A Urokinase Receptor-associated Protein with Specific Collagen Binding Properties*  

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The plasminogen activation cascade system, directed by urokinase and the urokinase receptor, plays a key role in extracellular proteolysis during tissue remodeling. To identify molecular interaction partners of these trigger proteins on the cell, we combined covalent protein cross-linking with mass spectrometry based methods for peptide mapping and primary structure analysis of electrophoretically isolated protein conjugates. A specific tri-molecular complex was observed upon addition of pro-urokinase to human U937 cells. This complex included the urokinase receptor, pro-urokinase, and an unknown, high molecular weight urokinase receptor-associated protein. The tryptic peptide mixture derived from a cross-linked complex of pro-urokinase and the latter protein was analyzed by nanoelectrospray tandem mass spectrometric sequencing. This analysis identified the novel protein as the human homologue of a murine membrane-bound lectin with hitherto unknown function. The human cDNA was cloned and sequenced. The protein, designated uPARAP, is a member of the macrophage mannose receptor protein family and contains a putative collagen-binding (fibronectin type II) domain in addition to 8 C-type carbohydrate recognition domains. It proved capable of binding strongly to a single type of collagen, collagen V. This collagen binding reaction at the exact site of plasminogen activation on the cell may lead to adhesive functions as well as a contribution to cellular degradation of collagen matrices.

Proteolytic degradation of the extracellular matrix is essential for the processes of tissue remodeling. These processes take place in a number of distinct physiological events in the healthy organism, such as trophoblast invasion, mammary gland involution, and skin wound healing, but also represent a crucial step in cancer invasion and metastasis (1).

The plasminogen activation system has an important position among the extracellular proteases engaged in these degradation reactions (2). This system is organized as a proteolytic cascade with active proteases and their pro-enzymes, protease inhibitors, and cellular and extracellular binding proteins. The urokinase-type plasminogen activator (uPA)1 has a triggering role in the system in the ability to release the large proteolytic potential of active plasmin from the abundant pro-enzyme, plasminogen. uPA and its pro-enzyme, pro-uPA, become localized at discrete areas on specific cell types by the interaction with the urokinase receptor (uPAR). This receptor serves to concentrate proteolytic activity at areas of cell substratum contact and furthermore participates in a complicated activation-acceleration mechanism in conjunction with plasminogen-binding components on the cell. Recent reviews provide details on the molecular and functional properties of the uPA-uPAR system and its role in extracellular matrix degradation (3, 4).

It is generally considered that the major function of the uPA-plasmin system is directed against fibrin and the noncollagen constituents of the extracellular matrix (2, 5, 6), whereas collagen degradation is undertaken mainly by the collagenase and gelatinase members of the matrix metalloproteinase (MMP) family (7). On the other hand it is also clear that these proteolytic systems can cooperate in some cases, an important example being the plasmin-mediated activation of some MMP proenzymes (8) and the coordinated organization of some of these activation reactions on cell surfaces where uPAR seems to play an important role (9).

During recent years evidence has accumulated that uPAR also takes part in other protein interactions, relevant not only to proteolysis but also to cell adhesion and signal transduction. uPAR thus binds to vitronectin (10), and it has been proposed that this interaction takes part in a balanced attachment and release scenario, directed by the plasminogen activator inhibitor type 1, which competes with uPAR in the vitronectin binding process (11). A further, indirect, role of uPAR in adhesion is provided by interactions with certain integrins, influencing the binding properties of the latter (12). In some cell types, intracellular protein tyrosine phosphorylation has been observed as a function of ligand binding to uPAR in processes that seem unrelated to proteolysis but that require interactions with unidentified, actively signal transducing membrane proteins (13).

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1 The abbreviations used are: uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; uPARAP, uPAR-associated protein; BSA, bovine serum albumin; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; DSS, N,N'-diisuccinimidylsuberate; mAb, monoclonal antibody; MALDI, matrix-assisted laser desorption ionization; RT, reverse transcription; PCR, polymerase chain reaction; pro-uPA, proenzyme for uPA; PAGE, polyacrylamide gel electrophoresis; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; MMP, matrix metalloproteinase.

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Common to most or all of the functions of uPAR is the necessity of interactions with other proteins on the cell surface. This fact has focused the interest on the identification of the interaction partners of uPAR on the cell. We have previously demonstrated a uPAR-dependent interaction between pro-uPA and a specific high molecular weight protein expressed by the monocyte-like cell line, U937 (14). In the present work we have used a novel analytical approach that combines protein cross-linking and mass spectrometry to identify the interacting molecules in this protein complex. Following this strategy we have characterized the uPAR-associated membrane protein, cloned its cDNA, and identified a strongly binding ligand in the extracellular matrix.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Human pro-uPA and human vitronectin were the generous gifts of Dr. J. Henkin (Abbott Laboratories, IL) and Dr. K. T. Preisner (Kerckhoff-Klinik, Bad Nauheim, Germany), respectively. Pro-uPA was labeled with $^{125}$I as described (15). The following purified proteins were purchased from commercial sources: human collagen types I, III, IV, and V and bacterial collagennase type III from Clostridium histolyticum (Calbiochem, Bad Soden, Germany), tissue transglutaminase from guinea pig liver and artificially glycosylated albumin derivatives (Sigma), rat uterine pro-uPA (mAb 5) performed using mouse tumor cell line U937, recombinant human uPA, mAb R2 and R3 against human uPA, and a control mAb against trinitrophenol were from clones described previously (16–18).

**Protein Cross-linking Assays**—Cells of the promyeloid histiocyte cell line U937 were cultured in RPMI 1640 medium supplemented with penicillin, streptomycin, and 5% fetal calf serum. Vascular smooth muscle cells from human aorta were purchased from American Type Culture Collection (product specification CRL-1999). These cells were grown in Ham's F-12K medium supplemented with penicillin, streptomycin, 10 mM HEPES, 10 mM TES, 50 mg/ml ascorbic acid, 10% fetal calf serum, and the following growth supplement mixtures: insulin-transferrin-selenium (Sigma product I-1884, providing final concentration of 10 $\mu$g/ml bovine insulin, 10 $\mu$g/ml human transferrin, and 10 ng/ml sodium selenite, respectively), and endothelial cell growth factor supplement mixture (Sigma product E-9640, used at a final 100-fold dilution). Assays for binding and cross-linking of $^{125}$I-labeled pro-uPA by enzymatic or chemical methods were done as described (14). Briefly, cells were suspended at a density of 5 $\times$ $10^6$ cells/ml in binding buffer (50 mM HEPES, 100 mM NaCl, 5 mM CaCl$_2$, 0.1% bovine serum albumin (BSA), pH 7.4) and incubated at 4 °C with 1.5 nM of $^{125}$I-labeled pro-uPA. After washing the cells, enzymatic cross-linking was performed by addition of tissue transglutaminase to a final concentration of 20 $\mu$g/ml and incubation for 30 min at 37 °C. For chemical cross-linking, the samples were treated with 2 nM of N,N'-dicyclohexylcarbodiimide (DSS) for 15 min at room temperature instead of the transglutaminase incubation step. In some experiments, potential competitor proteins were included at specification steps as indicated. The cells were washed again and lysed in lysis buffer (phosphate-buffered saline with 1% CHAPS, 10 mM EDTA). Before electrophoretic analysis, lysates were clarified by centrifugation. As a positive control for transglutaminase activity in the presence of the competitor proteins investigated, the enzyme was tested for its capability to form cross-linked vitronectin conjugates (19). For this purpose, human vitronectin (final concentration, 400 $\mu$g/ml) was dissolved in 0.1 M Tris/HCl, 6 mM CaCl$_2$, pH 7.4, and incubated for 30 min at 37 °C in the presence or absence of 20 $\mu$g/ml of tissue transglutaminase and the potential competitor proteins indicated. The reaction was stopped by addition of 20 $\mu$l of EDTA, after which the formation of covalent vitronectin conjugates was demonstrated by SDS-PAGE and Coomassie staining. Following this strategy we have identified a strongly binding ligand in the extracellular matrix.

**Cell Cultures**—The transglutaminase-mediated pro-uPA cross-linking was performed as above except that unlabeled pro-uPA (20 nM) was used instead of the labeled protein and that 1 mM phenylmethylsulfonylfluoride was added to the lysis buffer. The lysate of 6 $\times$ $10^6$ cells was applied on a column with immobilized anti-uPA clone 5 (16), which was subsequently washed with a series of washing buffers (phosphate-buffered saline with 0.05% CHAPS and NaCl concentrations of 140, 1000, and 50 mM, respectively). The column was eluted with elution buffer (0.1 M acetic acid, 0.5 M NaCl, 0.05% CHAPS, pH 2.5). Eluate fractions were immediately titrated to pH 7.4 by addition of the apro-
uPAR-associated Protein 195

RESULTS

A Complex of pro-uPA with Two Proteins on the Cell Surface—To demonstrate various interactions of pro-uPA with proteins on the cell surface, we added the radiolabeled proenzyme to human U937 cells and subjected the samples to protein cross-linking by different methods (Fig. 1A). Chemical cross-linking with DSS, an amino-directed homobifunctional cross-linker, led to the formation of an Mₙ > 100,000 labeled protein conjugate (lane 1), known from several studies to represent ligand labeling of uPAR of Mₙ > 55,000 (32–34). In contrast, enzymatic cross-linking using tissue transglutaminase led to the labeling of an unknown protein of Mₙ > 200,000, as reflected by the formation of an Mₙ > 250,000 conjugate with pro-uPA (lane 3). The latter conjugate has also been observed previously, including the following findings: (a) This product does not include uPAR in the covalent complex according to Western blotting experiments; (b) the reaction of pro-uPA with the unknown protein is nevertheless uPAR-dependent as pretreatment of the cells with specific uPAR-blocking reagents abolishes the enzymatic cross-linking reaction; and (c) there is a strong selectivity for the pro-form of uPA as little or no high molecular weight conjugate is formed when active two-chain uPA or diisopropylfluorophosphate-inactivated two-chain uPA is used instead of pro-uPA (14).

The same cross-linking experiment was performed with human vascular smooth muscle cells instead of U937 cells. Also with this cell type, uPAR and the high molecular weight conjugate could both be demonstrated (Fig. 1A, lanes 4 and 6, respectively).

These findings suggested that, at least with these cell types, irrelevant antibody (mAb against trinitrophenol; lanes 3 and 4). The samples were analyzed by electrophoresis and autoradiography as in A, except that a 5% gel was used for optimal separation of the high molecular weight proteins. Arrows indicate the high molecular weight conjugate formed by combined enzymatic and chemical cross-linking. C, antibody labeling of the trimolecular complex on the intact cell. U937 cells were incubated with ¹²⁵I-pro-uPA as above (panels I and III) or under the same conditions in the presence of 400 nM of unlabeled uPA as a competitor (panel II). All samples were then subjected to enzymatic cross-linking with tissue transglutaminase (TG) or parallel incubation with buffer alone (lanes 2 and 5), or enzymatic cross-linking using tissue transglutaminase (TG) or parallel incubation with buffer alone (lanes 7 and 8).

Fig. 1. A ternary complex with pro-uPA on the cell surface. A, ligand labeling of two cellular proteins. U937 cells (lanes 1–3) or human vascular smooth muscle cells (lanes 4–6) were incubated with ¹²⁵I-labeled pro-uPA followed by either chemical cross-linking with DSS (lanes 1 and 4), incubation with buffer alone (lanes 2 and 5), or enzymatic cross-linking with tissue transglutaminase (TG, lanes 3 and 6). The cells were washed and lysed with detergent, and the clarified lysates were analyzed by 6–16% SDS–PAGE followed by autoradiography of the gel. The electrophoretic mobilities of Mₙ marker proteins are indicated. B, cross-linking of a trimolecular complex. U937 cells were incubated with ¹²⁵I-pro-uPA followed by enzymatic cross-linking as above (TG; lanes 1–6) or parallel incubation with buffer alone (lanes 7 and 8). The samples shown in lanes 2, 4, 6, and 8 were subsequently treated with DSS for chemical cross-linking. Detergent lysates of the cells were analyzed directly (lanes 1, 2, 7, and 8) or subjected to immunoprecipitation (IP) using mAb R2 against human uPAR (lanes 5 and 6) or an irrelevant antibody (mAb against trinitrophenol; lanes 3 and 4).

The samples were analyzed by electrophoresis and autoradiography as in A, except that a 5% gel was used for optimal separation of the high molecular weight proteins. Arrows indicate the high molecular weight conjugate formed by combined enzymatic and chemical cross-linking. C, antibody labeling of the trimolecular complex on the intact cell. U937 cells were incubated with ¹²⁵I-pro-uPA as above (panels I and III) or under the same conditions in the presence of 400 nM of unlabeled uPA as a competitor (panel II). All samples were then subjected to enzymatic cross-linking. After washing the cells, the following mAbs were added: anti-uPAR R2 (lanes 2), anti-uPAR R3 (lanes 3), anti-trinitrophenol (irrelevant antibody; lanes 4), or buffer alone (lanes 1). The cells were washed again and lysed in a detergent-containing buffer as above (panels I and II) or in the same buffer including 400 nM of unlabeled uPA (panel III). The clarified lysates were subjected directly to precipitation with Protein A-Sepharose. Electrophoretic analysis and autoradiography were done as in A.
pro-uPA, uPAR and the unknown protein take part in a common noncovalent complex on the cell surface. The selectivity of the two cross-linking techniques opened the possibility to visualize any ternary complex present by combination of the two methods. Indeed, when pro-uPA cross-linking with transglutaminase was followed by a second cross-linking step with DSS on otherwise unmodified U937 cells, a labeled adduct was formed that had a higher apparent $M_r$ than that formed with transglutaminase alone (Fig. 1B, lane 2). This product was not formed if the enzymatic cross-linking step was omitted (lane 8). The presence of uPAR in this latter conjugate was confirmed by immunoprecipitation. Thus, the product could be immunoprecipitated using a monoclonal antibody (mAb R2) against human uPAR (lane 6), whereas no labeled product was precipitated with an irrelevant antibody (lane 4). Furthermore, the conjugate formed by enzymatic cross-linking alone, consisting of pro-uPA and the unknown protein, was also immunoprecipitated with the anti-uPAR antibody (lane 5), indicating that uPAR was (noncovalently) associated with this product even in the absence of chemical cross-linking.

The hypothesis of a uPAR-containing complex was further tested by addition of antibodies to intact cells on which the enzymatic pro-uPA cross-linking had been carried out. After washing and lysing the cells, the labeled complexes were then precipitated without any further addition of antibody, using protein A-Sepharose alone (Fig. 1C). The antibody R2 against human uPAR recognized a protein complex that included the high molecular weight protein and that was sufficiently stable to persist after cell lysis and protein A precipitation (panel I, lane 2). This product was still precipitated after cell lysis in the presence of an excess (400 nM) of unlabeled uPA (panel III), excluding the possibility that the complex arose from a secondary binding reaction between uPAR and pro-uPA after solubilization of the plasma membrane. In contrast, the same concentration of uPA completely blocked complex formation when added from the start of the experiment (panel II). The specificity of the precipitation experiment was tested by the inclusion of other antibodies. No labeled product was precipitated when the cells had been incubated with an irrelevant antibody (anti-trinitrophenol, lane 4) or an anti-uPAR antibody (mAb R3) that only recognizes the uncomplexed receptor (18) (lane 3). Together, these experiments demonstrated the existence of a trimolecular complex on the pro-uPA-treated cells, consisting of pro-uPA itself, uPAR, and the unknown high molecular weight uPAR-associated protein that we tentatively designated uPARAP.

**Purification and Microcharacterization of the Cross-linked Protein Conjugate—**Enzymatic cross-linking to pro-uPA was used to provide a tag for purification of uPARAP. Pilot studies indicated that, whereas cells grown under standard conditions as shown in Fig. 1 were convenient for a qualitative demonstration of the protein conjugate, a 3–4-fold increase in the yield of the cross-linked product could be achieved by stimulation of the U937 cells with cAMP for 48 h before the cross-linking assay (result not shown). Consequently, cAMP-stimulated U937 cells were incubated with 20 nM of unlabeled uPA followed by transglutaminase-mediated cross-linking. The cells were lysed with detergent, and the lysate was used for immunopurification, using mAb clone 5 against human pro-uPA. The concentrated eluate was subjected to preparative SDS-PAGE. A peptide mapping of the isolated conjugate by MALDI mass spectrometry. The high molecular weight protein conjugate was excised from the Coomassie-stained gel (inset, arrow) and treated with trypsin using in-gel digestion technique. The resulting peptide mixture was analyzed directly by MALDI mass spectrometry, and monoisotopic peptide masses were determined. Ion signals matching tryptic peptides as predicted from the amino acid sequences of human pro-uPA (circles), and the subsequently cloned uPARAP (squares) are indicated (see Table I). B, amino acid sequencing by nanoelectrospray tandem mass spectrometry. The tryptic peptide mixture derived from the protein conjugate was desalted and subjected to nanoelectrospray tandem mass spectrometry. The order of the first two amino acids could not be unambiguously determined. Note that C corresponds to S-carbamidomethylcysteine.

![Fig. 2. Isolation and characterization of the $M_r$ 250,000 pro-uPA conjugate.](image)

**FIG. 2.** Isolation and characterization of the $M_r$ 250,000 pro-uPA conjugate. CAMP-stimulated U937 cells were treated with 20 nM of pro-uPA followed by transglutaminase-mediated cross-linking. The cells were lysed with detergent, and the lysate was used for immunopurification, using mAb clone 5 against human pro-uPA. The concentrated eluate was subjected to preparative SDS-PAGE. A, peptide mapping of the isolated conjugate by MALDI mass spectrometry. The high molecular weight protein conjugate was excised from the Coomassie-stained gel (inset, arrow) and treated with trypsin using in-gel digestion technique. The resulting peptide mixture was analyzed directly by MALDI mass spectrometry, and monoisotopic peptide masses were determined. Ion signals matching tryptic peptides as predicted from the amino acid sequences of human pro-uPA (circles), and the subsequently cloned uPARAP (squares) are indicated (see Table I). B, amino acid sequencing by nanoelectrospray tandem mass spectrometry. The tryptic peptide mixture derived from the protein conjugate was desalted and subjected to nanoelectrospray tandem mass MS. The fragment ion pattern observed in the tandem mass spectrum of the doubly charged peptide ion at $m/z$ 663.3 is shown. This pattern was used to determine the sequence (D,C)S(L/I)A(L/I)PYVCK. The order of the first two amino acids could not be unambiguously determined. Note that C corresponds to S-carbamidomethylcysteine.

from the gel and was unambiguously identified as the cross-linked pro-uPA conjugate by two independent means: (a) the corresponding electrophoretic band was reactive with antibodies against human pro-uPA in Western blotting, even when employing an antibody (mAb clone 6) different from the one used for immunopurification (result not shown) and (b) a high accuracy MALDI mass spectrum obtained from a tryptic peptide mixture derived from the Coomassie-stained protein band
displayed more than 30 peptide signals (Fig. 2A). Nine of these signals could be assigned to human pro-uPA tryptic peptides (Table I).

Data base searching with the list of remaining tryptic peptide masses, an established method to identify multiple components in a protein band (25), did not retrieve any significant protein matches. This was strong evidence that the other component of the cross-linked complex, uPARAP, was indeed an unknown protein.

Identification of the Protein and cDNA Cloning—An aliquot of the tryptic in-gel digest of the cross-linked protein conjugate was analyzed by nanoelectrospray tandem mass spectrometry, allowing amino acid sequencing of individual peptides in the mixture (26). This experiment was successful in determining four internal uPARAP sequences of 6–13 amino acid residues each (Fig. 2B and Table I, boldface sequences; note that this method does not allow distinction between leucine and isoleucine because of the identical molecular mass of these amino acids). These peptide sequences were used to query biological sequence data banks, including protein and expressed sequence tag data bases, by the peptide tag approach (28) or by homology searching using BLAST (29). The amino acid sequence DCS/L/I/A/L/I/YPYVCK (Fig. 2B) was sufficient for the unambiguous identification of a single entry in the data base. This was the amino acid sequence derived from a murine cDNA, GenBank™ accession code mmu56734, in which residues 349–359 match this sequence with 100% identity (Fig. 3A). The sequences TP/L/I/W/L/I/G/L/I/A and GFSYHN were also recognized in mmu56734 (residues 1180–1187 and 765–770, respectively), whereas a fourth sequence, T/L/I/G/D/Q/L/I/S/L/I/L/I/L/I/GAR, was not found.

The finding of three human peptides completely matching the murine mmu56734 sequence strongly suggested that uPARAP was the human homologue of the latter product. This hypothesis was confirmed by RT-PCR-based cloning of the complete human cDNA and determination of the nucleotide and derived amino acid sequence (Fig. 3; see “Experimental Procedures” for details). The three peptide sequences forming the basis for the identification of the murine product were thus refound at the same positions in the human sequence with 100% identity (Fig. 3A, boxed residues). Furthermore, the fourth sequence mentioned above, which was absent from the murine protein, was indeed present in the human sequence (residues 125–137). Finally, examination of the MALDI spectrum of the original peptide mixture (Fig. 2A and Table I) identified 22 tryptic peptide masses matching the uPARAP sequence in Fig. 3A, ultimately confirming the identity of the cloned cDNA as encoding the protein complexed with pro-uPA in our purified material. Alignment of the derived amino acid sequences of the human uPARAP and the murine mmu56734 product showed an identity of 89% (Fig. 3A), clearly demonstrating the close relatedness of the two proteins.

uPARAP is a Lectin-like Membrane Protein That Belongs to the Macrophage Mannose Receptor Protein Family—The cloned
cDNA encodes a protein of 1479 amino acid residues. As pointed out already in the case of the murine mmu56734 (35), the sequence identified is clearly related to lectins of the macrophage mannose receptor family (see under "Discussion"). These lectins are multi-domain proteins with a characteristic domain composition as shown in Fig. 3B. Some or all of the proteins of this family can bind to certain glycoproteins, a property that has been studied systematically using artificially glycosylated albumin derivatives as the ligands (36–39). To learn whether uPARAP would share this binding capability and whether this type of binding would interfere with complex formation with pro-uPA, a blocking experiment was carried out. U937 cells were preincubated with different glycosylated albumins, washed, and subsequently subjected to the enzymatic cross-linking procedure with radiolabeled pro-uPA (Fig. 4). It was clear that some, but not all, of the albumin derivatives markedly hindered the formation of the labeled protein conjugate (lanes 2 and 7), pointing to some degree of carbohydrate specificity. The strongest inhibition was noted with the derivative, BSA-galactosamide (lane 2).

2 The uPARAP cDNA sequence data have been submitted to the DDBJ/EMBL/GenBank™ data bases under accession number AF107292. During the writing of this manuscript the sequence of this cDNA was independently deposited in GenBank™, accession code AB014609, as part of a sequencing program focused on large transcripts in the human brain (69).
no effect on the uPAR-mediated, primary binding of pro-uPA to the cells, seen as unconjugated pro-uPA on the gel. The fact that the cells were washed before the cross-linking assay dis favored the possibility that the glycoproteins could exert their effect merely by inhibiting the enzymatic activity of transglutaminase. This possibility was excluded completely by studies on BSA-galactosamidase and transglutaminase, using a different protein for cross-linking. For this purpose, we chose vitronectin, which is known to be a substrate for tissue transglutaminase (19). We found that incubation of human vitronectin with transglutaminase led to covalent cross-linking of vitronectin dimers, migrating with an apparent Mr of --150,000 in SDS-PAGE. This reaction was completely unaffected by the glycoprotein (results not shown). The blocking effect noted above was thus indeed directed against the interaction between uPAR-bound pro-uPA and the lectin-like uPARAP, consistent with a steric blocking of the latter. This finding further supported the resemblance of the novel protein with the other protein family members.

Binding to Collagen Type V—In addition to the carbohydrate recognition domains, we paid attention to the occurrence of a fibronectin type II domain in the structure of the proteins of this group (Fig. 3B). In fibronectin as well as several other proteins, this domain type has been found to take part in collagen binding reactions (40), and therefore it was tempting to test whether collagens could influence the interactions of uPARAP with uPAR and pro-uPA. U937 cells were subjected to enzymatic cross-linking with radiolabeled pro-uPA in the presence of various types of purified collagens as competitors. To compare the effect of different collagens quantitatively, the cross-linked uPARAP-pro-uPA conjugate obtained in each sample was excised from the gel after electrophoresis and subjected to γ-counting (Fig. 5).

Strikingly it turned out that a single type of collagen, collagen V, was a strong inhibitor of complex formation. Only a weak effect was found with type IV and type I collagens. Collagen type III also showed little or no inhibition (result not shown).

The inhibitory property of collagen V on protein complex assembly was studied in more detail. In the next experiment, we varied the design to allow the competitor to be present during discrete steps in the binding and cross-linking procedure (Fig. 6A). This experiment showed that preincubation of the cells with collagen V, even when followed by thorough washing, was sufficient for blocking the subsequent assembly of the labeled complex (lane 2). This blocking effect was not directed against the initial binding of pro-uPA to uPAR because the amount of nonconjugated pro-uPA bound by the cells was not affected by collagen V. The lack of interference with the pro-uPA-uPAR interaction was substantiated even further in a purified system where we found that collagen V had no effect on the DSS-mediated cross-linking between pro-uPA and recombinant soluble uPAR (result not shown). This observation opened the possibility that the collagen could prevent the interaction between uPARAP and pro-uPA even when added after the initial binding of pro-uPA to uPAR on the cells. This was indeed the case as efficient inhibition of conjugate formation occurred also in the sample where collagen V was added to the cells just before the enzymatic cross-linking step (Fig. 6A, lane 4).

All of these observations were consistent with a rather long-lived binding of collagen V to uPARAP, resulting in efficient blocking of the interaction of uPARAP with uPAR-bound pro-uPA. Even though the present detection method would not allow a stringent determination of the affinity, a strong interaction was indeed supported by a titration-inhibition experiment (Fig. 6B). Thus, the IC₅₀ for the inhibition of complex formation was about 0.2 µg/ml (0.6 nM) of collagen V.

Additional control experiments were performed to ascertain that the inhibitory phenomenon was not artifactual (results not shown). Firstly, we found that the inhibitory potency of collagen V was retained after desalting the protein by gel filtration, thus excluding an effect of salts or low molecular weight constituents in the purified collagen preparation that might have escaped detection by electrophoretic analysis. Secondly, treatment of collagen V with bacterial collagenase, which led to complete degradation of the purified collagen, totally abolished the inhibitory activity. Thirdly, collagen V had no effect on the enzymatic activity of transglutaminase as shown with vitronectin as the substrate as above, excluding that the inhibition was directed against the enzymatic cross-linking step.

**DISCUSSION**

This work has shown that a novel lectin-like membrane protein, uPARAP, is involved in interactions with uPAR on certain cell types, including monocyte-like U937 cells and vascular smooth muscle cells. A trimeric complex including both uPARAP and uPAR was formed when pro-uPA was added. This molecular interaction was demonstrated by covalent cross-linking on the intact cells, thus reflecting the actual existence of a molecular contact on the cell surface and not just a potential binding capability.

The amount of pro-uPA that ultimately could be cross-linked into a covalent conjugate with uPARAP typically constituted 5–8% of the total amount of pro-uPA present on the cells (Figs. 1, 5, and 6). Even though part of the complexes present might escape covalent fixation in the enzymatic cross-linking procedure, it thus seems likely that only a fraction of the uPAR-bound pro-uPA is engaged in the interaction with uPARAP. The detailed organization of membraneous subcompartments on the cell may govern the degree of co-localization of the two membrane proteins. An open question relates to the previously
reported selectivity for the pro-form of uPA in the formation of the cross-linked uPARAP conjugate (14). It is indeed possible that only pro-uPA takes part in the ternary complex on the cell surface. However, a thorough analysis of the utility of uPA and pro-uPA as transglutaminase substrates would be necessary to rule out that the preference for the pro-form could be related to the cross-linking method.

The strategy for identification of the novel component was based on microcharacterization of a protein conjugate formed by enzymatic cross-linking. This cross-linking technique proved valuable in the protein isolation step because it enabled us to use immunoaffinity purification based on available antibodies against pro-uPA and had the necessary chemical selectivity to allow the subsequent mass spectrometric peptide analysis. The low amount of protein conjugate isolated, less than 1 μg as judged by comparison with Coomassie-stained bands of known amounts of bovine serum albumin, was not sufficient to allow protein sequencing by conventional methods. Instead, we utilized mass spectrometry for characterization of the purified product. The MALDI mass spectrum of a tryptic peptide mixture derived from the protein conjugate ascertained that it contained human pro-uPA and an unknown protein component. Amino acid sequencing of several of the unknown peptides by nanoelectrospray tandem mass spectrometry enabled identification of the mouse homologue through data bank homology searches and subsequent cloning of the human cDNA, based on available expressed sequence tag sequences. The identity of the cloned DNA as encoding the second component of the human pro-uPA conjugate was then unambiguously confirmed by the initial MALDI mass spectrum (Fig. 2A and Table 1). This principle of combining a gentle cross-linking technique with sensitive peptide analysis by mass spectrometry enabled useful in other studies on specific protein interactions.

Like the murine mmu56734 gene product (35), uPARAP is a member of the macrophage mannose receptor protein family as evident from a clear sequence homology with the other family members. This family is comprised by a group of highly molecular weight, lectin-like type 1 membrane proteins (Fig. 3B) with highly diverse functions. The previously known family members are the macrophage mannose receptor, which is engaged in endocytosis of a number of glycoproteins (41); the cellular internalization receptor for secretory phospholipase A₂ (39, 42); and the receptor DEC-205, which functions in the internalization of antigens for processing and presentation by dendritic cells of the immune system (43). The actual amino acid sequence identities with uPARAP were 35, 35, and 33% for the human macrophage mannose receptor, the human secretory phospholipase A₂ receptor and the murine DEC-205, respectively (result not shown); closely similar values were reported after alignment with the murine mmu56734 sequence (35). This degree of homology is very close to the mutual homologies between the established family members (44).

Collagen type V bound to uPARAP as shown by efficient competition with pro-uPA complex formation. Only weak effects were noted with other collagens tested, suggesting a marked specificity with respect to collagen subtypes. A complete evaluation of this fine specificity, though, will have to await further studies because at this point it has only been possible to test a limited number of collagen subtypes and because it has been noted that subtle variations in the native state of some purified collagens may affect their binding properties in other receptor systems (45, 46).

The interaction with collagen V was remarkably strong with an IC₅₀ of competition below 1 nm. Based on the involvement of fibronectin type II domains of several other proteins in collagen binding reactions (40, 47, 48), it seems likely that this domain in uPARAP is likewise engaged in the interaction with collagen V. The phospholipase A₂ receptor, belonging to the same protein family as uPARAP, has also been found to bind certain collagens but loses this binding capability upon NH₂-terminal truncation, thus supporting a role of the fibronectin type II domain (49). In addition, it is possible that one or more of the C-type carbohydrate recognition domains contribute to the interaction because the triplehelical domain of collagen V contains a high number of galactosyl-hydroxylysines and glucosyl-galactosyl-hydroxylysines (50). Cross-linking/competition experiments indicated that, like the other members of this protein family, uPARAP bound to artificially glycosylated albumin derivatives with certain preferences among the derivatives tested. It is still an open question how many of the protein family members do have functions in recognition of natural
glycoproteins because critical sequence motifs in the potential carbohydrate recognition domains of some of the members vary markedly from those of established carbohydrate-binding proteins. In the mmu56734 gene product, however, the first and second C-type carbohydrate recognition domains were indeed considered likely candidates for a carbohydrate binding function, based on sequence comparison with the mannose-binding protein (for a discussion, see Ref. 44).

The identification of collagen-binding cellular components, including specific binding proteins for collagen V, is an important area of investigation. Studies on the adhesion and spreading of various cultured cells on collagen V containing matrices have shown that α1β1 and α2β1 integrins are dominant collagen V-binding components (51, 52). Interactions between collagen V and various proteoglycans have also been described (for review see Ref. 53), but the role of these reactions in cellular adhesion is not known. Interactions between platelets and collagen V may be mediated through thrombospondin, which specifically binds this type of collagen (54). Furthermore, it was recently shown that two receptor tyrosine kinases, DDR-1 and DDR-2, which belong to the discoidin domain receptor family, bind to and become activated by various collagens, including collagen V (45, 46).

The strong interaction between collagen V and the protein characterized in this work would fit with a role in cellular adhesion. In addition to this binding function, however, the association of the protein with uPAR may provide a mechanism for presentation of the bound collagen for proteolytic degradation. The collagen thus did not interfere with the primary binding reaction between pro-uPA and uPAR, and it is well recognized that uPAR is the preferred site of plasminogen activation on the cell (55, 56). We have found that, even in solution, collagen V has some sensitivity to plasmin-mediated cleavage,3 in accordance with the previously noted sensitivity of collagen V to trypsin-like enzymes (57). Such a mechanism may be amplified even further through plasmin-mediated activation of the matrix metalloproteinase zymogen, pro-MMP-9 (pro-gelatinase B; Ref. 8), because active MMP-9 is known as one of the rather few other proteases capable of degrading this type of collagen (58). It has been proposed that the sensitivity of collagen V to trypsin-like proteases is due to the presence of a molecular region of reduced helical stability in which the MMP-9-mediated cleavage also takes place (59).

Collagen V belongs to the fibrillar collagens and is in most cases associated with collagen I fibrils in the lamina reticularis, whereas another type of collagen, collagen IV, is part of the lamina densa in the basement membrane (50, 60). The intact structure of the interface between the lamina densa and the lamina reticularis is obviously essential for the integrity of the extracellular matrix. Studies by electron microscopy have suggested that interactions between collagens IV and V are dominant in this interface (50, 61), underlining the importance of cellular pathways for degradation of collagen V. Importantly, processes of tissue remodeling involve both the formation of cell to matrix contacts and the subsequent degradation of the extracellular proteins involved (7). Therefore, a combined mechanism of binding and degradation as suggested here may be crucial in a number of physiological and pathophysiological events, including those of cancer invasion and metastasis.

In addition to the binding of collagen V, it is quite likely that uPARAP takes part in other functions on the cell. All of the other protein family members are engaged in ligand internalization reactions as discussed above. A comparison of sequences in the intracellular domains within this family shows, however, that the motif NSYY that is critical for the internalization function of the rabbit phospholipase A2 receptor (62) is not present in uPARAP (Fig. 3A, residues 1450–1453). It is not known whether the latter sequence is allowed as a signal for internalization, and the role of uPARAP in this context is thus an open question. Distinct proteins engaged in uPAR-dependent ligand internalization have been identified previously. An extensive work has elucidated an interplay between uPAR and the low density lipoprotein receptor-related protein, or various related membrane proteins, in the internalization of uPA inhibitor complexes (for review see Ref. 63). uPAR-bound pro-uPA also becomes internalized, and on some cell types this seems to be mediated by the cation-independent mannose-6-phosphate receptor (64). These proteins belong to families different from uPARAP.

3 N. Behrendt, unpublished observations.
The composite nature of the functions of uPAR has also been studied by several other groups, and this work has led to identification of certain additional partner proteins at the functional level (for review see Ref. 4). However, as exemplified in the following, it is still unknown in most cases whether these proteins exert their functions through direct molecular interactions with uPAR or whether the functions in question arise from concerted actions within even larger molecular assemblies. This opens important questions concerning a putative role of uPARAP in these mechanisms. The above-mentioned functional interplay between uPAR and certain integrins has aroused great interest, and several recent studies have been focused on the molecular mechanism of these interactions (12, 65). The findings of uPAR-dependent signal transduction call for identification of actively signal-transducing “adapter” proteins (13) because uPAR, being anchored in the plasma membrane by a glycosyl-phosphatidylinositol anchor has no intracellular domain (66). Integrins may be involved in this function in some cases (for review see Ref. 67), but the exact molecular pathway of signal transduction remains elusive. Also unknown is the molecular assembly responsible for uPAR-dependent acceleration of plasminogen activation on the cell surface (55). Finally, the specific localization of uPAR at sites of focal cell substrate contact on certain cell types (68) indicates molecular contact formation with transmembrane proteins that interact directly or indirectly with the cytoskeleton. This points to yet another function where uPARAP may play a role.

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