Sequences within Domain II of the Urokinase Receptor Critical for Differential Ligand Recognition*

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The receptor for urokinase-type plasminogen activator (uPAR) plays important roles in a number of physiological and pathological processes by virtue of its interactions with urokinase-type plasminogen activator (uPA), vitronectin (Vn), and several other proteins. The uPA binding site spans all three domains (D1 to D3) of uPAR. However, the nature of the Vn binding site within uPAR is still not clear. In this study, we conducted homolog-scanning mutagenesis on uPAR by switching 14 individual segments of 4–8 residues to their counterpart sequences of a uPAR homolog CD59. All 14 mutants were well expressed, reacted with a panel of monoclonal antibodies, and exhibited correct molecular weights. Of these 14 mutants, six mutants were defective in both uPA and Vn binding. Most importantly, we found two unique uPAR domains: (1) uPA(uPAR)− and uPA(uPAR)137-HNNDTFHFLKC and 171-CNTTCKNEGPILELENLPQ) that are involved differentially in its recognition of uPA and Vn. These 14 mutants, six mutants were defective in both uPA and Vn binding, but completely lost Vn binding, indicating that these two sequences constitute a novel Vn binding site.

Indeed, two peptides, P1 (153-CPGSNGFHNNDTFHFLKC) and P2 (171-CNTTCKNEGPILELENLPQ), derived from the sequences of the identified uPA and Vn binding pockets within D2, respectively, behaved like bona fide ligand binding sites: peptide P1 bound uPA but not Vn, whereas peptide P2 bound Vn and inhibited uPAR-mediated cell adhesion, but did not interact with uPA. Altogether, our data demonstrated that uPAR D2 contains two distinct ligand binding sites for uPA and Vn. Such information will help us better understand the complex roles of uPAR in cell adhesion, migration, and tumor metastasis.

The receptor for urokinase-type plasminogen activator (uPAR) is a glycosylphosphatidylinositol-linked membrane protein, expressed on a wide range of cell types. uPAR plays important roles in cell adhesion and migration (1), chemotaxis (2), as well as tumor metastasis (3), by virtue of its interactions with the urokinase-type plasminogen activator (uPA), vitronectin (Vn), and several different integrins (4, 5). As Vn accumulates predominantly in extracellular matrices that are associated with acute injury and tissue repair, simultaneous binding of uPA and Vn to uPAR therefore concentrates the proteolytic activity of uPA and its activation product, plasmin, on the cell surface as well as close to the provisional matrix (6). uPAR/Vn interaction can be enhanced by uPA and attenuated by the plasminogen activator inhibitor-1, which binds to the somatomedin B domain of Vn (7). In addition, uPAR is capable of modulating cell adhesion by activating cells directly via a G-protein-coupled receptor (8), by sequestering caveolin (9), and by affecting intracellular signaling events (10). In support of these in vitro studies, uPAR-deficient mice were found to respond poorly to Pseudomonas aeruginosa infection, because of impaired neutrophil infiltration and reduced monocyte adhesion (11). Clinically, up-regulation of uPAR expression within tumor cells was found to correlate inversely with the survival rate of breast cancer patients (3). In mice, blocking uPAR functions by a polyclonal anti-uPAR antibody was found to markedly decrease tumor growth and metastases (12). Altogether, these data attest to the importance of uPAR in a variety of physiologic and pathogenic processes.

uPAR belongs to the Ly-6 protein family, and contains three domains (D1 to D3) that are homologous to CD59, Ly6E, and α-neutrotoxins (13). These domains fold into a central 3-stranded β-sheet and a globular, disulfide-rich core (14). The uPA binding site within uPAR has been studied extensively using a number of different approaches, including site-directed photoaffinity labeling, chemical cross-linking, alamine scavenging, as well as synthetic peptides, and the functional residues have been located within the α-helix and the three connecting loops, especially the second and third loops of all three domains (13, 15–18). In contrast to the well characterized uPA binding site, the Vn binding site within uPAR remains elusive. Recently, it was reported that deletion of D1 destroys uPAR binding to Vn (19), suggesting a role for this domain in Vn recognition. However, such interpretation is complicated by the fact that truncation of the entire D1 domain also abrogates uPA binding, which could lead to defective Vn binding as well.

To better understand the molecular basis governing Vn binding to uPAR, we have employed the homolog-scanning mutagenesis strategy (20) in this study and identified a region within uPAR that is involved differentially in its recognition of uPA and Vn. Our results demonstrate that two novel sequences (172-NTTK and 183-ELEN) within the uPAR D2 domain are critical for Vn binding but dispensable for uPA binding. In addition, we have identified two synthetic peptides (153-CPGSNGFHNNDTFHFPLKC and 171-CNTTCKNEGPILELENLPQ) that recapitulate the two identified ligand binding sites within the uPAR D2 domain: the former peptide bound uPA but not Vn, whereas the latter peptide interacted specifically with Vn but not uPA. Our study has for the first time unequivocally iden-
Vitronectin Binding Site within uPAR

EXPERIMENTAL PROCEDURES

Materials—Human kidney 293 cells were obtained from Dr. F. J. Castellino (Notre Dame, IN). The cDNA of human uPAR (16) was kindly provided by Dr. Doug Cines (Philadelphia, PA). Rabbit polyclonal antibody to human uPAR was obtained from Dr. Dudley Strickland (American Red Cross, Rockville, MD), and a similar antibody against uPAR (39R) was also obtained from American Diagnostics (Greenwich, CT). Human recombinative vitronectin was from the Enzyme Research Lab (South Bend, IN). mAbs 3931, 3932, 3936, 3937, 3471, and the high molecular weight form of uPA (HMW uPA) were from American Diagnostics; mAbs 10G7 and LM609 were from Chemicon (Temecula, CA); mAb VIM5 was from BD Pharmingen (San Diego, CA); and mAb TE3 was from the ATCC (Rockville, MD).

Site-directed Mutagenesis and Establishment of Stable Cell Lines—The detailed procedures for homolog-scanning mutagenesis and establishment of stable cell lines have been published elsewhere (21). Briefly, selected sequences of uPAR were switched individually to their counterpart sequences of CD59 (a uPAR homolog) using overlapping PCR. The presence of the mutation and the correctness of the remaining sequence were verified by DNA sequencing. The mutant uPAR cDNA was inserted in the expression vector pcDNA3 (Invitrogen) and then transfected into human 293 cells using LipofectAMINE (Invitrogen). Stable cell lines were established by selection with 418 (800 μg/mL). To obtain cell lines that express receptor numbers equivalent to wild-type uPAR, each mutant cell line was immuno-panned using a rabbit polyclonal antibody against human uPAR. Equivalent receptor expression for the wild-type and all 14 mutants was confirmed by FACS analysis.

FACS Analysis—A total of 10⁶ cells expressing wild-type or mutant uPAR in Hanks’ balanced salt solution (HBSS) were incubated with 1 μg of mAb for 30 min at 4°C. A subtype-matched mouse IgG served as a control. After washing with PBS, cells were mixed with fluorescein isothiocyanate goat anti-mouse IgG (F(ab’₂)₂ fragment (1:200 dilution) (Zymed), and then incubated for 30 min. Cells were then washed with PBS, and resuspended in 500 μL of Dulbecco’s phosphate-buffered saline (DPBS). The FACS analysis was performed using FACSscan (BD Biosciences), counting 10,000 events. Mean fluorescence intensities were quantified using the FACSscan program.

Ligand Binding Assays for uPAR—Binding activity of uPAR toward its two ligands uPA and Vn was assessed as previously described in the published procedures (22) with minor modifications. Binding of soluble HMW uPA was performed by FACS analysis. Briefly, a total of 10⁶ cells expressing wild-type or mutant uPAR in HBSS were incubated with different concentrations of uPA (50 to 200 nM) for 30 min at room temperature. After washing with DPBS, cells were mixed with a rabbit anti-human uPA antibody (number 389), and incubated for 30 min. Bound uPA was then detected with a fluorescein isothiocyanate conjugate of goat anti-rabbit IgG (F(ab’₂)₂ fragment (1:1000 dilution) (Zymed Laboratory), and kept at 4°C for another 30 min. Cells were then washed with PBS, and resuspended in 500 μL of Dulbecco’s phosphate-buffered saline (DPBS). The FACS analysis was performed using FACSscan (BD Biosciences), counting 10,000 events. Mean fluorescence intensities were quantified using the FACSscan program. The titration curve was fitted to a single binding site model using Sigma Plot (SPSS, Chicago, IL). For cell adhesion, native Vn (10 μg/mL) was used to coat the center of each well in a 24-well non-tissue culture polystyrene plate. After blocking with 300 μL of 1% BSA in DPBS, a total of 2 × 10⁶ cells in HBSS in the presence or absence of different concentrations of synthetic peptides or 20 μg/mL mAb VIM5 (against uPAR) were added to each well and incubated at 37°C for 20 min. The unbound cells were removed by three washes with DPBS and the adherent cells were quantified by staining with crystal violet. To exclude potential binding by endogenous integrins (αvβ3, α5β1, or αvβ1), all cell adhesion experiments were conducted in the presence of 2 μM EDTA. In addition, we have added the RGD peptide (1 μM), an αv-specific mAb LM609 (20 μg/mL), or a β3-specific mAb 7E3 (20 μg/mL) in some of our cell adhesion experiments and similar results were obtained.

Biotinylation of Synthetic Peptides—Synthetic peptides were biotinylated via their free sulphydryl (-SH) groups, using the EZ-link PEO-maleimide-activated biotin kit (Pierce), based on the product instructions. The efficiency of biotinylation was determined by immobilizing 0.2 μM of the biotinylated peptides on a maleic anhydride-activated polystyrene strip plate (also from Pierce) overnight, which reacts with the free NH₂ group in the peptides. This procedure resulted in more than 99% of the peptides being captured on the plate. After washing with PBS and blocking with BSA, the amount of biotin group was determined using an alkaline phosphatase-avidin conjugate, measuring the absorption at 405 nm. Similar biotinylation efficiencies were obtained for all peptides.

Solid Phase Binding Assays—To test the interaction between the identified sequences of uPAR and its two ligands (uPA and Vn), wells of a 96-well microtiter plate (Immulon 4E, Dynex Technologies Inc., Chantilly, VA) were coated with the different synthetic peptides at 2 mg/mL overnight at 4°C and postcoated with 3% BSA for 2 h at room temperature. uPA (10 μg/mL) or Vn (10 μg/mL) in DPBS containing 0.1% BSA was added to the wells and incubated for 2 h at 37°C. After washing with DPBS, bound ligands were detected with a rabbit anti-human uPA antibody (number 389), or a sheep anti-human Vn antibody, followed by alkaline phosphatase conjugates of a goat anti-rabbit or a donkey anti-sheep IgG, respectively. Bound conjugates were measured by reaction with p-nitrophenyl phosphate. Reciprocally, the plate was coated with 100 μL of 10 μg/mL HMW uPA, blocked with 3% BSA, and incubated with different concentrations (0 to 500 μM) of biotinylated peptides. After washing, bound peptides was detected with an alkaline phosphatase-avidin conjugate, measuring absorption at 405 nm.

RESULTS

Homolog-scanning Mutagenesis of uPAR—To identify the functional sequences within uPAR for Vn binding, we employed the homolog-scanning mutagenesis strategy (20), which has been used successfully by us in the past to determine the ligand binding sites within several receptors (21, 23). In this work, we substituted the corresponding segments (between 4 and 8 residues) of uPAR with their counterpart sequences of CD59, based on the premise that uPAR and CD59 are homologous but recognize completely different ligands. Given the dependence of the uPAR/Vn interaction on uPA, we speculated that the bind-
ing sites for uPA and Vn might reside close to each other. Because the functional residues for uPA binding have been mapped to loops 2 and 3, as well as the α-helical region within each domain (15–18), we have initially targeted these regions for homolog-scanning mutagenesis (Fig. 1A). Guided by the three-dimensional structure of CD59 (14), we constructed nine mutants that would cover the targeted regions within all three domains (Table I and Fig. 1A). Upon completion of site-directed mutagenesis, the presence of the expected mutations and the correctness of the rest of the uPAR sequence were confirmed by DNA sequencing analysis. Stable cell lines were then established for wild-type and mutant uPAR by G418 selection, and similar surface expression levels of uPAR were verified by FACS analyses using a rabbit anti-human uPAR antibody (data not shown). In addition, Western blot analyses using mAb VIM5 showed that eight of the nine mutant receptors migrated as 46-kDa proteins on SDS-PAGE, similar to the wild-type receptor (Fig. 1B). These data indicated that segment replacements did not affect protein processing of these mutant receptors. One mutant, uPAR(Glu37–Leu40), was not included in Fig. 1B, as its VIM5 epitope was disrupted by mutation (see below).

Ligand Binding by the Homolog-scanning Mutants—To see if substitution of these nine segments had any effect on uPAR binding to uPA and Vn, we conducted binding assays as described under “Experimental Procedures,” based on modifications of the published procedures (22). As shown in Fig. 2, wild-type uPAR bound uPA in a dose-dependent and saturable manner. The uPA titration data could be fit to a single binding site model using non-linear regression analysis. The calculated $K_d$ for uPA binding to wild-type uPAR is 1.6 nM, which is very close to the published value of 2.1 nM (24). Verifying specificity, mock transfected 293 cells did not show any detectable uPA binding (Fig. 2). In addition, uPA binding to the uPAR-expressing cells could be blocked by addition of mAb VIM5 (data not shown). Next, we carried out similar uPA binding experiments for the nine homolog-scanning mutants, and the $K_d$ values were calculated from the titration curves for each mutant. The uPA binding affinity ($1/K_d$) toward each mutant was then compared with that of wild-type uPAR and expressed as percentage of the wild-type binding activity. As shown in Fig. 3A, of the nine mutants, two mutants (Asn233–Gly360, His369–Asn375) had no detectable uPA binding. The most dramatic loss of uPA binding was observed for five mutants, all of which exhibited less than 0.5% uPA binding compared with wild-type. These mutants included Arg139–Gly142, Arg142–Asn146, and Asn162–Phe167. The first two mutants (Arg139–Gly142 and Leu161–Leu166) are located within D1, and the last three are located within D2. These data suggested that these five segments contributed significantly to the formation of the uPA binding site.

To confirm that all nine uPAR mutants had correct conformation, we conducted FACS analysis using a panel of different uPAR-specific mAbs, including VIM5, 3931, 3932, 3936, and 3937. All nine mutants reacted well with these mAbs, except for those where their epitopes reside within the switched segments (data not shown). For example, the functional mutant uPAR(Glu37–Leu40), which was active in both uPA and Vn binding (Fig. 3, A and B), was not recognized by mAb VIM5, indicating that segment 37ELEL epitope within D1 represents part of the epitope for this mAb. Taking these data together, we concluded that loss of uPA binding by these five uPAR mutants resulted from direct alterations of the uPA binding site, rather than from gross structural changes within these mutants. Such a conclusion was supported further by our observation that a synthetic peptide (P1), corresponding to the identified residues (Gly155–Asn161 and Asn162–Phe167) within D2, bound uPA directly (see below).

Next, we evaluated the effects of the nine uPAR mutations on Vn binding, using cell adhesion assays (22). For these experiments, we used native Vn to coat non-tissue culture 24-well plates. After blocking with BSA, the uPAR-expressing 293 cells were added to each well in the absence of Ca$^{2+}$ and Mg$^{2+}$ (to block integrin-mediated cell adhesion; see “Experimental Procedures”), and the number of adherent cells were determined. As shown in Fig. 3B, the five mutants that were defective in uPA binding were also defective in Vn binding. These mutants include Arg139–Gly142, Leu161–Leu166 within D1, and Lys139–Arg142, Gly155–Asn161, and Asn162–Phe167 within D2. Their Vn binding activities were reduced by 11-, 9-, 6-, and 11-fold, respectively. Specificity of the Vn binding was confirmed by the

**Table I**

| Position | From | To | Mutagenic primers (5’ to 3’) |
|----------|------|----|-----------------------------|
| 1        | 37-40| ELEL | AGLQ                        |
| 2        | 53-60| RTL3YRTG | FNDVTTR          |
| 3        | 61-66| LKTTSL | LRENEL         |
| 4        | 139-142| KDDR | AGLQ                        |
| 5        | 155-161| GSNFGHN | FNDVTTR        |
| 6        | 162-167| NDTRRH6 | LRENEL        |
| 7        | 233-236| NQGSY | AGLQ                        |
| 8        | 249-255| RAHLGDA | FNDVTTR         |
| 9        | 256-261| FSNMNH | LRENEL         |

**Fig. 2.** uPA binding to wild-type uPAR. HMW uPA (0 to 100 nM) in HBSS was mixed with 2 × 105 uPAR-expressing cells (●) for 30 min. After three washes, a uPA-specific polyclonal antibody (number 389) was added and incubated for 30 min. The amount of cell-bound uPA was detected with a fluorescein isothiocyanate conjugate of goat anti-rabbit IgG by FACS analysis. The dissociation constant ($K_a$) was calculated from the titration data, based on a single binding site model, using a non-linear regression program in SigmaPlot. Mock transfected cells (△) did not show detectable binding of uPA. Data shown are the mean ± S.D. of five independent experiments.
ability of mAb VIM5 to block cell addition by the wild-type and all functional uPAR mutants (data not shown). Taking these data together, we found that two of three mutations in D1 and three of three mutations within D2 had dramatic effects on ligand binding by uPAR. These data underscored the critical roles of D2 in uPAR recognition of both ligands.

Differential Ligand Recognition by the uPAR D2 Domain—Given the importance of the D2 domain in both uPA and Vn binding, we chose to focus our subsequent study on this domain alone. To probe the entire hydrated surface of the D2 domain, we constructed an additional five mutants (Ser90–Glu94, Ser101–Ser104, Arg116–Glu120, Asn172–Lys175, and Glu183–Asn186), in addition to the three D2 mutants described in Fig. 3. Of these five new mutants, residues Ser90–Glu94 are located within the NH2 terminus of D2, Ser101–Ser104 are located within the loop 1 region, Arg116–Glu120 are located in the middle, and finally, Asn172–Lys175 and Glu183–Asn186 are located within the COOH terminus of D2. Upon establishing these five stable cell lines, the effect of these mutations on Vn and uPA binding was determined as described above. As shown in Fig. 4A, all five additional mutants bound uPA well, exhibiting 70 to 120% wild-type uPA binding activities. In contrast, differential effects of these five mutations were observed on Vn binding (Fig. 4B): three mutants, uPAR(Ser90–Glu94), uPAR(Ser101–Ser104), and uPAR(Arg116–Glu120), exhibited normal Vn binding, whereas two mutants (uPAR(Asn172–Lys175) and uPAR(Glu183–Asn186)) failed to recognize Vn. These latter two mutants possessed intact conformations, as evidenced by their high affinity uPA binding with $K_d$ values of 1.9 and 1.6 nM, respectively, which are essentially identical to the $K_d$ of 1.6 nM for the wild-type receptor (Fig. 2). Thus, loss of Vn binding by uPAR(Asn172–Lys175) and uPAR(Glu183–Asn186) is likely because of a direct perturbation of the Vn binding site within D2.

Synthetic Peptides of the D2 Domain Blocked Vn Binding to uPAR—To understand the underlying mechanism for the differential involvement of the two novel sequences (Asn172–Lys175 and Glu183–Asn186) in Vn and uPA binding, we constructed a three-dimensional model of D2, based on its homology to CD59 (14). As shown in Fig. 5, residues that were critical to Vn binding (Asn172–Lys175 and Glu183–Asn186) clus-
ter on the opposite site of those that were critical to uPA binding (Lys\(^{139}\)–Arg\(^{142}\), Gly\(^{155}\)–Asn\(^{161}\), and Asn\(^{162}\)–Phe\(^{167}\)), suggesting a possibility that the uPAR D2 domain may contain two separate ligand binding sites for uPA and Vn. To test this hypothesis, we prepared two synthetic peptides, P1 (\(153\text{CPG-SNGFHNNDTFHFLKC}\)) and P2 (\(171\text{CNTTKCNEGPILELENLPQ}\)), representing the predicted ligand binding sites for uPA and Vn, respectively. The P1 peptide corresponds to mutants uPAR(Gly\(^{155}\)–Asn\(^{161}\)) and uPAR(Asn\(^{162}\)–Phe\(^{167}\)), which were defective in both uPA and Vn binding, and the P2 peptide corresponds to mutants uPAR(Asn\(^{172}\)–Lys\(^{175}\)) and uPAR(Gly\(^{180}\)–Asn\(^{183}\)), which were defective only in Vn binding. In addition, we prepared scrambled P1 and P2 peptides, sP1 (GHGNFPCSTKFNCNHLDF) and sP2 (NQLCNEIEFLQKTENPL). To see if these peptides could inhibit Vn binding by wild-type uPAR, we performed cell adhesion experiments in the presence or absence of these peptides. As shown in Fig. 6, the P2 peptide inhibited cell adhesion in a dose-dependent manner. Addition of 0.1 and 0.5 mM P2 blocked 55 and 75% uPAR-mediated cell adhesion to Vn, respectively. The P1 peptide and the two scrambled peptides (sP1 and sP2) had no significant effect on Vn binding by uPAR, verifying the specificity of these assays.

Direct Binding of the D2 Domain Peptides to uPA and Vn—We next evaluated the ability of the D2 peptides to bind uPA and Vn by ELISAs. In these experiments, 96-well microtiter plates were coated with different concentrations of peptides P1, P2, and their scrambled controls. After blocking with BSA, binding of Vn and the HMW uPA to the peptides was assessed using antibodies specific for these two proteins. As shown in Fig. 7A, peptide P2 bound Vn strongly, whereas its scrambled control sP2 did not show significant binding. In contrast, neither peptide P1 nor its scrambled control sP1 showed any significant Vn binding. To see if these peptides could interact with HMW uPA directly, similar ELISA experi-
FIG. 7. Direct binding of Vn and uPA to synthetic peptides of the D2 domain. A, Vn binding. Direct interactions between the synthetic peptides and Vn were measured by ELISA. Briefly, 96-well microtiter plates were coated with different synthetic peptides (2 μg/ml) overnight, and then blocked with 3% BSA. Native Vn (10 μg/ml) was added and incubated for 30 min at 22 °C. Bound Vn was detected using a sheep anti-Vn and an alkaline phosphatase conjugate of donkey anti-sheep IgG. p-Nitrophenyl phosphate was used as the substrate, and absorbance at 405 nm was measured with a ELISA reader. Data shown are the mean ± S.D. of three independent experiments. B, uPA binding. HMW uPA (10 μg/ml) in HBSS was added to the wells that were pre-coated with a series of different concentrations (0 to 10 mM) of synthetic peptide P1 (●), P2 (○), sP1 (■), and sP2 (◇), followed by incubation at 22 °C for 30 min. After washing, bound ligands were detected with a rabbit anti-uPA antibody, and an alkaline phosphatase conjugate of goat anti-rabbit IgG, measuring absorbance at 405 nm. The background of the three-step ELISA, obtained using BSA-coated wells, or in the presence of the uPA-specific mAb 3471, was ~40% of the total binding at 10 mM peptide P1. Data shown are the mean ± S.D. of three independent experiments. C, reciprocally, the plate was coated with 100 μl of 10 μg/ml HMW uPA, blocked with 3% BSA, and incubated with different concentrations (0–500 μM) of biotinylated peptide P1 (●), P2 (◇), sP1 (■), and sP2 (○). After washing, bound peptides were detected with an alkaline phosphatase-avidin conjugate, measuring absorption at 405 nm. Addition of the uPA-specific mAb 3471 (●) completely blocked P1 binding to uPA, verifying specificity of the assay. Data shown are the mean ± S.D. of three independent experiments.
alkaline phosphatase-avidin conjugate. As shown in Fig. 7C, only peptide P1 bound uPA with high affinity, whereas peptide P2 and the two scrambled peptides sP1 and sP2 did not have significant binding. The binding data could be fitted to a single binding site model with a \( K_d \) of 1.6 \( \mu \)M. We have verified that all four peptides had similar efficiency of biotinylation. Moreover, P1 binding to uPA could be completely blocked by addition of a mAb against the amino-terminal fragment of uPA (number 3471), demonstrating the specificity of the binding assay.

**Discussion**

Given the importance of uPAR and Vn in a number of physiological and pathological processes, including cell adhesion, migration, and tumor metastasis, we have studied the molecular basis underlying uPAR recognition of Vn, using the homolog-scanning mutagenesis approach (20). In particular, we have successfully separated Vn binding from uPA binding by uPAR, and identified a novel sequence \((172^{NTTKCNEGPILELENLPQ})\) within the COOH terminus of the D2 domain that is critically involved in Vn but not uPA binding. Our conclusion that this sequence represents a novel Vn binding site in uPAR is supported by the following observations: 1) mutations of the above sequence completely destroyed Vn binding but did not affect uPA binding; 2) its corresponding synthetic peptide (P2) blocked uPAR/Vn interaction; and 3) the P2 peptide bound specifically to purified Vn but not HMW uPA.

Previously, we employed the homolog-scanning mutagenesis approach in our studies of several cell surface receptors and successfully identified a number of different ligand binding sites (21, 23, 25). For the current study, similar approaches were used to delineate the ligand binding sites of uPAR, based on sequence similarity but functional disparity between uPAR and its homolog CDS59 (13). To preserve maximally the gross structure of uPAR, none of the mutations in this study involved the conserved residues that likely play essential roles in protein folding. As established from our earlier studies, the homolog-scanning mutations, including those that destroyed ligand binding, did not significantly change the overall structure of the mutated receptor, and therefore loss of function was attributed to perturbations of the ligand recognition site (21, 23, 25). In this work, a total of 9 homolog mutants were constructed initially, which covered loops 2 and 3, as well as the helical regions within all three domains of uPAR. An additional 5 mutants were subsequently constructed that covered the remaining hydrated surface of the D2 domain. These 14 homolog-scanning mutants were correctly processed as evidenced by their correct molecular weights, expressed on the cell surface, and reacted well with a number of uPAR-specific mAbs, suggesting that all 14 uPAR mutants, including the seven defective receptors, possessed correct conformations. Therefore, we concluded that loss of ligand binding by these seven mutants was caused by direct perturbations of the binding pockets for Vn and uPA. Indeed, we found that two synthetic peptides (P1 and P2), corresponding to the two ligand binding sites identified in this study, behaved like bona fide ligand binding sites, and displayed proper selectivity for their respective ligands. That is, the P1 peptide, corresponding to the sequences \((\text{Gly}^{155}-\text{Asn}^{161} \text{ and Asn}^{162}-\text{Phe}^{167})\) that are critical for uPA binding, interacted with uPA but not Vn; whereas the P2 peptide, corresponding to the sequences \((\text{Asn}^{172}-\text{Lys}^{175} \text{ and Glu}^{180}-\text{Asn}^{186})\) that are critical for Vn binding, bound Vn but not uPA (Fig. 7).

The uPA binding site within uPAR has been studied extensively in the past several years, using a number of different approaches (15–18). In good agreement with the published data, we found that the uPA binding site occupies a broad region that encompasses all three domains, with the most critical residues located in D1 and D2. These include residues 53 to 66 within D1 and 139–142 and 155–167 within D2 (Fig. 3). In contrast, little is known about the Vn binding site within uPAR. In this regard, our current study has provided very strong evidence for a direct role of D2 in Vn binding. This conclusion is based on our novel observation that a unique sequence \((172^{NTTKCNEGPILELENLPQ})\) located at the COOH terminus of D2 is critical to Vn binding but dispensable to uPA binding. Furthermore, a synthetic peptide (P2) derived from this sequence inhibited uPAR-mediated adhesion to Vn (Fig. 6), and when coated on microtiter wells, bound Vn directly but did not interact with uPA. In addition, we found another peptide (P1), derived from our identified sequences of the adjacent uPA binding site, bound uPA but not Vn (Fig. 7). Taking these data together, we concluded that the D2 domain of uPAR possesses two separate ligand binding sites with specificity for uPA and Vn, respectively. In a three-dimensional model, constructed from the crystal structure of CD59 (14), the uPA and Vn binding sites reside on opposite sides of the D2 domain (Fig. 5). The uPA binding site is composed of residues Lys139–Arg142 Glu155–Asn161, and Asn162–Phe167, whereas the Vn binding site is composed of amino acids Asn172–Lys175 and Glu183–Asn186. These two ligand binding sites are linked intimately via a 2-amino acid residue linker (Leu168–Lys169). Therefore, it is possible that ligand binding to one site could affect the conformation of the other site. Consistent with this notion, we found that: 1) although uPA was not required, it did promote Vn binding to uPAR (–1.5-fold) (data not shown; and 2) all mutants defective in uPA binding also failed in Vn binding (Fig. 3). Nevertheless, it must be stressed that uPA could augment Vn binding by other mechanisms. For example, uPA could promote Vn binding by inducing oligomerization of soluble uPAR (26). Additional experiments will be required to differentiate these mechanisms.

In summary, using the homolog-scanning mutagenesis approach, we have successfully separated Vn binding from uPA binding by uPAR, and identified a novel sequence \((172^{NTTKCNEGPILELENLPQ})\) within the COOH terminus of its D2 domain, which is critically involved in Vn but not uPA binding. In addition, we have located two distinct ligand binding sites with uPAR. A synthetic peptide (P1), corresponding to the identified uPA binding site, bound uPA, but not Vn. Another synthetic peptide (P2), corresponding to the Vn binding site, bound Vn directly and blocked uPA-mediated cell adhesion, but did not interact with uPA. Protein modeling showed that these two ligand binding sites reside in separate regions of the D2 domain but are connected with a short linker, suggesting a possibility that uPA binding to the uPAR D2 domain could induce conformational changes within the Vn binding site, leading to optimal Vn binding. Given the critical role of uPAR in a number of physiological and pathological processes, the results from this study could help us better understand how uPAR recognizes its different ligands, and most importantly, how these interactions contribute to cell adhesion, chemotaxis, and tumor metastasis.

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**References**

1. Nguyen, D. H., Hussaini, I. M., and Gonias, S. L. (1998) *J. Biol. Chem.* 273, 8502–8507

2. Gysko, R. E., Todd, B. F., Wilkinson, C. C., and Sitrin, R. G. (1994) *J. Clin. Invest.* 93, 1380–1387

3. Memarzadeh, S., Kozak, K. R., Chang, L., Natarajan, S., Shintaku, P., Reddy, S. T., Fairies-Emmer, R., and Memarzadeh, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 10647–10652

4. Simon, D. I., Rao, N. K., Xu, H., Wei, Y., Majdic, O., Ronne, E., Kobilka, K., and Chapman, H. A. (1998) *Blood* 88, 3185–3194

5. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1996) *Science* 273, 1551–1555

6. Chavakis, T., Kanse, S. M., Yutzy, B., Lijnen, H. R., and Preissner, K. T. (1998)
Vitronectin Binding Site within uPAR

7. Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. (1996) *J. Cell Biol.* **134**, 1563–1571
8. Liu, D., Aguirre, G. J., Estrada, Y., and Ossowski, L. (2002) *Cancer Cell* **1**, 445–457
9. Wei, Y., Yang, X., Liu, Q., Wilkins, J. A., and Chapman, H. A. (1999) *J. Cell Biol.* **144**, 1295–1299
10. Nguyen, D. H., Catling, A. D., Webb, D. J., Sankovic, M., Walker, L. A., Somlyo, A. V., Weber, M. J., and Gonias, S. L. (1999) *J. Cell Biol.* **146**, 149–164
11. Gyetko, M. R., Sud, S., Kendall, T., Fuller, J. A., Newstead, M. W., and Standiford, T. J. (2000) *J. Immunol.* **165**, 1513–1519
12. Rabbani, S. A., and Gladu, J. (2002) *Cancer Res.* **62**, 2390–2397
13. Ploug, M., Kjalka, M., Ronne, K., Weidle, U., Hoyer-Hansen, G., and Dano, K. (1993) *J. Biol. Chem.* **268**, 17539–17546
14. Kieffer, B., Driscoll, P. C., Campbell, I. D., Willis, A. C., van der Merwe, P. A., and Davis, S. J. (1994) *Biochemistry* **33**, 4471–4482
15. Gardevoll, H., Dano, K., and Ploug, M. (1999) *J. Biol. Chem.* **274**, 37995–38003
16. Bdeir, K., Kuo, A., Mazar, A., Sachais, B. S., Xiao, W., Gawlak, S., Harris, S., Higazi, A. A., and Cines, D. B. (2000) *J. Biol. Chem.* **275**, 28532–28538
17. Liang, O. D., Chavakis, T., Kanse, S. M., and Preissner, K. T. (2001) *J. Biol. Chem.* **276**, 28946–28953
18. Behrendt, N., Ronne, E., and Dano, K. (1996) *J. Biol. Chem.* **271**, 22885–22894
19. Sidenius, N., and Blasi, F. (2000) *FERS Lett.* **470**, 40–44
20. Cunningham, B. C., Jhurani, P., Ng, P., and Wells, J. A. (1989) *Science* **243**, 1330–1336
21. Zhang, L., and Plow, E. F. (1997) *J. Biol. Chem.* **272**, 17558–17564
22. Wei, Y., Waltz, D. A., Rao, N., Drummond, R. J., Rosenberg, S., and Chapman, H. A. (1994) *J. Biol. Chem.* **269**, 32380–32387
23. Zhang, L., and Plow, E. F. (1999) *Biochemistry* **38**, 8064–8071
24. Miles, L. A., Levin, E. G., Plescia, J., Collen, D., and Plow, E. F. (1988) *Blood* **72**, 628–635
25. Ugarova, T. P., Solovrov, D. A., Zhang, L., Leukinov, D. I., Yee, V. C., Medved, L. V., and Plow, E. F. (1998) *J. Biol. Chem.* **273**, 22519–22527
26. Sidenius, N., Andolfi, A., Fesce, R., and Blasi, F. (2002) *J. Biol. Chem.* **277**, 27982–27990
27. Guex, N., and Peitsch, M. C. (1997) *Electrophoresis* **18**, 2714–2723
