Mapping Part of the Functional Epitope for Ligand Binding on the Receptor for Urokinase-type Plasminogen Activator by Site-directed Mutagenesis*

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The urokinase-type plasminogen activator receptor (uPAR) is a glycolipid anchored multidomain member of the Ly-6/uPAR protein domain superfamily. Studies by site-directed photoaffinity labeling, chemical cross-linking, and ligand-induced protection against chemical modification have highlighted the possible involvement of uPAR domain I and particularly loop 3 thereof in ligand binding (Ploug, M. (1998) Biochemistry 37, 16494–16505). Guided by these results we have now performed an alanine scanning analysis of this region in uPAR by site-directed mutagenesis and subsequently measured the effects thereof on the kinetics of uPA binding in real-time by surface plasmon resonance. Only four positions in loop 3 of uPAR domain I exhibited significant changes in the contribution to the free energy of uPA binding (ΔΔG ≥ 1.3 kcal mol⁻¹) upon single-site substitutions to alanine (i.e. Arg⁵³, Leu⁵⁵, Tyr⁵⁷, and Leu⁶⁶). The energetic impact of these four alanine substitutions was not caused by gross structural perturbations, since all monoclonal antibodies tested having conformation-dependent epitopes on this domain exhibited unaltered binding kinetics. These sites together with a three-dimensional structure for uPAR may provide an appropriate target for rational drug design aimed at developing new receptor binding antagonists with potential application in cancer therapy.

The urokinase-type plasminogen activator receptor (uPAR)³ is a multifunctional membrane glycoprotein primarily involved in the regulation of pericellular proteolysis due to its high affinity interaction with the growth factor-like module of urokinase-type plasminogen activator (uPA) (1), but which has also been implicated in the promotion of cell adhesion due to its vitronectin and integrin binding properties (2), in signal transduction (3) and chemotaxis (4, 5). However, the uPA-uPAR interaction per se is intimately coupled to the latter “non-proteolytic” functions of uPAR, since this interaction either elicits or modulates these events. Cell surfaces expressing uPAR constitutes favored microenvironments for uPA-mediated plasminogen activation (6). Consequently, the uPA-uPAR interaction represents an attractive molecular target for the development of small receptor binding antagonists that may prove useful during treatment of certain diseases in which uPAR has been implicated, i.e. cancer invasion and metastasis (7–13).

uPAR is a glycosylphosphatidylinositol-anchored plasma membrane protein (14) having an extracellular part composed of three homologous domains belonging to the Ly-6/uPAR protein domain family,² as reviewed (15). This family is dominated by single domain proteins among which the glycolipid-anchored members are found primarily in mammalians (i.e. CD59, Ly-6, E48, and ThB) whereas the secreted members belong to either reptiles or amphibians (i.e. α-neurotoxins, fasciculins, cardiotoxins, and xenoxins). Intriguingly, a similar phylogenetic relationship exists for the few family members identified so far containing two Ly-6/uPAR domains, i.e. the glycolipid anchored BoBo-1 (16) and metastasis-associated C4.4 (17) isolated from rat versus the secreted phospholipase A2 inhibitor isolated from cobra blood (18). Comparison of the three-dimensional protein structures available for several single domain members of this protein family reveals a “three finger” consensus structure consisting of 3 loops, a central 3-stranded β-sheet, and a globular, disulfide-rich core (19–21). The individual domains of uPAR (numbered I, II and III) are thought to adopt a similar “three finger fold” (15).

The involvement of uPAR domain I (residues 1–87)³ in uPA binding is demonstrated by several lines of evidence. First, uPAR domain I can be specifically cross-linked to a receptor binding derivative of uPA using two different chemical cross-linking reagents (23, 24). Second, the uPA-uPAR interaction can be inhibited competitively by monoclonal antibodies recognizing epitopes on uPAR domain I (25–27). Third, uPAR domain I is also a target for the specific photoinsertion from a small peptide antagonist of uPA binding (28). Specific proteolytic cleavage after Tyr⁶⁷, situated in the linker region between domains I and II, is, however, also accompanied by a >1,500-fold reduction in the affinity for uPA (ΔΔG > 4 kcal/mol), which clearly emphasizes the necessity of the multidomain structure of uPAR for high affinity ligand binding (27, 28). Previous attempts at dissecting the ligand interaction site on uPAR have highlighted the importance of loop 3 in uPAR domain I, since Arg⁵³, Tyr⁵⁷, and Leu⁶⁶ reside at the receptor-ligand interface as assessed by either photoaffinity labeling (29) or by ligand-induced protection against chemical modification with tetrami-

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The abbreviations used are: uPAR, uPA receptor; ATF, amino-terminal fragment of uPA; MALDI-MS, matrix-assisted laser desorption ionization mass spectrometry; s-uPAR, soluble uPAR; uPA, urokinase-type plasminogen activator; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

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tromethane (30). Guided by these data we have performed an alanine scanning analysis of this region (residues 47–70) of uPAR by site-directed mutagenesis enabling us to discriminate between structural and functional epitopes of ligand-binding, i.e. residues present at the interface versus those also making a productive contribution to the free energy of ligand-binding (31–33).

MATERIALS AND METHODS

Chemicals and Reagents—Pfu DNA polymerase (EC 2.7.7.7) was from Stratagene (La Jolla, CA). HPLC-purified DNA oligonucleotides were purchased from DNA Technology (Aarhus, Denmark). Activated-enzyme chain uPA (EC 3.2.2.21) was purchased from Stratagene. Overnight cultures of E. coli were grown at 37 °C in a humid atmosphere with 5% CO2 in minimal medium with ribo- and deoxyribonucleotides and GlutaMAX (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 800 μg/ml G418. The recombinant pro-uPA expressed in mammalian expression vector (Promega) as a soluble truncated soluble form of human uPAR (denoted suPAR) and encompassing amino acids 1–283 was constructed utilizing two separate PCR performed by standard PCR techniques using Pfu DNA polymerase (Fig. 1). In the first reaction, generating a domain I encoding fragment, a SpeI site was introduced in front of the non-coding 5′-end, and an XhoI site was added at position 394 as above, and a TAA stop codon, followed by an EcoRI site, was introduced at position 962 in the 3′-end. Finally, the two PCR products from these reactions were cut with the appropriate restriction enzymes, ligated, and cloned into the Nhel (SpeI compatible) and EcoRI sites of the mammalian expression vector pCI-neo (Promega) as a SpeI-EcoRI fragment giving the plasmid pCI-neo/suPAR.

Mutations were introduced into suPAR domain I by PCR using a one-tube-based modification of the megaprimer procedure which employs Pfu DNA polymerase (34). In brief, the procedure requires three oligonucleotide primers: two flanking primers, which are upstream and downstream of the mutation site, and one mutagenic primer (Table I) designed to contain at least 10 perfectly matched bases at both the 5′- and 3′-end. To that end, uPAR domain I was flanked by SpeI and XhoI sites as above and cloned into pBluescript (Stratagene), as shown in Fig. 1. This plasmid (pBluescript/shuPAR-D1) was used as a template with T3 and T7 primers as flanking primers for the production of PCR containing the desired mutation. Mutated domain I PCR fragments were cloned as cassettes into the Nhel and XhoI sites of pCI-neo/suPAR depleted for wild-type domain I. The suPAR-W129A mutant in domain II was constructed using pCI-neo/suPAR wild-type DNA as template in a site-directed mutagenesis PCR using flanking primers residing in domain I and III, respectively. The W129A mutant PCR fragment was cloned as a cassette into XhoI and ApaI sites of pCI-neo/suPAR depleted for wild-type domain II. Prior to transfection PCR-generated sequences of all constructs were confirmed by DNA sequencing.

Transfection, Expression, and Purification of suPAR Mutants—Chinese hamster ovary cells were transfected with expression vectors using a calcium phosphate precipitation procedure (Stratagene). After 48 h, selection, clones were picked, propagated, and the conditioned medium tested for the production of recombinant suPAR by enzyme-linked immunosorbent assay (35) using anti-uPAR monoclonal antibody R2 recognizing domain III as detecting antibody. The cells were maintained as monolayers at 37 °C in a humid atmosphere with 5% CO2 in minimum essential medium supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 800 μg/ml G418. The harvest fluid contained between 0.01 and 0.5 μg/ml recombinant protein (Table I).

The suPAR mutants were purified by immunoaffinity chromatography from the conditioned media as described previously (36) followed by reverse-phase HPLC using a Brownlee Aquapore C4 column and a linear gradient (40 min) from 0 to 70% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate 250 μl min−1. Purified mutants were dissolved in phosphate-buffered saline after solvent evaporation and were either quantified spectrophotometrically using E280 nm = 9.2 (37) or by quantitative amino acid analysis (mutants containing tryptophan substitutions). Purity was assessed by SDS-PAGE followed by silver staining and judged to be >95%.

Verification of Mutations in suPAR Domain I by MALDI-MS—To ascertain that the desired mutation in suPAR domain I had occurred at the protein level and to analyze the consequence of the mutations on the processing of the glycans moieties attached to Asn57 (38), the NH2-terminal domain I (residues 1–87) was liberated from suPAR by limited proteolysis using chymotrypsin and subsequently purified by size exclusion chromatography using 0.1 M NH4HCO3 as solvent (36). The mass of purified domain I was determined by MALDI-MS before and after treatment with N-glycanase (5 milliunits/ml for 60 min at 37 °C) and compared with that of wild-type uPAR domain I (38), see Table I.

Real Time uPA Binding Kinetics of suPAR Mutants Measured by Surface Plasmon Resonance—The kinetics of the interaction between suPAR (wild-type as well as mutants) and immobilized uPA, pro-uPA, or various monoclonal anti-uPAR antibodies were measured in real-time by surface plasmon resonance using a BIAcore 2000™ equipment (Pharmacia Biosensor, Uppsala, Sweden). A carboxymethylated dextran matrix (CMS sensor chip) was preactivated with N-hydroxysuccinimide/N-ethyl-N3-(dimethylaminopropyl)carbodiimide according to the manufacturer’s recommendations. Random amine coupling of the respective ligands was achieved by subsequent injection of uPA, pro-uPA (each 20 μg/ml), or a monoclonal anti-uPAR antibody (50 μg/ml) in 10 mM sodium acetate, pH 5.0, at a flow rate of 5 μl min−1 for 2 min. Sensorgrams (resonance units versus time) were recorded by the BIA-
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| Mutant | Mutagenic primers* | Molecular mass | Glycosylation of Asn52 | Yield** |
|--------|--------------------|----------------|----------------------|---------|
| wt (1-283) | None | 9,754.78 (0.7) | + | 45 |
| W32A | ATCGTGCGCTTGAGCAGAAGAAGAGA | 9,638.66 (0.3) | + | 24 |
| H47A | AACAGGAGTCAGGCTGAGAAGAAGAGA | 9,691.99 (3.0) | + | 86 |
| E49A | CGACACTGTCAGGCTGAGAAGAAGAGA | 9,696.97 (0.9) | + | 113 |
| K50A | AGCACATGTCAGGCTGAGAAGAAGAGA | 9,709.97 (4.0) | + | 146 |
| T51A | CACTGTCAGGCTGAGAAGAAGAAGAGA | 9,721.83 (2.2) | + | 53 |
| T52Q | TACGAGGAGTCAGGCTGAGAAGAAGAGA | 9,766.81 (0.3) | + | 21 |
| R53A | GAGAAGGAGTCAGGCTGAGAAGAAGAGA | 9,666.64 (2.3) | + | 40 |
| T54A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,721.39 (1.7) | + | 72 |
| L55A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,710.80 (1.2) | + | 68 |
| S56A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,738.55 (1.5) | + | 128 |
| Y57A | AGAAGGTGTCAGGCTGAGAAGAAGAGA | 9,661.97 (3.0) | + | 79 |
| R58A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,668.96 (0.6) | + | 76 |
| T59A | CTAGAGGAGTCAGGCTGAGAAGAAGAGA | 9,725.14 (1.1) | + | 24 |
| L61A | TACGAGGAGTCAGGCTGAGAAGAAGAGA | 9,709.54 (2.5) | + | 174 |
| K62A | CGGACAGGAGTCAGGCTGAGAAGAAGAGA | 9,698.49 (1.5) | + | 42 |
| I63A | ACTGCTGTCAGGCTGAGAAGAAGAGA | 9,711.18 (0.8) | + | 36 |
| T64A | GGCTGTCAGGCTGAGAAGAAGAAGAGA | 9,724.40 (0.4) | + | 59 |
| S65A | TTAGACGTGACGCTGAGAAGAAGAAGAGA | 9,735.84 (0.8) | + | 219 |
| L66A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,711.99 (1.6) | + | 37 |
| T67A | AAGAACAGGAGTCAGGCTGAGAAGAAGAGA | 9,725.75 (1.7) | + | 6 |
| E68A | CACGCTGTCAGGCTGAGAAGAAGAAGAGA | 9,696.03 (0.7) | + | 15 |
| V69A | GCTCTGTCAGGCTGAGAAGAAGAAGAGA | 9,728.06 (2.1) | + | 115 |
| V70A | TATCGGCTGACGCTGAGAAGAAGAAGAGA | 9,724.29 (1.7) | + | 92 |
| W79A | CTCTGTCAGGCTGAGAAGAAGAAGAGA | 9,711.86 (0.8) | + | 88 |
| R53L | GAGAAGGAGTCAGGCTGAGAAGAAGAGA | 9,726.17 (1.2) | + | 152 |
| R53K | AGAAGACAAAGAGGAGTCAGGCTGAGAAGAAGAAGAGA | 9,770.98 (1.1) | + | 81 |
| L55V | AAGAACAGGAGTCAGGCTGAGAAGAAGAAGAGA | 9,742.60 (2.6) | + | 28 |
| Y75W | GAGGCTGTCAGGCTGAGAAGAAGAAGAGA | 9,779.90 (2.8) | + | 279 |
| Y75F | GAGGCTGTCAGGCTGAGAAGAAGAAGAGA | 9,727.07 (0.6) | + | 179 |
| Y75K | AGACCTGTCAGGCTGAGAAGAAGAAGAGA | 9,721.09 (2.0) | + | 318 |
| Y75E | AGACCTGTCAGGCTGAGAAGAAGAAGAGA | 9,720.80 (0.8) | + | 8 |
| Y75H | AGACCTGTCAGGCTGAGAAGAAGAAGAGA | 9,729.27 (1.2) | + | 162 |

* Sequences of the synthetic oligonucleotide primers are shown from 5’ to 3’ with the mutated codons underlined by underlined bold letters.

** The molecular masses were determined by MALDI-MS for chymotrypsin released domain I (residues 1–87) deglycosylated by N-glycanase. The mutants Asn52→Gln and Thr52→Ala were untreated since these point mutations compromise the sole N-linked glycosylation motif present in domain I (Asn-Arg-Thr). The deviation between the determined mass and the calculated average molecular mass of deglycosylated domain I carrying the respective mutations are shown in brackets.

− denotes that the carbohydrate profile on Asn52 determined by MALDI-MS for this mutant is comparable to that published previously for wild-type uPAR secreted by Chinese hamster ovary cells (38), while − indicates the absence of glycosylation on domain I.

pA, the amount of suPAR mutant protein secreted to the medium was measured by enzyme-linked immunosorbent assay.

ND, not determined.

RESULTS AND DISCUSSION

Choice, Design, and Expression of Soluble uPAR Mutants—In the present study we have selected non-glycine residues in loop 3 of uPAR domain I (residues 47–70) as the primary target for site-directed mutagenesis, since this region previously has been implicated in ligand-binding by either photoaffinity labeling (Arg53 and Leu196) (28, 29), ligand-induced protection against chemical modification (Try74) (30) or enzymatic deglycosylation (Asn52) (38). In addition, the only two tryptophan residues present in uPAR (Trp32 and Trp129) were also mutagenized, since tryptophan residues generally participate so prevalently in protein-protein interactions (33, 42), and a surface exposed aromatic/hydrophobic patch on uPAR correlates to the vacancy of the high-affinity uPA-bind-
ing site as demonstrated by 8-anilino-1-naphthalene sulfonate fluorescence spectroscopy (27).

We have chosen to perform the present alanine scanning mutagenesis analysis using a truncated, secreted uPAR variant (denoted suPAR), having uPA binding properties indistinguishable from those of the wild-type glycolipid-anchored receptor present on the cell surface (27). This truncated receptor can conveniently be produced by Chinese hamster ovary cells after transfection with a DNA carrying a deletion corresponding to the COOH-terminal signal peptide responsible for the attachment of the glycolipid anchor (14, 37, 43). A cassette expression vector was therefore constructed in such a manner that single-site mutations in suPAR domain I could be introduced without the necessity to confirm the DNA sequence encoding the entire receptor for each mutant created (Fig. 1). Expression of recombinant "wild-type" suPAR (encompassing residues 1–283) as well as mutants thereof were accomplished after stable transfection of Chinese hamster ovary cells and the secreted receptor proteins were isolated from the harvest fluid by immunoaffinity chromatography using the high-affinity monoclonal antibody R2 specific for uPAR domain III (25) followed by reversed-phase HPLC. The purity of these protein preparations were >95% as judged from silver-stained gels after SDS-PAGE of reduced and alkylated samples (data not shown).

To reassure that the correct mutation had been introduced into the purified protein and to reveal any impact thereof on the carbohydrate processing of Asn52, suPAR domain I of each individual mutant was excised by limited proteolysis (36). Analysis by MALDI-MS before and after removal of the N-linked carbohydrate on domain I by N-glycanase treatment revealed that the masses recorded were compatible with the respective alanine replacements and the carbohydrate processing at Asn52 was similar to that of the wild-type suPAR (38), the only exceptions being those point mutations that compromised the glycosylation motif itself i.e. Asn52 → Gln and Thr24 → Ala where no glycosylation were detected (Table I).

**Kinetic Analysis of the Interaction between uPA and Purified Single-site suPAR Mutants**—To study the impact of these single-site alanine mutations on the binding kinetics of the uPA-suPAR interaction, uPA, or its zymogen, pro-uPA was immobilized on a biosensor chip by random amine coupling and the interaction with various suPAR mutants measured in real-time by recording the changes in surface plasmon resonance upon injection of suPAR followed by buffer. Typical binding curves (sensorgrams) obtained at high receptor concentration (100 nM) are shown in Fig. 2A for wild-type suPAR and 4 selected mutants. Single-site mutations affecting the uPA-suPAR interaction by destabilizing the receptor-ligand complexes are readily recognized in such sensorgrams as they exhibit significantly increased off-rates, as illustrated in Fig. 2B. The kinetic rate constants of uPA binding were subsequently determined for each suPAR mutant after analysis of several receptor concentrations in the range of 2–200 nM (Table II).
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All rate constants are shown as the mean of three to six separate determinations (each including 8 different receptor concentrations from 2 to 200 nM) along with the standard deviations (in parentheses). Measurements were performed at 5°C in 10 mM Hepes, 150 mM NaCl, pH 7.4, containing 0.005% (v/v) surfactant P20. The $K_d$ was calculated from the means of the respective association and dissociation rate constants ($K_d = k_{off}/k_{on}$).

| Mutant | $k_{on} \times 10^{-4}$ | $k_{off} \times 10^5$ | $K_d$ | $\Delta G_{mut-wt} \Delta G$ |
|--------|-------------------------|----------------------|-------|-----------------------------|
| wt (1–283) | 8.11 (± 4.0) | 5.75 (± 1.6) | 0.71 | 0 |
| W32A | 5.59 (± 1.9) | 5.45 (± 1.2) | 0.97 | 0.17 |
| H47A | 11.1 (± 5.2) | 12.1 (± 2.4) | 0.98 | 0.18 |
| E49A | 13.2 (± 5.3) | 7.13 (± 1.5) | 0.54 | 0.15 |
| K50A | 12.8 (± 2.8) | 20.5 (± 4.4) | 1.60 | 0.45 |
| T51A | 7.86 (± 2.7) | 35.2 (± 6.9) | 4.48 | 1.02 |
| N52Q | 4.26 (± 2.3) | 19.4 (± 5.1) | 4.55 | 1.03 |
| R53A | 4.66 (± 2.9) | 37.3 (± 7.0) | 8.00 | 1.34 |
| T54A | 4.06 (± 2.4) | 12.1 (± 1.8) | 2.98 | 0.79 |
| L55A | 6.01 (± 3.7) | 55.3 (± 8.4) | 9.20 | 1.42 |
| S56A | 6.88 (± 1.7) | 6.13 (± 1.8) | 0.89 | 0.13 |
| Y57A | 7.61 (± 3.1) | 56.5 (± 11.9) | 7.42 | 1.30 |
| R58A | 5.19 (± 1.5) | 11.9 (± 1.5) | 1.7 (± 7.8) | 3.67 | 0.91 |
| T59A | 7.17 (± 2.3) | 13.8 (± 1.2) | 1.92 | 0.55 |
| L61A | 13.6 (± 4.8) | 6.47 (± 1.5) | 0.48 | 0.22 |
| K62A | 16.3 (± 7.0) | 13.6 (± 3.9) | 0.83 | 0.09 |
| I63A | 11.3 (± 4.3) | 10.8 (± 2.8) | 0.90 | 0.13 |
| T64A | 9.54 (± 2.5) | 18.4 (± 3.6) | 1.90 | 0.55 |
| S65A | 5.64 (± 1.6) | 6.13 (± 1.6) | 0.90 | 0.13 |
| L66A | 4.31 (± 0.8) | 37.5 (± 2.8) | 8.70 | 1.39 |
| T67A | 3.98 (± 1.7) | 11.9 (± 2.9) | 2.99 | 0.80 |
| E68A | 11.6 (± 0.8) | 7.31 (± 3.0) | 0.63 | 0.07 |
| V69A | 9.55 (± 2.7) | 9.75 (± 1.9) | 1.02 | 0.20 |
| V70A | 8.40 (± 4.3) | 11.3 (± 3.7) | 1.34 | 0.35 |
| W129A | 6.40 (± 2.8) | 8.29 (± 2.1) | 1.30 | 0.33 |
| R53L | 18.6 (± 9.9) | 153 (± 223) | 8.23 | 1.36 |
| R53K | 10.8 (± 2.2) | 19.6 (± 3.4) | 1.81 | 0.52 |
| L55M | 8.49 (± 1.4) | 7.77 (± 2.2) | 0.91 | 0.14 |
| L55V | 13.1 (± 4.4) | 29.2 (± 2.6) | 2.23 | 0.63 |
| Y57W | 11.8 (± 5.5) | 5.51 (± 1.3) | 0.47 | 0.23 |
| Y57F | 13.0 (± 4.7) | 7.36 (± 1.5) | 0.57 | 0.12 |
| Y57K$^a$ | 3.61 (± 2.6) | 394 (± 239) | 10.29 | 2.79 |
| Y57H$^a$ | 12.5 (± 5.3) | 116 (± 7.4) | 9.28 | 1.42 |
| Y57F$^a$ | 15.7 (± 5.5) | 35.4 (± 2.8) | 2.25 | 0.65 |

$^a$ Differences in the free-energy changes are calculated as the difference between $\Delta G_{mut}$ and wild-type receptor ($\Delta G = \Delta G_{mut} - \Delta G_{wt}$) and were derived from the kinetic rate constants determined by surface plasmon resonance and calculated as $RT \ln(K_d/\mu M/K_d/wt)$.

$^b$ When analyzed by the Bioevaluation 3.0 software the binding profiles observed for these mutants did not yield a perfect fit to a 1:1 Langmuir binding. Nevertheless, the best association and dissociation rate constants derived from such fitting attempts are shown.

**Table II**

Summary of the kinetics derived from surface plasmon studies on the interaction between immobilized uPA and recombinant soluble wild-type and mutant uPAR.

It is well documented in the literature that human uPA has Leu$^{66}$ have previously been assigned to the structural epitope on uPAR for ligand binding, i.e. present at the receptor-ligand interface (28–30). The data obtained in this study by alanine scanning therefore demonstrates that these residues together with Leu$^{55}$ also form part of the functional epitope for uPA binding, as they contribute significantly to the binding energetics of the receptor-ligand interaction.

Smaller effects were also consistently observed for the interaction between uPA and uPAR mutants carrying either a Thr$^{51}$ to Ala or Asn$^{52}$ to Gln mutation, both of which caused an approximately 6-fold increase in the $K_d$ ($\Delta G = 1$ kcal/mol). From a structural point of view the involvement of Thr$^{51}$ in uPA binding is interesting, since it is located at a position normally engaged in the formation of an otherwise strictly conserved disulfide bond in the Ly-6/uPAR domain family, but which is uniquely absent from the NH2-terminal domains of both uPAR and C4.4 (17). The structural importance of this disulfide bond is emphasized by the severe folding problems encountered when it is removed by site-directed mutagenesis in the single domain proteins CD59 (50) and $\alpha$-bungarotoxin (51). It has been speculated previously that the lack of this particular disulfide bond in uPAR domain I could be of functional consequence for the receptor (15).

Enzymatic removal of the carbohydrate from Asn$^{52}$ in uPAR domain I has previously been reported to have a moderate impact on the receptor binding kinetics for uPA causing a 5-fold increase in the $K_d$ (38). Consistently, the two mutations introduced in the present study preventing subsequent glycosylation of Asn$^{52}$ (i.e. Asn$^{52}$ to Gln and Thr$^{54}$ to Ala) do also cause a moderate increase in the $K_d$ for uPA binding (Table II). Neither of the two tryptophan residues in uPAR seems critical for uPA binding.

To validate the data obtained by surface plasmon resonance, a selection of suPAR mutants, excluding those involving lysine replacements, were subjected to a semiquantitative cross-linking analysis (41). Complexes between uPAR and $^{125}$I-labeled ATF were allowed to form at receptor concentrations close to the $K_d$ of the interaction for the wild-type proteins (i.e. 0.5 nM) before addition of a homobifunctional chemical cross-linker to covalently stabilize the preformed complexes. As shown by SDS-PAGE and autoradiography, alanine substitutions of any of the 4 amino acids previously assigned to the functional epitope for uPA binding by surface plasmon resonance (i.e. Arg$^{53}$, Leu$^{55}$, Tyr$^{57}$, and Leu$^{66}$) consistently led to reduced complex formation as compared with the wild-type (41). Likewise, a number of non-alanine suPAR mutants tested by chemical cross-linking also revealed complex levels that were in agreement with the kinetic data obtained by surface plasmon resonance (Fig. 4 versus Fig. 5A).

**Further Mutations of the Functional Epitope on uPAR for uPA Binding**—To further characterize the molecular interactions of the individual side chains of the key residues assigned to the functional epitope for uPA binding, we also mutated Arg$^{53}$, Leu$^{55}$, and Tyr$^{57}$ to other residues than alanine. uPAR mutants having Tyr$^{57}$ replaced with either Trp or Phe demonstrated uPA binding kinetics indistinguishable from those of the wild-type receptor (compared with the $\Delta G$ of 1.3 kcal/mol for the alanine mutation) highlighting the importance of an aromatic side chain at position 57 and also demonstrating that the hydroxyl group of Tyr$^{57}$ is energetically dispensable for the functional epitope (Table II and Fig. 5A). In contrast, introduction of either negatively or positively charged side chains at this position (Glu or Lys) renders the uPA-uPAR complex very unstable, causing large changes in the free energy of uPA binding (Table II).

It is well documented in the literature that human uPA has
a very low affinity for mouse cells and vice versa (52, 53). Since Leu55 is the only residue that differs between man and mouse among those assigned so far to the uPA binding epitope on human uPAR, we also changed Leu55 to Met, the equivalent residue present in the murine uPAR sequence (Fig. 5B). However, this conservative replacement did not affect the binding kinetics to human uPA, excluding Leu55 as a major determinant of the observed species specificity.

Finally, we created two additional substitutions at position 53 (Arg53 → Leu or Lys) both of which provide reasonable isosteric replacements of the relatively long aliphatic side chain of Arg53, but only the Lys substitution retains the additional potential for electrostatic interactions that is provided by the guanido group of Arg53. As shown in Table II and Fig. 5A a lysine substitution at Arg53 proved more permissible for high-affinity ligand binding than the corresponding alanine or leucine substitutions (ΔΔG ~ 0.5 kcal/mol versus 1.4 kcal/mol). It is therefore possible that the guanido group of Arg53 may

FIG. 3. Kinetic rate constants determined by surface plasmon resonance for the interaction between immobilized uPA and various single-site alanine mutants of uPAR. Panel A shows the dissociation rate constants determined for the various suPAR mutants. The broken line corresponds to a 5-fold enhancement in the dissociation rate constant compared with that of the wild-type suPAR. Panel B shows the corresponding association rate constants determined in parallel. Data is transferred from Table II.

FIG. 4. Chemical cross-linking analysis of preformed complexes between ATF and soluble uPAR mutants. Purified uPAR mutants (0.5 nM) and [125I]-labeled ATF (1 nM) were incubated at 4 °C for 60 min before the formed uPAR-ATF complexes were conjugated chemically by addition of 1 mM disuccinimidyl suberate. Shown is the autoradiogram obtained after SDS-PAGE of these samples.
FIG. 5. Secondary mutations introduced at positions in uPAR domain I previously identified as part of the functional epitope for ligand binding by alanine scanning mutagenesis. Panel A, dissociation rate constants for the interaction between purified, soluble uPAR mutants and immobilized uPA were determined by surface plasmon resonance at 5 °C. Panel B, sequence alignment of loop 3 in human and murine uPAR domain I as well as human CD59. Note that CD59 contains a consensus disulfide bond in this region uniquely lacking in uPAR domain I, but not in uPAR domains II and III (15, 36). Residues assigned to the functional epitope for ligand binding by alanine scanning mutagenesis are highlighted. Residues critical for the inhibitory properties of the glycolipid anchored CD59 on the assembly of autologous complement membrane attack complex determined by alanine scanning mutagenesis are also highlighted (55).

FIG. 6. Assessment of the overall structural integrity of the various receptor mutants relative to the wild-type uPAR probed by monoclonal antibodies. The interaction between various immobilized, monoclonal anti-uPAR antibodies (R3, R5, and R9) and soluble uPAR mutants were measured by surface plasmon resonance. The dissociation rate constants are shown. The bars represent the standard deviations obtained after at least five independent determinations. The asterisks highlight residues belonging to the functional epitope determined here for uPA binding.
contribute to ligand binding via electrostatic interactions.

Single-site Substitutions do Not Result in Gross Structural Perturbations within uPAR Domain I as Probed by Monoclonal Antibodies—The structural integrity of the alanine mutants used to delineate the functional epitope for uPA binding was subsequently assessed by measuring their binding activities toward three different mouse monoclonal antibodies all having conformation-dependent epitopes on uPAR domain I (R3, R5, and R9). As shown in Fig. 6 binding of the four alanine mutants defining the functional epitope for uPA binding (i.e. Arg53, Leu55, Tyr57, and Leu66) to domain I-specific monoclonal antibodies was essentially unchanged, indicating that the structural integrity of domain I is maintained in these mutants. A similar lack of effect was also observed with the non-alanine mutations (data not shown).

Part of the functional epitope for one of the monoclonal antibodies (R3) was identified during the present alanine scanning mutagenesis of uPAR, and being centered on Leu61 and Lys62 it is located in close sequence proximity to the uPA-binding site (Fig. 6, lower panel). This epitope mapping concurs with the functional properties of the R3-uPAR interaction: the antibody being a competitive inhibitor of uPA binding (25, 54), preventing photochemical insertion of a photosensitive peptide antagonist of uPA binding (28), blocking the specific uPAR-induced enhancement of ANS fluorescence (27) and not recognizing murine uPAR (for sequence comparison see Fig. 5B).

CONCLUSIONS

Due to the involvement of the uPA system in tissue remodeling, including cancer invasion and metastasis (1, 57, 58), the high affinity interaction between uPA and uPAR represents an obvious target for experimental drug development ($K_d$; $0.6 \text{ nM}$; $\Delta G = -11.7 \text{ kcal/mol}$). To assist future attempts on rational design of low molecular weight uPAR antagonists, we have in the present study used site-directed mutagenesis to localize and characterize structural elements comprising the functional epitope on uPAR for binding. Kinetic data obtained here by surface plasmon resonance for 33 single-site uPAR mutants have defined 4 positions in loop 3 of uPAR domain I as critically involved in uPA binding, i.e. Arg53, Leu55,

\[ \Delta \Delta G (\text{kcal/mol}) \]

- $\geq 1.3$
- $< 1.0$
- $1.0 - 1.2$
- Untested

Fig. 7. Localization of the functional epitope for uPA binding on human uPAR. The primary sequence of human uPAR domain I is shown along with residues assigned to the functional epitope for uPA binding. For comparison the three-dimensional structure of the homologous, single domain human CD59 (20) is also shown with the residues in red corresponding to its functional epitope for complement inhibition determined by alanine scanning mutagenesis (55). The three loops of the individual Ly-6/uPAR type modules are indicated by Arabic numerals. The secondary structure of CD59 is highlighted as follows: $\beta$-sheets (green arrows), $\alpha$-helix (red cylinder), and disulfide bonds (yellow).

4 K. List, unpublished data.
Tyr67, and Leu66 (illustrated in Fig. 7). A comparison to some of the single domain members of the Ly-6/uPAR superfAMILY, for which three-dimensional structures as well as mutagenesis data are available, reveals that the position of the functional epitope in this domain family varies. The glycolipid anchored CD59 has a functional epitope for inhibition of autologous complement attack located on loop 3 (Fig. 7) (55), whereas the functional epitope on erabutoxin a for the nicotinic acetylcholine receptor contains 10 residues that are primarily located on loops 1 and 2 (19). The functional epitope on uPAR for uPA binding possibly includes additional residues besides those we have assigned to loop 3 of domain I. Accordingly, we have identified a composite structural epitope for a decamer peptide antagonist of uPA binding involving loop 3 of both uPAR do-
mains I (Arg53 and Leu66) and domain III (His74) (28, 29). Furthermore, both uPA and the peptide antagonist require the full 3-domain receptor protein for a productive high-affinity interaction (27, 28). We are therefore currently searching for additional residues of the functional epitope for uPA binding by single-site mutagenesis in both uPAR domains I and III.

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