Characterization of the Functional Epitope on the Urokinase Receptor

COMPLETE ALANINE SCANNING MUTAGENESIS SUPPLEMENTED BY CHEMICAL CROSS-LINKING*1

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The high affinity interaction between the serine protease urokinase-type plasminogen activator (uPA) and its glycolipid-anchored receptor (uPAR) represents one of the key regulatory steps in cell surface-associated plasminogen activation. On the basis of our crystal structure solved for uPAR in complex with a peptide antagonist, we recently proposed a model for the corresponding complex with the growth factor-like domain of uPA (Llinas et al. (2005) EMBO J. 24, 1655–1663). In the present study, we provide experimental evidence that consolidates and further develops this model using data from a comprehensive alanine scanning mutagenesis of uPAR combined with low resolution distance constraints defined within the complex using chemical cross-linkers as molecular rulers. The kinetic rate constants for the interaction between pro-uPA and 244 purified uPAR mutants with single-site replacements were determined by surface plasmon resonance. This complete alanine scanning of uPAR highlighted the involvement of 20 surface-exposed side chains in this interaction. Mutations causing $\Delta \Delta G \geq 1$ kcal/mol for the uPA interaction are all located within or at the rim of the central cavity uniquely formed by the assembly of all three domains in uPAR, whereas none are found outside this crevice. Identification of specific cross-linking sites in uPAR and pro-uPA enabled us to build a model of the uPAR/uPA complex in which the kringle domain of uPA was positioned by the constraints established by the range of these cross-linkers. The nature of this interaction is predominantly hydrophobic and highly asymmetric, thus emphasizing the importance of the shape and size of the central cavity when designing low molecular mass antagonists of the uPAR/uPA interaction.

The urokinase-type plasminogen activator receptor (uPAR) is a glycosylphosphatidylinositol-anchored membrane glycoprotein (1) that has a primary role in focalizing plasminogen activation at the cell surface through its specific high affinity interaction with the urokinase-type plasminogen activator (uPA). Besides facilitating the generation of plasmin activity in the vicinity of uPAR-expressing cells, which is directly or indirectly involved in remodeling of the extracellular matrix (2, 3), the uPAR/pro-uPA interaction also assists in regulating other aspects of cell adhesion and migration. Among these molecular processes is the direct interaction with matrix-deposited vitronectin (4); the modulation of integrin function, in particular $\alpha_5\beta_2$, $\alpha_v\beta_3$, and $\alpha_v\beta_1$ (5–8); and the activation of the chemotactic FPR1 receptor (9). As an increased expression level of uPAR is often found in the invasive areas of various human cancers and correlates with poor prognosis (10), the uPAR/uPA interaction and uPA catalytic activity are considered relevant molecular targets for drug development (11–13). Intervention strategies developed for targeting the uPAR/uPA interaction with a view to cancer therapy include recombinant fusion proteins containing the receptor-binding module of uPA (14, 15), anti-uPAR monoclonal antibodies (16), synthetic peptide antagonists (17–19), down-regulation of uPAR expression by small interfering RNAs (20, 21), and a modified anthrax toxin that is specifically activated by the enzymatic activity of receptor-bound uPA (22).

Although the uPAR/uPA interaction thus plays an active role in orchestrating these processes at the biochemical level, an accurate experimental description of the three-dimensional structure of this complex has yet to be solved by x-ray crystallography. During the last decade, a number of studies have reported on the assignment of the uPA-binding site on uPAR using a plethora of different techniques, including chemical

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2 The abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; uPA, urokinase-type plasminogen activator; ATF, amino-terminal fragment; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; NHS, N-hydroxysuccinimide; DST, disuccinimidyl suberate; DGG, disuccinimidyl glutarate; DST, disuccinimidyl tartrate; HPLC, high pressure liquid chromatography; MALDI, matrix-assisted laser desorption ionization time-of-flight; GFD, growth factor-like domain; uPAR*, wild-type urokinase-type plasminogen activator receptor; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.
Mapping the uPAR/uPA Interface

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—The zero-length cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC); N-hydroxysuccinimide (NHS); and the homobifunctional amino-reactive chemical cross-linkers disuccinimidyl suberate (DSS), disuccinimidyl glutarate (DSG), and disuccinimidyl trtarate (DST) were from Pierce. Modified porcine trypsin was from Promega Corp. (Madison, WI); N-glycosidase F was from Roche Applied Science (Mannheim, Germany); and 18O-labeled H2O (>95% purity) was from Spectra Stable Isotopes (Columbia, MD). Anti-uPA (clone 6) and anti-uPAR (R2, R3, S1, S2, and KOR-1) monoclonal antibodies were made in-house, and the anti-pen-tahistidine monoclonal antibody was from Invitrogen.

**Expression and Purification of Soluble Human Recombinant uPAR Mutants**—Soluble forms of human uPAR were expressed in and secreted by Drosophila melanogaster Schneider 2 (S2) cells, which were stably transfected with pMTC/uPAR (residues 1–283)3 (34). These proteins are secreted to the conditioned medium because of a deletion of the carboxy-terminal signal sequence that is required for glycolipid anchoring (1, 26). Single-site alanine replacements were introduced into pMTC/uPAR by site-directed mutagenesis using a previously designed three-gene cassette approach (34), and the corresponding 244 soluble uPAR mutants were expressed in S2 cells. All constructs were verified by DNA sequencing using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, CA). The majority of the secreted uPAR mutants were purified from the conditioned medium by immunoaffinity chromatography using an immobilized anti-uPAR monoclonal antibody (R2), followed by reverse-phase HPLC using a Vydac C4 column (0.46 × 25 cm) and a linear gradient (40 min) from 0 to 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. Immunoaffinity purification of uPAR mutants changed at positions 274–277 (covering the epitope for antibody R2) was performing using anti-uPAR monoclonal antibody R3, the functional epitope of which resides on uPAR domain I. As judged by SDS-PAGE of ~5 μg of reduced and alkylated sample, the purity of these uPAR preparations were generally >95% (supplemental Fig. S1). The identities of individual purified uPAR mutants were also verified at the protein level by peptide mass mapping and tandem mass spectrometric sequencing after in-gel digestion of reduced and carbamidomethylated protein using an endoprotease (modified porcine trypsin, chymotrypsin, Lys-C, or Asp-N) before mass assignment by an Autoflex II134 MALDI-TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) (35). If the mutated residue was located in a glycopeptide, the corresponding in-gel digest was also subjected to deglycosylation by N-glycosidase F. Protein concentrations of the purified uPAR mutants were determined spectrophotometrically using an extinction coefficient (ε1%/H18528) of 9.2 (36), except for those mutants involving Trp12 or Trp129, for which an estimated ε1%/H18528 of 6.8 was used.

**Expression and Purification of Murine uPAR and Murine uPA, Pro-uPA Mutants, and the Carboxyl-terminally His6-tagged Growth Factor-like Domain (GFD)**—Human pro-uPA (residues 1–411)3 with its active-site Ser356 replaced with alanine was produced by Drosophila S2 cells stably transfected with the pMTB/uPAS356A vector. Expression of pro-uPA543K was induced by 0.5 mm Cu2SO4 as described previously (34). The S2 medium also contained 10 μg/ml aprotinin to prevent proteolytic cleavage at the activation site of pro-uPA. Pro-uPA was isolated by immunoaffinity chromatography using an immobilized anti-uPAR monoclonal antibody (clone 6). The purity of this pro-uPA was >95%, and the preparation showed only negligible conversion to two-chain uPA as judged by SDS-PAGE (supplemental Fig. S2). The identity of the purified pro-uPA543K was verified by electrospray ionization mass spectrometry, which revealed two components with a mass of either 3The numbering of amino acid residues in uPA and uPAR refers to the cDNA-derived sequences, omitting the signal sequences. The nomenclature for the secondary structure elements in uPAR follows the conventions established for snake venom α-neurotoxins (58) and more explicitly clarified for the modular domains in uPAR (33).
Mapping the uPAR/uPA Interface

47,511.6 or 47,365.8 Da, corresponding to the disulfide-bonded polypeptide chain of pro-uPA356A with one N-linked glycosylation at Asn307 (1039.0 Da) and, in ~60% of the molecules, one additional O-linked fucose at Thr18 (1461.1 Da). Following the same protocol, 12 different pro-uPA mutants with single-site replacement of lysine with alanine within the amino-terminal region were produced (supplemental Fig S2). The protein concentration of a stock solution of pro-uPA356A was determined accurately by amino acid composition analysis (37), and the various lysine mutants were quantified using an E280 nm of 18.5.

The GFD (residues 1–48) of human uPA was expressed as a carbonyl-terminally His6-tagged protein in Pichia pastoris strain X-33 using the yeast expression vector pPICZαA/GFD-His according to the protocols of Invitrogen. The secreted GFD-His6 was purified from the medium by adsorption onto an Ni2+ chelate column (HiTrapTM chelating HP), followed by reverse-phase HPLC using a Vydac C4 column (0.46 × 25 cm) and a linear gradient (40 min) from 0 to 35% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The identity of the purified GFD was verified by MALDI mass spectrometry (average mass of 6189.6 Da; no fucosylation present at Thr18), and the protein concentration was determined using an estimated E280 nm of 11.8.

Assessing uPAR Binding to Immobilized Pro-uPA by Surface Plasmon Resonance—All real-time interaction studies were carried out on a Biacore 3000TM (Biacore International AB, Uppsala, Sweden) in running buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% (v/v) surfactant P20, pH 7.4). Human pro-uPA356A (0.2–0.5 μg/ml) was immobilized covalently at pH 5.0 on a carboxymethylated dextran matrix (CM5 sensor chip) using NHS/EDC as described previously (24), which yielded coupling levels in three different flow cells covering the range of 200–1000 resonance units, corresponding to 0.2–1 ng/mm2 pro-uPA. For comparative evaluation of the kinetics of the uPAR/pro-uPA interaction, serial 2-fold dilutions of the various uPAR mutants (0.8–100 nM in running buffer) were analyzed in parallel for uPA binding at a flow rate of 20 μl/min at 20 °C. Association was recorded for 300 s, followed by a dissociation phase of 775 s, and the derived sensorgrams for such an interaction analysis using the wild-type receptor are shown in supplemental Fig S3. After each run, the sensor chip was regenerated by two consecutive injections of 10 μl of 0.1 M acetic acid and 0.5 M NaCl. Data processing was accomplished by double referencing, during which each sensorgram was corrected by subtraction of the signal from both a mock-coupled flow cell and an appropriate buffer run. The kinetic rate constants k on and k off were derived by nonlinear fitting of the association and dissociation phases to a simple bimolecular interaction model (uPA + uPAR ↔ uPARuPA) using BIAevaluation Version 4.1 software (Biacore International AB). In brief, k off values were initially derived by fitting the collected data to dr/dt = −k off R, and the corresponding k on was subsequently fitted to dr/dt = k on[uPAR](Rmax − R) − k off R, assuming pseudo first-order reaction kinetics essentially as described (38). The curve fitting to the recorded data for the wild-type uPAR (uPARwt)/uPA interaction is shown in supplemental Fig S3 along with the corresponding residual plot. The change in free energy (ΔΔG) caused by each mutation was calculated from ΔΔG = ΔG mut − ΔG wt = RT ln(Kd(mut)/Kd(wt)), where Kd values were derived from the ratio of the kinetic constants k on and k off, R = 1.99 cal/mol K, and T = 293 K.

Because of the limitations enforced by the logistics of this experiment, it was impossible to analyze freshly prepared protein samples for each of the large number of mutants. The entire library of uPAR alanine mutants was therefore kept frozen at −80 °C, and individual mutants were thawed and diluted in running buffer immediately before analysis. At appropriate time intervals, uPARwt was analyzed in parallel and served as a reference for calculation of ΔΔG. A number of uPAR mutants, including some of those that exhibited severely impaired kinetics for the pro-uPA interaction, were subjected to size exclusion chromatography (Superdex 200TM in 10 mM phosphate and 0.1 M NaCl, pH 7.4) to separate uPAR monomer from any aggregated protein before reanalysis of the monomer fraction by surface plasmon resonance without prior freezing. Generally, the k off values did not change significantly in this second analysis, but the k on values occasionally increased by up to 2-fold after size exclusion chromatography.

Covalent Conjugation of uPAR-Ligand Complexes by Bifunctional Chemical Cross-linkers—Covalent chemical conjugation via ε-amines of optimally positioned lysine residues in preformed uPA-pro-uPA or uPAR-ATF complexes was introduced by various homobifunctional NHS esters with variable spacer arm lengths (DSS, ~11.4 Å; DSG, ~7.7 Å; and DST, ~6.4 Å). Concentrated stock solutions of these chemical cross-linkers were prepared in dry Me2SO and were diluted in Me2SO to the working solutions (5–20 mM) immediately prior to use. Noncovalent complexes between 4 nM 125I-labeled ATF and either 20 nM uPARwt or single-site lysine mutant uPAR were allowed to preform during a 60-min incubation at 4 °C in cross-linking buffer (0.1 M Tris, pH 8.1, containing 0.2% (w/v) CHAPS). Covalent cross-linking of the preformed complexes was accomplished by a 10–15 min incubation with the respective cross-linker (0.1–1 mM), after which the reaction was quenched by addition of 100 mM CH3COONH4. In other experiments, 4 nM 125I-labeled uPAR was subjected to chemical cross-linking to either pro-uPA356A or one of the 12 different single-site lysine mutants of pro-uPA. Cross-linking efficiency was visualized by SDS-PAGE of the reduced and alkylated samples, followed by autoradiography.

Zero-length Cross-linking of uPAR-GFD Complexes Using Carbodiimide—Monomeric uPAR (10 μM; prepared by size exclusion chromatography as describe above) was mixed with 90 μM GFD for 1 h at 4 °C to allow the formation of noncovalent uPAR-GFD complexes. Zero-length cross-linking between optimally positioned amino and carboxylate groups was achieved by additional incubation overnight at 20 °C with 5 mM EDC added from a freshly prepared stock solution in H2O. Cross-linking efficiency was assessed by SDS-PAGE of reduced and carbamidomethylated samples. The relevant Coomassie Brilliant Blue-stained bands were excised from the dried gel and subjected to in-gel trypsin digestion in parallel using either 18O- or 16O-enriched H2O as solvent. This solvent-induced “isotope coding” of tryptic peptides, which causes a specific mass shift of 8 Da for cross-linked peptides with two carboxyl termini (39),
facilitated the identification of these particular peptides by subsequent mass spectrometric analyses by nanoflow reverse-phase liquid chromatography connected to a quadrupole time-of-flight mass spectrometer (Q-Tof Ultima, Micromass, Manchester, UK). The further identification of such cross-linked peptides was enabled by the high mass accuracy and precision obtained using a 7-Tesla linear quadrupole ion trap-fourier transform ion cyclotron resonance mass spectrometer (LTQ FT\textsuperscript{TM}, Thermo Electron Corp., Bremen) with a modified nanoelectrospray ion source (Proxene Biosystems, Odense, Denmark).

Molecular Modeling of uPAR Complexes—Molecular modeling of uPAR-GFD complexes based on a convergence in the receptor-binding motif between the β-hairpin in the GFD of uPA and the α-helix of a linear peptide antagonist was performed essentially as described (33). In brief, a high-temperature molecular dynamics procedure was applied in which ambiguous constraints were introduced between the side chains of (i) Tyr\textsuperscript{24} in GFD and Val\textsuperscript{125}, Leu\textsuperscript{168}, His\textsuperscript{251}, Leu\textsuperscript{252}, Ala\textsuperscript{255}, and His\textsuperscript{466} in uPAR; (ii) Phe\textsuperscript{25} in GFD and Leu\textsuperscript{123}, Val\textsuperscript{125}, Leu\textsuperscript{250}, Leu\textsuperscript{468}, and His\textsuperscript{471} in uPAR; (iii) Ile\textsuperscript{28} in GFD and Leu\textsuperscript{55}, Leu\textsuperscript{123}, Arg\textsuperscript{53}, Leu\textsuperscript{123}, Leu\textsuperscript{150}, and Lys\textsuperscript{130} in uPAR; and (iv) Trp\textsuperscript{30} in GFD and Val\textsuperscript{29}, Leu\textsuperscript{31}, Leu\textsuperscript{55}, Tyr\textsuperscript{57}, and Leu\textsuperscript{66} in uPAR. 600 structures of complexes were generated, and the 300 with the best atom of GFD was introduced. 30 structures were refined, and Val\textsuperscript{29}, Leu\textsuperscript{31}, Leu\textsuperscript{55}, Tyr\textsuperscript{57}, and Leu\textsuperscript{66} in uPAR. 600 structures of complexes were generated, and the 300 with the best atom of GFD was introduced. 30 structures were refined, and the 300 with the best atom of GFD was superimposed onto that of the corresponding uPAR-GFD model. Distance constraint values were changed to serine, whereas Lys\textsuperscript{39} from uPAR was introduced in MODELLER Version 6.0 to position the kringle domain in the complex. The coordinates for the NMR-derived solution structure for human ATF (residues 6–135) and the crystal structure for human uPAR (residues 1–283) were used for these modeling studies (Protein Data Bank accession codes 1URK (43) and 1YWH (33), respectively).

RESULTS
Several laboratories have previously shown that a secreted form of human uPAR can be produced by various eukaryotic expression systems, e.g. Chinese hamster ovary cells (24, 44) and D. melanogaster S2 cells (18, 25, 34), after deletion of the carboxyl-terminal signal sequence, which is instrumental for the in vivo tethering of uPAR to the cell surface by a glycosphosphatidylinositol membrane anchor (1). This soluble recombinant uPAR (residues 1–283\textsuperscript{3}) represents a convenient surrogate for the glycosphosphatidylinositol-anchored uPAR, as in a range of biochemical assays, it retains many of the functional properties inherent to the native protein, including its high affinity for uPA (24, 32). In this study, we exploited Drosophila S2 cells as a host for the stable expression of a large number of uPAR mutants with defined single-site mutations covering almost 90% of the fully processed uPAR sequence (residues 1–283). To avoid compromising residues in uPAR that are of structural importance, we focused our mutagenesis efforts on residues that are not included in the consensus sequence defining the Ly-6/uPAR/α-neurotrophin protein domain family (26, 33, 45), i.e. all cysteines and the conserved Asn residue that immediately follows the last cysteine of each domain were omitted. The resultant 244 uPAR mutants were expressed well by the S2 cells, and the purities and yields of the mutant proteins were comparable with those of uPAR\textsuperscript{wt} (supplemental Fig. S1).

Impact of Single-site Mutagenesis in uPAR on Pro-uPA Binding Kinetics—To address the functional importance of specific amino acid side chains in human uPAR, we produced a comprehensive uPAR mutant library in which defined side chains of the soluble receptor were individually deleted beyond the C\textsuperscript{β} atom by alanine substitution. Native alanine residues in uPAR were changed to serine, whereas N-linked glycosylation sites were replaced with glutamine. The impact of these specific side chain deletions on the uPA interaction were assessed by measuring the kinetic rate constants for the interaction between immobilized pro-uPA\textsuperscript{SSS6A} and the respective purified uPAR single-site mutants by surface plasmon resonance. A complete inventory of these experimentally determined rate constants is provided in supplemental Table S1. During the course of this study, we observed that repeat determinations of the dissociation rate constant for the interaction between uPAR and pro-uPA\textsuperscript{SSS6A} were very reproducible over time and insensitive to cycles of freeze-thawing of the purified protein preparation. In contrast, the association rate constant could vary by as much as 2-fold under the same conditions. Because of the logistics of the present experimental setup comparing a very large number of purified uPAR mutants, it was, however, impossible to ensure...
Mapping the uPAR/uPA Interface

exact identical handling of all samples. In the following data evaluation, we therefore initially focused on $k_{\text{off}}$ values, which seemed reasonable, as this rate constant furthermore yielded the largest contribution to the global change in the Gibbs free energy of binding ($\Delta G$). The impact of specific side chain deletion primarily on $k_{\text{off}}$ rather than on $k_{\text{on}}$ is concordant with reports for other protein/protein interactions probed by alanine scanning mutagenesis (46, 47). The quality of the data evaluation for these interactions is illustrated in supplemental Fig. S3, showing the actual fits to the data of sensorgrams obtained for the interaction between uPARwt and immobilized pro-uPA356A. The dissociation time recorded for this particular interaction was considered adequate to identify uPAR mutants with impaired $k_{\text{off}}$ values caused by specific alanine replacements.

The $k_{\text{off}}$ values determined for the 244 different uPAR-pro-uPA complexes are illustrated by the histograms shown in Fig. 1 (uPAR domain I), Fig. 2 (uPAR domain II), and Fig. 3 (uPAR domain III). Each histogram shows threshold levels for the effects of the mutations, arbitrarily set to 2.5, 5, and 10 times the dissociation rate of uPARwt. As evident from Fig. 1, nine residues in uPAR domain I displayed a $\geq 2.5$-fold increase in the dissociation rate constant upon alanine substitution (i.e. Arg25, Thr27, Leu50, Lys50, Thr51, Arg53, Leu55, Tyr57, and Leu66), and two residues exhibited a $\geq 5$-fold increase in $k_{\text{off}}$ (Leu55 and Leu66). According to the crystal structure solved for uPAR in complex with a uPA antagonist peptide (33), all nine residues exhibit a surface accessibility of $>15 \, \text{Å}^2$ in the unoccupied receptor after removal of the antagonist. The involvement of some of these residues in uPA binding (Thr51, Arg53, Leu55, Tyr57, and Leu66) has been identified previously by alanine scanning mutagenesis and chemical protection analysis (23, 24).

A similar scanning of uPAR domain II identified 21 different residues with a $\geq 2.5$-fold increase in $k_{\text{off}}$ upon alanine replacement (Fig. 2 and supplemental Table S1). However, 10 of these residues (Ser97, Ser100, Leu113, Asp124, Leu144, Arg145, Gly146, Gly148, Phe165, and His166) do not pass the minimum threshold of 15 $\, \text{Å}^2$, which we have set for an acceptable surface accessibility. Of the remaining 11 residues in uPAR domain II that, according to these criteria, are important for uPA binding (Asp102, Ser104, Glu106, Val125, Thr127, Asp140, Asp141, His143, Leu150, Pro151, and Leu168), seven displayed a $\geq 5$-fold increase in $k_{\text{off}}$ (shown in boldface), and two of these displayed as much as 23-fold (Asp102) and 36-fold (Leu150) increases in $k_{\text{off}}$. This analysis clearly emphasizes the important role of uPAR domain II in uPA binding.

In contrast to uPAR domains I and II, very few residues in uPAR domain III were found to contribute to the stability of the uPAR-pro-uPA complexes (i.e. Met219, Gly227, and Phe256). In addition, these effects were at the lower end of the arbitrary threshold ($\sim 2.5$-fold increase in $k_{\text{off}}$) (Fig. 3) and were most likely due to local perturbations of uPAR domain III because none of these residues occupies a surface-exposed position in the structure ($<15 \, \text{Å}^2$ of surface accessibility). A compilation of these data highlighting residues in uPAR that we have identified as being important for uPA binding by single-site alanine scanning mutagenesis and surface plasmon resonance is shown in Table 1. This functional epitope for uPA binding is also highlighted in the surface representation of the uPAR structure shown in Fig 4.
Positioning the Kringle Domain in the uPAR/H18528 Pro-uPA Complex by Chemical Cross-linking—As chemical cross-linking has developed into a valuable tool for low resolution structural modeling (48, 49), we used this technique to establish distance constraints within the uPAR/H18528 pro-uPA complex.

Covalent conjugation of human uPAR to radiolabeled pro-uPA by the amine-specific NHS-based cross-linker DSS has been used as a sensitive tool for the detection of uPAR in various biological fluids and detergent lysates from neoplastic cell lines (1, 28, 50, 51). However, this cross-linking reaction is not observed when using murine proteins, despite an equivalently high affinity uPAR/uPA interaction (18, 50),
Mapping the uPAR/uPA Interface

TABLE 1
Kinetic rate constants for selected uPAR mutants with an impaired interaction with immobilized pro-uPA<sup>356A</sup> as determined by surface plasmon resonance

The interactions between immobilized pro-uPA (200–1000 resonance units) and the respective purified uPAR mutants were measured at 20 °C for serial 2-fold dilutions ranging from 1 to 100 nM uPAR and analyzed in three flow cells with different levels of immobilized ligand. Shown are the means for the rate constants $K_u$ and $K_d$ derived from these data by nonlinear least-squares curve fitting using BIA evaluation Version 4.1 software, but only residues experiencing a $\geq 2.5$-fold increase in $K_u$ and having a surface accessibility of $>15$ Å$^2$ are included. The $K_d$ was calculated from the means of the corresponding rate constants ($K_d = K_{calc}/K_u$). The change in the Gibbs free energy was calculated as $\Delta G = RT \ln(K_{calc}/K_u)$. For comparison, the accessibility derived from the crystal structure of uPAR is shown (33).

| uPAR     | $k_u$ ($10^{-5}$ M$^{-1}$ s$^{-1}$) | $k_d$ ($10^{-4}$ s$^{-1}$) | $K_u$ (nM) | $K_d$ (nM) | $\Delta G$ (kcal/mol) | Access (Å$^2$) |
|----------|---------------------------------|--------------------------|------------|------------|----------------------|----------------|
| wt       | 3.98 ± 0.98                     | 1.77 ± 0.31              | 0.46 ± 0.11| 11.4 Å     |                      |                |
| R25A     | 2.72                            | 6.95                     | 2.56       | 1.00       | 38.9                 |                |
| T27A     | 3.12                            | 6.69                     | 2.14       | 0.90       | 21.8                 |                |
| L40A     | 2.24                            | 4.38                     | 1.96       | 0.85       | 84.0                 |                |
| K50A     | 4.24                            | 5.76                     | 1.36       | 0.63       | 45.3                 |                |
| T51A     | 5.60                            | 8.58                     | 1.53       | 0.70       | 49.9                 |                |
| R53A     | 4.22                            | 8.68                     | 2.06       | 0.87       | 43.6                 |                |
| L55A     | 3.02                            | 9.04                     | 3.00       | 1.09       | 41.0                 |                |
| Y57A     | 1.78                            | 6.23                     | 3.49       | 1.18       | 50.5                 |                |
| L66A     | 2.26                            | 10.4                     | 4.70       | 1.35       | 59.9                 |                |
| D102A    | 5.07                            | 40.4                     | 7.16       | 1.66       | 120.4                |                |
| S104A    | 6.88                            | 8.89                     | 1.29       | 0.60       | 70.5                 |                |
| E106A    | 3.41                            | 6.20                     | 1.82       | 0.80       | 125.3                |                |
| V125A    | 3.33                            | 5.79                     | 1.74       | 0.78       | 25.1                 |                |
| T127A    | 4.13                            | 10.3                     | 2.50       | 0.99       | 17.2                 |                |
| D140A    | 3.79                            | 15.5                     | 4.10       | 1.28       | 124.5                |                |
| D141A    | 2.58                            | 6.97                     | 2.70       | 1.03       | 60.0                 |                |
| H143A    | 8.90                            | 12.0                     | 1.35       | 0.63       | 39.7                 |                |
| L150A    | 8.41                            | 64.0                     | 7.61       | 1.64       | 20.0                 |                |
| P151A    | 5.37                            | 7.17                     | 1.26       | 0.59       | 29.4                 |                |
| L168A    | 6.13                            | 9.92                     | 1.62       | 0.73       | 20.6                 |                |

suggesting the absence of optimally positioned lysine side chains in the noncovalent complex.

The observation that mutation of any of the 10 lysine residues in human uPAR did not have a pronounced impact on the affinity for pro-uPA (supplemental Table SI) enabled us to identify the lysine residues involved in the cross-linking reaction. In these experiments, complexes were formed using saturating amounts of the unlabeled mutant component (in this case, uPAR). The noncovalent uPAR-ATF complexes were conjugated by a short exposure to various NHS-based cross-linkers with different spacer arm lengths (DSG, ~11.4 Å; DSG, ~7.7 Å; and DST, ~6.4 Å). The short exposure to the active NHS ester ensured that only optimally positioned lysines were cross-linked. The autoradiograph in Fig. 5 shows that all mutants except uPAR<sup>R43K</sup> formed covalent complexes with 125I-labeled ATF under these conditions with DSG as cross-linker. Similar results were also obtained with DSS and DST (data not shown), illustrating that the e-amino group of Lys<sup>R43</sup> in uPAR domain I can be positioned within 6.4–11.4 Å of its target amine in ATF. The difference in susceptibility among the various uPAR lysine mutants for chemical conjugation to ATF cannot be ascribed to different levels of noncovalent complexes, as the mutants have comparable $K_d$ values for pro-uPA (supplemental Table SI), and preincubation using concentrations well above the $K_d$ ensured that equal levels of noncovalent complexes were formed.

Identification of Lys<sup>R43</sup> as the specific cross-linking site in human uPAR provides a rational structural basis for the observed cross-species barrier in the cross-linking potential of amine-reactive esters because a non-reactive arginine residue occupies the equivalent position in murine uPAR. To confirm this, we introduced a lysine at this position in murine uPAR and created a gain-of-function mutant, as the resultant murine uPAR<sup>R43K</sup> now efficiently formed a covalent complex with murine ATF using DSS (Fig. 6, eighth lane versus seventh lane). The affinity of murine uPAR<sup>R43</sup> and uPAR<sup>R43K</sup> for murine pro-uPA was comparable as judged by surface plasmon resonance studies (data not shown). Although very faint, the band in the autoradiograph in Fig. 6 (fourth lane) clearly shows that murine uPAR<sup>R43K</sup> also gained some cross-linking potential for human ATF. It is possible that the chemical conjugation efficiency between murine uPAR<sup>R43K</sup> and human ATF even resembles the one obtained in the pure human system, as saturation is by far reached under these conditions because of a >300-fold reduction in affinity for the noncovalent complex formation in the mixed-species experiment (18). This suggests that the corresponding target site in ATF probably is conserved between these two species and that the species specificity in cross-linking is entirely governed by the lysine-to-arginine substitution at position 43 in the receptor.

To identify the corresponding target site(s) in pro-uPA for these amine-reactive cross-linkers, we expressed and purified 12 pro-uPA single-site mutants, targeting all lysine positions in the non-catalytic modular part of uPA corresponding to ATF (supplemental Fig. S2). All these pro-uPA mutants displayed unaltered binding kinetics for immobilized uPAR<sup>wt</sup> as assessed by surface plasmon resonance, with the exception of pro-uPA<sup>R23A</sup>, which exhibited a moderately reduced affinity (52) (data not shown). Cross-linking experiments were performed as described above using excess unlabeled mutants (in this case, pro-uPA). As shown in Fig. 7, pro-uPA<sup>K98A</sup> clearly encountered the more severe impact on its cross-linking competence as evidenced by both reduced complex formation and accumulation of unreacted 125I-labeled uPAR. As previously inferred from the reverse cross-linking experiments described above, the target site in pro-uPA (Lys<sup>98</sup>) is indeed conserved between man and mouse.

Cross-linking uPAR-GFD Complexes by EDC—To provide additional and independent intermolecular distance constraints within the uPAR-GFD complex, we also performed zero-length conjugation of preformed complexes between uPAR<sup>wt</sup> and GFD-His using EDC. The traditional stabilization with NHS was omitted in this case to avoid stable “dead-end” modifications of carboxylate groups in either of the proteins. As evident from the experiment shown in Fig. 8 (lanes 1), monomeric uPAR<sup>wt</sup> underwent extensive oligomerization during EDC-induced cross-linking. Occupancy of uPAR by GFD-His completely prevented this receptor oligomerization, which was replaced with a specific conjugated uPAR-GFD complex (Fig. 8, lanes 2). These complexes contained one molecule of each protein as revealed by MALDI mass spectrometry (data not shown). Limited chymotrypsin treatment of the cross-linked complexes, leading to cleavage between uPAR domains I and II after Tyr<sup>R47</sup> (28, 44), clearly revealed that uPAR domain I was by far the major partner for the intermolecular cross-linking (Fig. 8, lanes 3). Specific cross-linking sites were subsequently identified by mass spectrometry of the paired samples after parallel in-gel hydrolysis by trypsin using solvents enriched in 18O- and 18O-labeled H$_2$O. As evident from the masses deter-
mined for tryptic uPAR peptides 31–43 and 117–139 (containing several glutamic and aspartic acids), the present EDC cross-linking procedure did not create unproductive dead-end modifications of the carboxylates (Table 2). So far, we have assigned a single intermolecular cross-link between uPAR and GFD. This was introduced between the $^{/}H9251$-amino group of Ser1 in GFD and the carboxylate.

**FIGURE 4.** Surface topology of the functional epitope on uPAR for uPA binding. The structure of human uPAR determined by x-ray crystallography in complex with a peptide antagonist is shown as a surface representation after removal of the peptide (33). All residues subjected to alanine scanning are shown in *wheat*, whereas the residues defining the consensus sequence for the Ly-6/uPAR modules are shown in *white*. To highlight the functional epitope on uPAR for uPA binding, the residues selected as being important for this interaction (Table 1) are shown in *red* ($\Delta\Delta G > 1$ kcal/mol) and *blue* ($\Delta\Delta G = 0.5–1$ kcal/mol). The carbohydrate moieties defined by the crystal packing in the x-ray structure are shown in *green*. A shows the front of uPAR with the distinct ligand-binding cavity; B shows this cavity at a higher magnification; and C shows the back of uPAR. The glycolipid attachment site is indicated (glycosylphosphatidylinositol (GPI)). These images were generated with PyMOL (DeLano Scientific) using Protein Data Bank code 1YWH for uPAR molecule E in the unit cell for the octamer (33).

**FIGURE 5.** Defining the target lysine in uPAR for chemical cross-linking of uPAR-ATF complexes using homobifunctional NHS esters. This autoradiogram shows a comparison of the cross-linking capacity of purified uPAR$^{wt}$ and the 10 single-site alanine mutations that individually target all lysines present in human uPAR. Preformed complexes between 4 nM $^{125}$I-labeled ATF (residues 1–135) and 20 nM uPAR (residues 1–283) were exposed to 400 $\mu$M DSG (range of 7.7 Å) for 10 min at room temperature before the reaction was quenched. After reduction and alkylation, these samples were subjected to SDS-PAGE, followed by autoradiography.

**FIGURE 6.** Structural basis for the species-specific cross-linking selectivity of homobifunctional NHS esters. Shown is a comparison of the capacities of human (*h*) and murine (*m*) uPAR$^{wt}$ and uPAR$^{K43A}$ to cross-link human ATF (residues 1–135) (*left panel*) and murine ATF (residues 1–143) (*right panel*). In this case, 8 nM $^{125}$I-labeled ATF and 40 nM uPAR were incubated for 60 min on ice before the chemical cross-linking was initiated by addition of 1.5 mM DSS (range of 11.4 Å), and conjugation was allowed to proceed for 15 min at room temperature. Reduced and alkylated samples were analyzed by SDS-PAGE, followed by autoradiography. Traces of dimeric and trimeric murine ATFs are evident in the *right panel*. 

}(\Delta\Delta G = 0.5–1 \text{ kcal/mol})
The latter was subjected to limited proteolysis by 12 nM chymotrypsin to tryptic fragment in our purified conjugates (Table 2). Molecular cross-links must be present in the uPAR domain I (Fig. 8). However, additional unidentified inter-domain linkers were found in both our cross-linked samples containing either intact uPAR or uPAR domain I (21 residues), thus providing a structural basis for the composite nature of the uPA-binding site in uPAR. Although the alanine scanning mutagenesis did not reveal any important role for uPAR domain III in uPA binding, this domain nevertheless appears to play an auxiliary role in this high affinity interaction. This supposition is based on the observation that neither uPAR domain I (32) nor uPAR domains I and II (53) can form very stable complexes with GFD as opposed to either intact uPAR or a mixture containing uPAR domain I and uPAR domains II and III. This phenomenon most likely reflects a stabilizing role of uPAR domain III in the assembly of a functional ligand-binding cavity in the three-domain uPAR.

A significant body of biochemical data exists in the literature to highlight the role of uPAR domain I in uPA binding (23, 24, 28, 32, 44). This relationship is further substantiated by this study, which identified new residues involved in uPA binding (Arg25, Thr27, and Leu40) and which validated the importance of previously assigned residues (Thr31, Arg35, Leu55, Tyr57, and Leu66). Interestingly, the side chains of these residues line a significant fraction of the “right” wall of the central ligand-binding cavity of uPAR (Fig. 4) and are defined by the crystal structure of uPAR in complex with a peptide antagonist (33).

The present alanine-scanning mutagenesis also unexpectedly uncovered that uPA binding is particularly sensitive to mutagenesis in uPAR domain II (Fig. 2), where 11 surface-exposed residues were considered to be important. The involvement of this domain in uPA binding was previously inferred primarily from indirect evidence. Proteolytic cleavage of the linker region between uPAR domains I and II thus led to a >1500-fold reduction in affinity for uPA equivalent to $\Delta \Delta G \approx 4$ kcal/mol (32). In a mutagenesis study, Bdeir et al. (25) focused on uPAR domain II, but only interrogated five residues in this domain and reported that the double mutation K139A/H143A caused a 5-fold reduction in $k_{\text{off}}$. This finding is concordant with our data that uPARK139A/H143A displayed a similar decrease in $k_{\text{off}}$. Bdeir et al. also reported that the triple mutation R137A/R142A/R145A had a severe impact on uPA binding, which was predominantly enforced by a reduction in $k_{\text{on}}$. With a view to our present data, this effect is most likely caused by structural perturbation because, of the corresponding single mutants, only R145A had a significant effect on uPA binding (Fig. 2 and supplemental Table S1) and because Arg455 has a surface accessibility of only 0.5 Å² in our x-ray structure (33).
The importance of uPAR domain II in coordinating the assembly of a functional ligand-biding cavity in the intact uPAR is also highlighted by the observation that several buried residues in this domain have a severe impact on uPA binding when mutated individually (Fig. 2). One β-strand in particular exhibits a very low tolerance to mutagenesis, i.e. βIIID in uPAR domain II (residues 142–149). We have previously proposed that this bent β-strand plays a key role in the internal dynamics of this modular receptor by acting as a hinge centered on the RGC sequence and that this hinge enables movement between domains I and II; the angle between these domains varies from 107.8° to 112.2°, as observed in our crystal structure (33). Impairment of this interdomain flexibility through structural perturbations imposed by mutagenesis in βIID is therefore likely to affect the architecture of the central cavity in uPAR, with resultant effects on uPA binding to the composite functional epitope comprising residues from both uPAR domains I and II.

Examination of the ΔΔG values presented in Table 1 reveals that no single side chain has the predominant contribution to the free energy of uPA binding, i.e. typical “hot spots” (54) are absent from the receptor interface with uPA. This property may be related in part to the architecture of the uPA-binding site, where the engagement of the deep central cavity is governed by a flexible surface comprising hydrophobic and aliphatic residues. Alanine scanning mutagenesis of uPAR will therefore not significantly affect the hydrophobic nature of this ligand interface and will be accompanied by compensatory effects that may attenuate the impact observed on the ΔΔG values. Nevertheless, the present alanine scanning mutagenesis clearly demonstrated the important role of several hydrophobic amino acid residues from uPAR domains I and II, which line the central cavity (Fig. 4). As illustrated in Fig. 4B, two tracks of residues involved in uPA binding seem to irradiate from Leu150 at the floor of the cavity to either Asp102 or Asp340 at the rim of this gorge. Notably, no residue causing a shift in ΔΔG > 1 kcal/mol is found exposed on the opposite side of uPAR, and the only residue in this region causing an intermediate effect (T51A) is localized close to the entrance of the binding cavity (Fig. 4C).

On the basis of these biochemical data, we have now built a model for human uPAR in complex with the ATF of uPA in which the kringle domain is positioned by the low resolution distance constraint established by our cross-linking experiments (Fig. 9). This model merges the information derived from the present experiments with our previous proposal of a convergent receptor-binding site displayed by the β-hairpin in the GFD of uPA and a linear peptide antagonist developed by combinatorial chemistry (18, 33). The long β-hairpin of GFD engages the deep central cavity in uPAR, and the resultant interface buries 1629 Å² of accessible surface area of uPA and 1318 Å² of uPAR in the complex. Remarkably, in this model, the complementary receptor/ligand interface of the uPAR-GFD complex is highly asymmetrical. In uPAR, 27 residues participate in the formation of this interface, but none of the individual contributions actually exceed 6% of the total buried accessible surface area. As opposed to this, only four residues in GFD provide ~40% of the buried surface (Lys27, Tyr24, Phe25, and Trp30). This distinction in geometry may have a bearing on the determined ΔΔG values after mutagenesis, where no real hot spots are found among the many residues forming the large hydrophobic cavity in uPAR.

The intimate engagement of the β-hairpin of GFD in the interaction with the central cavity of uPAR provides a plausible binding mechanism that amalgamates the majority of the biochemical data reported in the literature for this interaction into one unifying model. First, the location of the GFD-binding site at a composite interface involving several residues in both uPAR domains I and II lining the central cavity explains why the maintenance of the three-domain structure of uPAR is required to yield high affinity uPA binding (32, 53). This architecture also provides an explanation for the observation that the isolated GFD module can induce the specific assembly of a stable trimolecular complex comprising uPAR domain I, GFD, and uPAR domains II and III, whereas all the corresponding bimolecular complexes are very short-lived. A similar property has been reported for ATF (55). Second, the model buries the amino acid side chains in both uPAR and uPA that become protected against chemical modification upon complex formation at the interface of the complex, i.e. Tyr27 in uPAR and Lys273 and Tyr24 in uPA (23, 26). Third, residues highlighted as being important for this interaction by single-site mutagenesis are also confined to the β-hairpin of GFD (52) or, in the case of

### TABLE 2

| uPAR sequence | GFD sequence | Determined peptide mass | Calculated peptide mass | Δm | Δm (18O/16O) | Cross-link (uPAR-GFD) | Identified in sample |
|---------------|--------------|-------------------------|-------------------------|-----|---------------|----------------------|---------------------|
| 8–13          | 1–23         | 3294.3990               | 3294.3869               | 0.0121 | 8            | Asp11-Ser3           | 1, 2                |
| 117–139       | 2723.2742    | 2723.2730               | 0.0011                 | 4    | None          | None                 | 1, 3                |
| 31–43         | 1602.7885    | 1602.7897               | -0.0012                | 2    | None          | None                 | 1–4                 |

* Accurate peptide masses were determined by nanoflow liquid chromatography/electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry.
* Mass differences observed between peptides derived from in-gel trypsin cleavage in 18O- and 16O-enriched solvents were determined by nanoflow liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry.
Mapping the uPAR/uPA Interface

uPAR, are located within or at the rim of the central ligand-binding cavity (Fig. 4) (24). Two exceptions to this are Asp\textsuperscript{102} and Asp\textsuperscript{140}, which are located somewhat distantly from the docked GFD module at the rim of the central cavity in uPAR (Fig. 9). One likely explanation for this apparent inconsistency relates to the mobility of these residues because both exhibit B-factors above the average for uPAR, and Asp\textsuperscript{102} in particular is located in a flexible region of loop 1 in uPAR domain II that is relatively less well defined in the structure (33).

The kringle domain of uPA is positioned on the outside of uPAR domain I in this model, causing ATF to virtually embrace this domain in a “loose-fitting sandwich” (Fig. 9). This conformation places the ε-amino groups of uPAR Lys\textsuperscript{43} and uPA Lys\textsuperscript{98} within the deduced cross-linking distance. However, it should be emphasized that the entire range of spacer arm lengths for the homobifunctional cross-linkers (6.4–11.4 Å) used in this study leads to a specific productive conjugation of uPAR to pro-uPA, which indicates certain flexibility in the position of the kringle domain of uPA in relation to uPAR. Such a flexible and transient nature of the interplay between the kringle domain and uPAR is compatible with the observation that GFD and the kringle domain behave as completely independent modules in the NMR-derived structure of ATF (43) and with our previous observation that GFD and ATF exhibit comparable kinetic rate constants for the interaction with uPAR (56). The proximity of the flexible amino terminus of GFD to Asp\textsuperscript{11} in uPAR domain I established by zero-length cross-linking is also compatible with the proposed model of the uPAR-ATF complex. Finally, our model of the uPAR-ATF complex provides insight into the molecular basis for the competitive inhibition exerted by anti-uPAR monoclonal antibody R3 on the uPAR/uPA interaction. Although the functional epitope for antibody R3 (i.e. Glu\textsuperscript{33}, Leu\textsuperscript{61}, and Lys\textsuperscript{62}) (24, 26) does not coincide with that determined here for uPA, it is clear from the model that the corresponding structural epitopes must overlap to such an extent that steric hindrance would preclude concomitant binding of ATF and antibody R3 (Fig. 9).

During the revision of this manuscript, the crystal structure of human uPAR in complex with ATF and a Fab fragment of an anti-uPAR monoclonal antibody was published (57). This structure nicely confirms the overall positioning of uPAR and ATF in our model, where the β-hairpin of GFD engages the central ligand-binding cavity of uPAR and where the kringle domain is located close to uPAR domain I. Notably, the distance in the crystal structure between Lys\textsuperscript{43} in uPAR and Lys\textsuperscript{98} in the kringle domain of ATF (10.8 Å) is indeed within the range defined by the chemical cross-linkers employed in this study. However, one important distinction between the crystal structure of uPAR in complex with ATF and a Fab fragment of an anti-uPAR monoclonal antibody was published (57). This structure nicely confirms the overall positioning of uPAR and ATF in our model, where the β-hairpin of GFD engages the central ligand-binding cavity of uPAR and where the kringle domain is located close to uPAR domain I. Notably, the distance in the crystal structure between Lys\textsuperscript{43} in uPAR and Lys\textsuperscript{98} in the kringle domain of ATF (10.8 Å) is indeed within the range defined by the chemical cross-linkers employed in this study. However, one important distinction between the crystal structure of uPAR in complex with ATF and a Fab fragment of an anti-uPAR monoclonal antibody was published (57). This structure nicely confirms the overall positioning of uPAR and ATF in our model, where the β-hairpin of GFD engages the central ligand-binding cavity of uPAR and where the kringle domain is located close to uPAR domain I.
Combined with the recently published crystal structure of the uPAR-ATF complex, the present functional study thus provides some of the structure-function relationships that are required to understand the molecular mechanisms controlling the interactions between uPAR and its biological ligands such as pro-uPA, vitronectin, and certain integrins. This information may also prove essential for "decoding" the well established species barrier of both the uPAR/uPA interaction and the selective inhibition of this interaction by peptide antagonists. The pronounced species barrier between man and mouse is a ""...""
Mapping the uPAR/uPA Interface

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