (glyco)lipids, and protein kinases has been recently described by us (27, 33) and others (34–37). To test whether uPA-R complex has similar properties, we determined its size by fractionation of monocyte lysate employing gel filtration on a Sepharose 4B column (27). In the first set of experiments, we analyzed resulting fractions for uPA-R/protein kinase association. When we isolated uPA-R using its natural ligand, uPA, we detected kinase activity only in fractions 4 and 5, and the pattern of phosphoproteins was identical to that obtained from unfractionated lysate (Fig. 2). Because of the calibration of the column with erythrocytes, IgM and IgG, the fractions 4 and 5 correspond to the column void volume. Inasmuch as the exclusion limit of Sepharose 4B is in the range of tens of millions of daltons, this indicates a relatively large size of the uPA-R complex. To follow the distribution of the total uPA-R, and to analyze whether complexes of different composition or free forms exist, the particular fractions were subjected to SDS-PAGE and immunoblotting using anti-uPA-R mAb H2. All detectable uPA-R was found in void volume fractions, demonstrating that at least a majority of uPA-R is present in the complexes of large size (Fig. 3 A). In contrast, several other abundant monocyte surface proteins, such as CD43 (Fig. 3 B), CD18, CD32, and CD45RA (not shown) were detected in fractions corresponding both to the large complexes, and to single molecules (or simple complexes).

Identification of uPA-R-associated PTKs. We (27, 33, 38) and others (25, 35, 36, 39–41) have previously described association of several GPI-anchored proteins with members of

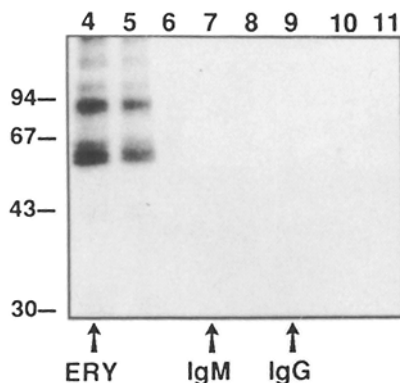


Figure 2. Determination of the size of uPA-R complex. Monocyte lysate was size fractionated using a Sepharose 4B column. uPA-R was affinity-isolated on its ligand, uPA, from the individual fractions, and analyzed by *in vitro* kinase assay. Presence of kinase activity was detected by SDS-PAGE and autoradiography. The fraction numbers are indicated at the top. The elution volumes of erythrocytes (ERY), IgM, and IgG are shown at the bottom. Molecular mass standards are indicated in kD.

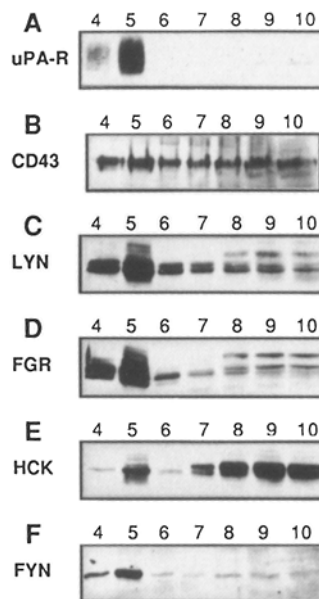


Figure 3. Distribution of monocyte antigens in size-fractionated monocyte lysate. Monocyte lysate was size-fractionated using a Sepharose 4B column and the resulting fractions (indicated on the top) were analyzed by immunoblotting with the following Abs: (A) H2 to uPA-R; (B) MEM-59 to CD43; (C) anti-Lyn; (D) anti-Fgr; (E) anti-Hck; (F) anti-Fyn. A similar distribution as for CD43 was found for CD18, CD32 and CD45RA.

the Src-family of PTKs. Among them, p60^{lyn}, p53/56^{lyn}, p58/64^{hck}, and p59^{fgr} are expressed by monocytes, and autophosphorylated or phosphorylated *in vitro* (25, 42). Thus, the presence of the 55–60 kD *in vitro* phosphorylated proteins in the uPA-R complex (Fig. 1) suggested identity with some of those. When we analyzed size-fractionated monocyte lysate (see above) by immunoblotting, we found a different distribution of the individual PTKs in the particular fractions (Fig. 3 C–F). However, all of them were detected in void volume fractions, indicating presence of subpopulations of these kinases in complexes of the similar size to that found for the uPA-R complex. Indeed, the PTKs p60^{lyn}, p53/56^{lyn}, p58/64^{hck}, and p59^{fgr} were clearly demonstrated by reprecipitation of *in vitro* phosphorylated material from the uPA-R complex by means of specific antibodies (Fig. 4 A), as well as by immunoblotting analysis of isolated uPA-R complex (Fig. 4 B).

Stimulation Via uPA-R Complex Involves Tyrosine Phosphorylation. Our finding that several members of the Src-family of PTKs are associated with uPA-R prompted us to examine possible tyrosine phosphorylation upon stimulation of monocytes with uPA. Indeed anti-phosphotyrosine immunoblotting analysis of monocyte lysates revealed that both active and DFP enzymatically inactivated uPA (DFP-uPA) induced tyrosine phosphorylation on several proteins in the range of 26–30 and 38–40 kD as compared to the medium control (Fig. 5). We did not detect phosphoproteins of similar size by the anti-phosphotyrosine immunoblotting analysis of the uPA-R complex, which was isolated from these monocyte lysates in parallel by using mAb E4 directed against a non-ligand-binding domain of the uPA-R (data not shown). These phosphoproteins in the range of 26–30 and 38–40 kD were also not seen in the *in vitro* kinase assay on isolated uPA-R (Figs. 1 and 2). Therefore, these findings suggest that the molecules tyrosine phosphorylated after the engagement of

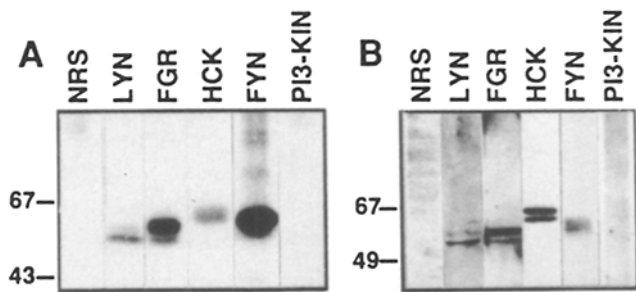


Figure 4. Identification of PTKs coisolated with uPA-R. (A) uPA-R was affinity isolated on uPA-Sepharose from void volume fractions of monocyte lysate, and subjected to *in vitro* kinase assay. PTKs were reprecipitated employing the indicated Abs, and the resulting precipitates were analyzed by SDS-PAGE and autoradiography. (B) uPA-R was affinity-isolated on uPA-Sepharose from void volume fractions of monocyte lysate, and analyzed by immunoblotting with the indicated Abs. Normal rabbit serum (NRS) was used as control. Molecular weight standards are indicated in kD.

uPA-R are downstream components of the signaling pathways rather than immediate associated substrates of the PTKs present in the uPA-R complex.

Identification of Further Components of the uPA-R Complex.

The PI3-K is an important signaling molecule, whose 85-kD subunit associates with several cell surface receptors, and can be tyrosine phosphorylated (43). However, neither by reprecipitation nor immunoblotting were we able to demonstrate the identity of this enzyme subunit to the *in vitro* phosphorylated 85-kD protein present in the uPA-R complex (Fig. 4, A and B).

Analysis of the size-fractionated cell lysate by immunoblotting indicated the presence of monocyte GPI-linked proteins CD14, CD58, and CD59 in the Sepharose 4B void volume fractions corresponding to the large complexes (data not shown). Due to our previous experience with the simultaneous presence of considerable amounts of several GPI-anchored proteins in large complexes (27, 33), we analyzed the uPA-R complex by immunoblotting using CD14, CD58, and CD59 mAbs. The result was negative (Fig. 6 A), indicating that none of these molecules, or only a small amount below the detection level of our assay, were present.

Recent reports have shown a relationship between the

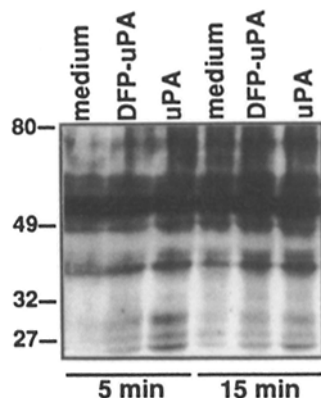


Figure 5. Induction of tyrosine phosphorylation upon incubation of monocytes with uPA. Monocytes were incubated with uPA, DFP-inactivated uPA, or with medium alone for 5 and 15 min. Tyrosine phosphorylated proteins were visualized by immunoblotting using mAb 4G10. Molecular weight standards are indicated in kD.

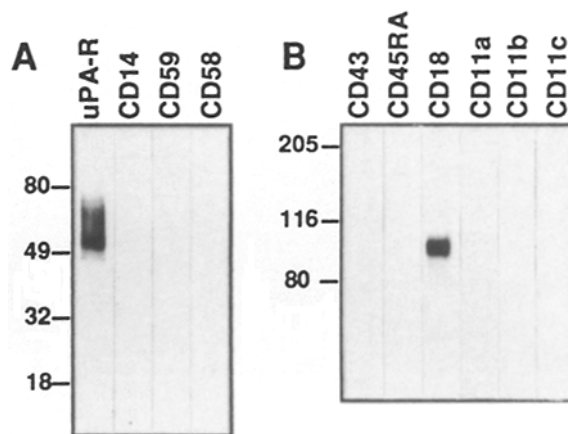


Figure 6. Identification of proteins coisolated with uPA-R. uPA-R was affinity isolated on uPA-Sepharose from void volume fractions of monocyte lysate, and analyzed by SDS-PAGE and immunoblotting with the following mAbs: (A) H2 to uPA-R, MEM-18 to CD14, MEM-43 to CD59, TS2/9 to CD58; analysis was performed using a 12.5% SDS-polyacrylamide gel. (B) MEM-59 to CD43, MEM-56 to CD45RA, MEM-48 to CD18, MEM-95 to CD11a, PEN-2 to CD11b, Ki-M1 to CD11c; analysis was performed using a 7.5% SDS-polyacrylamide gel. Molecular weight standards are indicated in kD.

detergent-resistant membrane domains enriched in GPI-anchored proteins and the plasma membrane invaginations termed caveolae in epithelial cells. Since the caveola coat protein caveolin is a v-Src substrate and seems to have a transmembrane topology, caveolin has been suggested to be involved in transmembrane signaling via GPI-anchored proteins in these cells (44). Therefore, we wanted to evaluate the role of caveolin in signaling via uPA-R in monocytes. However by using two anti-caveolin Abs in immunoblotting, we were unable to detect caveolin in detergent lysates of monocytes, while it was readily found in human fibroblast lysates under the same conditions (data not shown).

Subpopulations of several transmembrane monocyte antigens, including CD43, CD18, CD32, and CD45RA were found in Sepharose 4B void volume fractions (Fig. 3 B, and data not shown). From them, only CD18, the common subunit of β 2-integrins, was identified in uPA-R complex by immunoblotting (Fig. 6 B).

uPA-R Complex Contains the β 2-integrins LFA-1 and CR3.

Initially we tried to determine which of the β 2-integrins, LFA-1 (CD11a/CD18), CR3 (CD11b/CD18), CR4 (CD11c/CD18), are involved in the uPA-R complex by direct identification of the α -chains (CD11) using immunoblotting. For this, 12 mAbs against CD11a, 10 mAbs against CD11b, and 6 mAbs against CD11c were screened for immunoblotting reactivity with monocyte lysate. mAbs MEM-95 to CD11a, PEN-2 to CD11b, and Ki-M1 to CD11c were selected as the best reacting. Even with these mAbs we were unable to identify which CD11 molecule is present in the uPA-R complex (Fig. 6 B). Therefore, a reverse approach was taken. We immobilized mAbs to CD18, CD11a, CD11b, and CD11c on Eupergit C1Z microparticles, and used these immunosorbents to isolate the subpopulations of β 2-integrins from the Seph-

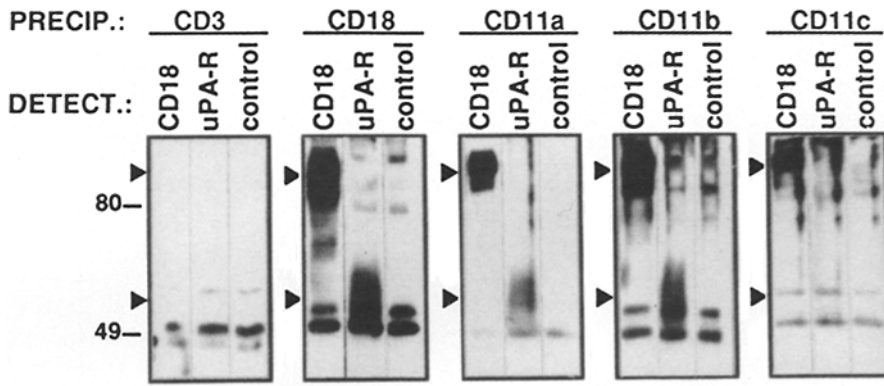


Figure 7. Coprecipitation of uPA-R with β 2-integrins. β 2-integrins were precipitated from Sepharose 4B void volume fractions of monocyte lysate using the following mAbs immobilized on Eupergit C1Z particles: MEM-48 to CD18, MEM-83 to CD11a, MEM-170 to CD11b, BU-15 to CD11c, and MEM-57 to CD3 (control). Resulting precipitates were analyzed by immunoblotting with mAb MEM-48 to CD18, H2 to uPA-R, and B2M-02 to β 2-microglobulin (control). Positions of uPA-R and CD18 are indicated by arrows. Molecular weight standards are given in kD.

arose 4B void volume fractions of monocyte lysate, where the uPA-R complex was present. The resulting material was analyzed by immunoblotting with anti-uPA-R mAb H2. As shown in Fig. 7, we were able to demonstrate the specific 50–65-kD zone corresponding to uPA-R in the immunoprecipitates of CD18, CD11a, CD11b, but not CD11c, although also here, the considerable amount of detected CD18 indicated the efficiency of precipitation.

mAbs to β 2-integrins Induce Cocapping and Modulation of uPA-R. Our biochemical data indicated that only subpopulations of the β 2-integrins and the Src-PTKs are present in the uPA-R complex (Fig. 3). Therefore, it could be expected to be technically difficult to demonstrate the uPA-R complex in the intact monocyte. Nevertheless, two of the performed approaches provided further evidence for its existence on the surface of monocytes.

First, we were able to induce CR3 capping on ~70% of the monocytes by cross-linking of CD11b mAb MEM-170 with FITC-conjugated F(ab')₂ fragments of sheep anti-mouse Ig and shifting the cells for 5 min to 37°C (Fig. 8A). When we stained these cells with biotinylated uPA and TRITC-conjugated streptavidin, a clear codistribution of uPA-R and CR3 was observed on 40–50% of the capped cells (Fig. 8B). This result is in good agreement with the recently reported

cocapping of uPA-R and CR3 on granulocytes (45). The effect was less pronounced with CD18 capped cells and hardly seen with CD11a capped cells. As a control, no codistribution of uPA-R was observed after capping of the monocyte surface protein CD31 (Fig. 8, C and D). Using a similar approach, we were unable to demonstrate unequivocally cocapping of the Src-family PTKs p60^{bcn}, p53/56^{bcn}, p58/64^{bcn}, and p56^{lck} with uPA-R or CR3. Although there was a somewhat stronger accumulation of the PTKs in the uPA-R or CR3 caps, most of them remained homogeneously distributed. This is most likely because only minor fractions of the PTKs are engaged within the complex.

The second approach was undertaken after we found down-regulation of monocyte β 2-integrins by incubation with CD18 mAb KIM127. Then we estimated the influence on uPA-R by staining the β 2-integrin modulated cells with PE-conjugated mAb VIM5 and FACS[®] (Becton Dickinson and Co., Mountain View, CA) analysis. The results were similar for each of the three experiments, one of which is shown in Fig. 9. The MFI of the cells stained by PE-labeled anti-uPA-R mAb VIM5 modestly but reproducibly decreased after the mAb KIM127 treatment by 14 ± 4% (mean ± SD) in comparison to control mAb incubated cells. Binding of CD11b mAb VIM12 was diminished by 12 ± 3% and that of CD11a

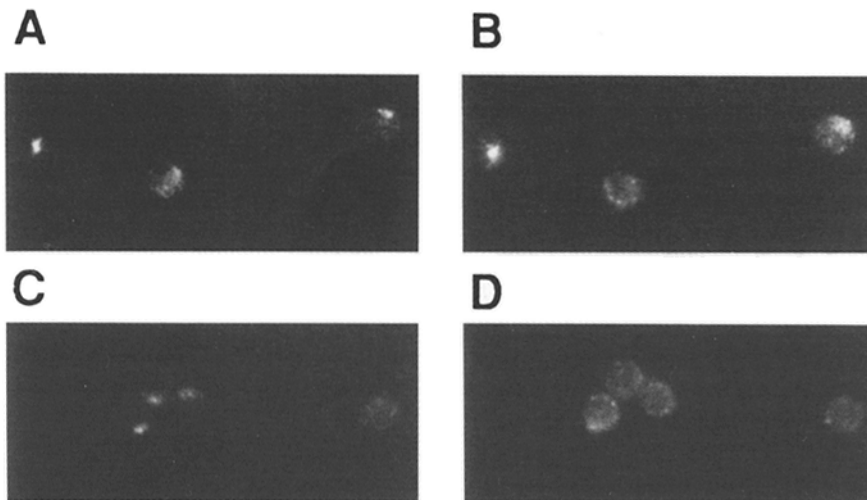


Figure 8. Colocalization of uPA-R and CR3. Representative fluorescence photomicrographs of cocapping experiments are shown. (A) CR3 was first capped using mAb MEM-170 to the α -chain CD11b and FITC-conjugated F(ab')₂ fragments of a sheep anti-murine Ig antiserum. (B) The monocytes were then labeled with biotinylated uPA and TRITC-conjugated streptavidin. As a control, (C) CD31 mAb 7E4 capped cells (D) that were probed with uPA.

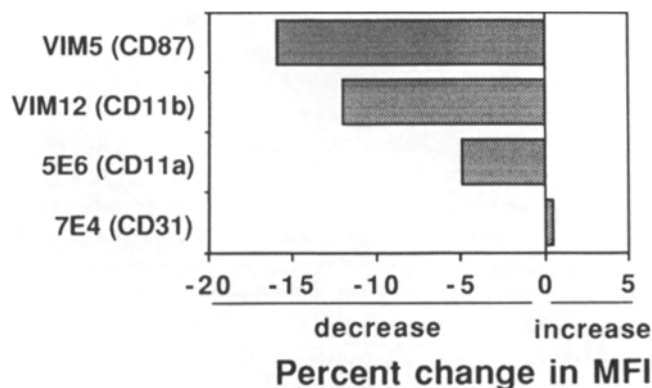


Figure 9. Modulation of uPA-R by β 2-integrin β -chain mAb KIM127. Cells were incubated with CD18 mAb KIM127 at 37°C for 15 min, stained with PE-labeled mAbs (VIM5 to uPA-R, VIM12 to CD11b, 5E6 to CD11a, 7E4 to CD31), and analyzed by flow cytometry. The alterations in binding of the direct-labeled mAbs due to the KIM127 preincubation are expressed as percent change in MFI in comparison to cells incubated with control mAb VIAP.

mAb 5E6 by $7 \pm 3\%$. On the other hand, no change in the staining of CD31 mAb 7E4 was detected. We interpret this effect as comodulation of uPA-R with β 2-integrins. An alternative explanation based on possible steric hindrance of the mAbs is unlikely as no such effects were found when the experiment was done with prefixed monocytes. Taken together, these results support the coisolation experiments considering that a part of the β 2-integrins is physically associated with uPA-R on the surface of intact human monocytes.

Discussion

In the present study, we found uPA-R as a component of a relatively large receptor complex in human monocytes including β 2-integrins, and four PTKs of the Src-family. The proximity of molecules involved in adhesion, extravasation, chemotaxis, pericellular proteolysis, as well as intracellular signal transduction may obviously provide opportunities for their mutual interactions. Therefore, it is tempting to speculate that this uPA-R complex is a “switchboard” involved in regulation of monocyte migration.

Interaction of ligand binding receptor elements and protein tyrosine kinases, resulting in modulation of kinase activity and tyrosine phosphorylation represent the first steps in a cascade of events accompanying leukocyte activation. As we demonstrate, this seems to be true also for signaling via uPA-R. By isolation of the monocyte uPA-R using its natural ligand, uPA, we identified its association with the Src-family PTKs p60^{bcr}, p53/56^{lyn}, p58/64^{hck}, and p59^{fer} (Fig. 4). Involvement of these kinases in uPA-R signaling is supported by induction of tyrosine phosphorylation upon stimulation of monocytes with uPA, independently of its enzymatic activity (Fig. 5). This finding is in agreement with uPA-stimulated tyrosine phosphorylation in U937 cells (16). Indeed, we also coisolated phosphotransferase activity with uPA-R from lysates of these cells (not shown).

How can uPA-R be associated with PTKs, and how can

uPA-R be involved in uPA-induced cellular adhesion (12, 13) and chemotactic movement (14, 15)? These are particularly important questions, since uPA-R belongs to the family of GPI-anchored membrane proteins. Attached to the outer leaflet of the membrane via its lipid moiety, and thus devoid of transmembrane and cytoplasmic domains, uPA-R presumably cannot directly interact with PTKs which are restricted to the inner leaflet of the membrane through their covalently linked fatty acid residues (46). However, as our data and several previous reports show (25, 38–40), tight physical and functional interrelations between GPI-anchored proteins and PTKs do exist. Therefore, it was speculated that an adaptor, possible candidates for which could be specific (glyco)lipids or transmembrane proteins, must be present to mediate the contact (37, 47). Such a linker molecule could be a mere connector or bear various functional properties. Upon ligation, GPI proteins could interact and activate this structure and subsequently also downstream signaling cascades. Caveolin, a 22-kD protein with putative type II transmembrane orientation has been hypothesized to play such a role in epithelial caveolae and endothelial “caveolin-rich domains,” in which GPI-anchored proteins are concentrated (44). Since we were not able to detect any caveolin in monocytes, this indicates that caveolin may not be an essential component of at least some types of membrane domains enriched in GPI-anchored proteins. In this respect, our demonstration that uPA-R and PTKs are joint components of a single receptor complex containing the leukocyte-integrins LFA-1 and CR3 is of particular interest. Because of their transmembrane topology, proposed existence of allosteric modifications (48) and interaction with the cytoskeleton (49), LFA-1 and CR3 can principally provide uPA-R access to intracellular signaling cascades.

β 2-integrins are crucial molecules for leukocyte adhesion and migration. However, leukocyte integrins are not merely passive adhesion molecules (50, 51). It was shown that they are able to transduce signals in neutrophils (52, 53) and lymphocytes (54), which is accompanied by tyrosine phosphorylation of intracellular substrates (55). Cytoplasmic parts of integrins seem to be important for their signaling (48). Interestingly, an uncharacterized protein kinase activity associated with LFA-1 has been reported earlier (56). Indications that β 2-integrins functionally cooperate with GPI-anchored proteins were provided by several reports. Engagement of CD14 activates the adhesive capacity of CR3 on neutrophils (57), and that of LFA-1 on monocytes (58). In agreement with our data, antibody induced cocapping of uPA-R (45) and CD16 (Fc γ RIII) (59) with CR3 have been observed on neutrophils. This seems to be dependent on lectin-like interactions between the molecules involved. Importantly, disruption of this interaction leads to decreased CD16 signaling (52).

However, so far it has not been known what is the structural basis of this functional relationship between GPI-anchored proteins and β 2-integrins, i.e., whether these molecules are physically associated within some type of a membrane complex. By coisolation from lysates of freshly prepared human peripheral blood monocytes, we show here that the GPI-anchored protein uPA-R is associated with subpopulations

of $\beta 2$ -integrins and PTKs within one receptor complex of a relatively large size. This finding is supported by the observed mAb-induced colocalization of uPA-R and integrins on 40–50% of CR3 capped cells (Fig. 8). Although we cannot completely rule out the possibility that ligation of integrins by mAbs enhances these associations, these observations suggest that uPA-R complex is a constitutive receptor domain on monocytes rather than on a minor “activated” subpopulation of these cells. The occurrence in large complexes with limited solubility is not unique for uPA-R. Several other GPI-anchored proteins have been found to be components of very large, detergent-resistant complexes (27, 33, 36) corresponding presumably to membrane microdomains of specific lipid composition (34, 35, 37). Such accumulation of receptors in large complexes could be important for strengthening of effector functions (pericellular proteolysis, adhesion) by crowding effector molecules as well as for the channeling of signals. The compositions of these relatively large “GPI-domains” seem to differ in various cells but also in a given cell type. There is basically a single type of GPI-domain containing several GPI-anchored proteins joined together in T cells (27, 33). Here we show, that the uPA-R complex is a type of such large membrane complexes in monocytes, that clearly contains transmembrane proteins, i.e., the $\beta 2$ -integrins. The absence of at least substantial amounts of the abundant GPI-

anchored proteins (CD14, CD58, CD59) in these uPA-R complexes (Fig. 6 A), but their presence in Sepharose 4B void volume fractions (data not shown) suggests that monocyte membranes contain more than one type of large complexes (microdomains) involving different major GPI-anchored proteins. In monocytes we did not find caveolin, the coat protein of GPI protein-enriched domains in epithelial cells (44), which on the other hand lack $\beta 2$ -integrins. This further points to the heterogeneity of these membrane receptor domains. An unclear question is whether the composition of these complexes is changed upon cell activation. Inasmuch as uPA-R is a possible target in the treatment of tumor metastasis as well as inflammatory disorders, careful analysis of the composition and stoichiometry of these uPA-R complexes in individual cells under resting and activation state might provide a basis to develop specific therapeutic strategies.

We conclude, that the large, PTK-containing complex described here is most likely involved in signal transduction through two different types of receptors, uPA-R and $\beta 2$ -integrins. A coordinated action of extracellular proteolysis, adhesion, and cell activation at the right time and place is obviously required for cell migration. Therefore, it can be speculated that close proximity of these two types of receptors, and sharing of the common intracellular signaling molecules may be important for these processes.

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