Mimicry of the Regulatory Role of Urokinase in Lamellipodia Formation by Introduction of a Non-native Interdomain Disulfide Bond in Its Receptor

Background: The urokinase receptor (uPAR) acts as a modulator of lamellipodia formation on vitronectin-rich matrices.

Results: Constraining the flexibility of uPAR by an interdomain cross-link drives it into a constitutively active state.

Conformational dynamics of uPAR is important for its function and is regulated by uPA binding.

Significance: This flexibility needs to be considered when investigating and targeting the function of uPAR.

The high-affinity interaction between the urokinase-type plasminogen activator (uPA) and its glycolipid-anchored receptor (uPAR) plays a regulatory role for both extravascular fibrinolysis and uPAR-mediated adhesion and migration on vitronectin-coated surfaces. We have recently proposed that the adhesive function of uPAR is allosterically regulated via a “tightening” of its three-domain structure elicited by uPA binding. To challenge this proposition, we redesigned the uPAR structure to limit its inherent conformational flexibility by covalently tethering domains DI and DIII via a non-native interdomain disulfide bond (uPAR^{H47C-N259C}). The corresponding soluble receptor has 1) a smaller hydrodynamic volume, 2) a higher content of secondary structure, and 3) unaltered binding kinetics towards uPA. Most importantly, the purified uPAR^{H47C-N259C} also displays a gain in affinity for the somatomedin B domain of vitronectin compared with uPAR wt, thus recapitulating the improved affinity that accompanies uPA-uPAR wt complex formation. This functional mimicry is, intriguingly, operational also in a cellular setting, where it controls lamellipodia formation in uPAR-transfected HEK293 cells adhering to vitronectin. In this respect, the engineered constraint in uPAR^{H47C-N259C} thus bypasses the regulatory role of uPA binding, resulting in a constitutively active uPAR. In conclusion, our data argue for a biological relevance of the interdomain dynamics of the glycolipid-anchored uPAR on the cell surface.

The glycolipid-anchored urokinase-type plasminogen activator receptor (uPAR) is the single membrane protein responsible for focalizing urokinase-mediated plasminogen activation to cell surfaces both in vitro and in vivo (2–4). This is accomplished through a high-affinity interaction (Kd ∼ 0.5 nM) between uPAR and the N-terminal growth factor-like domain (GFD) of the urokinase-type plasminogen activator (uPA). Genetic abrogation of this bimolecular assembly in vivo by either wholesale gene ablations of the corresponding Plaur or Plau genes or by selectively abrogating the uPA-uPAR interaction sensu stricto, through redesigning the Plau gene to encode a uPA protein with a nonfunctional β-hairpin, all lead to pathological hepatic fibrin depositions associated with chronic inflammation (5, 6). These genetic dissections clearly emphasize a role for the uPA-uPAR axis in maintenance of extravascular fibrin homeostasis under normal physiological conditions. As both uPAR and uPA are expressed by either tumor or stromal cells in the invasive microenvironment of many human cancers, including colon (7), breast (8), and pancreatic (9) carcinomas, this enzyme system is also assumed to contribute to the pathogenesis of these diseases. Elevated levels of uPA and uPAR are accordingly important biomarkers of poor prognosis for such patients (10, 11), and both are considered attractive targets for drug development (12, 13).

Although uPA undisputedly is the bona fide protease ligand for uPAR, this receptor also cooperates with other proteins that are involved in cell adhesion and migration, e.g. vitronectin and certain integrins (14–18). The direct interactions between uPAR, uPA, and vitronectin have recently been thoroughly characterized both biochemically and structurally. Complementary functional epitopes on uPAR and its biological ligands (uPA and vitronectin) have been

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identified by systematic alanine scanning mutagenesis (5, 17, 19, 20), and the corresponding binding interfaces are well defined at high resolution by x-ray crystallography (5, 21, 22). The proposed molecular interplay between uPAR and integrins remains, however, to be defined at the structural level (15).

From extensive biochemical and structural studies, it is now clear that all three Ly6/uPAR/α-neurotoxin-like (LU) domains in uPAR cooperate to assemble the composite binding sites for both uPA and vitronectin, which encompass distinct surface-exposed interdomain junctions on the receptor (13, 23). Recently, we introduced a molecular model for uPAR function, which assumes that uPAR may explore different conformational states having distinct functional properties (24). According to this model, unoccupied human uPAR predominantly exists in an open conformation, which does not support uPAR-dependent induction of lamellipodia formation on vitronectin-coated surfaces. This model implies that ligand binding to the GFD of uPA shifts the conformational equilibrium in the GFD towards a more closed conformation, which renders uPAR active in supporting lamellipodia formation (24).

Based on the crystal structures available for uPAR in complex with the amino-terminal fragment (ATF) of uPA (5, 21) and a high-affinity antagonist peptide (25), we have now designed a stabilized uPAR variant, which cannot populate the allegedly open native conformation due to the constraint introduced by an engineered interdomain disulfide bond between uPAR domains I and III (DI and DIII). Whereas maintaining unaltered binding kinetics towards uPA, this engineered receptor gains a number of new functional properties due to this constrained conformation. Most importantly, we found that this particular receptor is constitutively active in stimulating lamellipodia formation in vitro (18).

Antibodies were produced in-house (R2, R3, R5, R8, R9, R21, and R35) and calibrated with the appropriate molecular mass markers (ribonuclease (13,700 Da), carbonic anhydrase (29,000 Da), ovalbumin (43,000 Da), and conalbumin (75,000 Da)). Sample emission was measured from 400 to 600 nm using slit widths of 5 nm. Measurements were performed in 20 mM sodium phosphate buffer, pH 7.2, 50 mM Na$_2$SO$_4$, and calibrated with the appropriate molecular mass markers (ribonuclease (13,700 Da), carbonic anhydrase (29,000 Da), ovalbumin (43,000 Da), and conalbumin (75,000 Da)). Sample volumes of 200 μl containing 3 mg/ml of the respective recombinant uPAR mutants were applied and analyzed at room temperature.

Circular Dichroism Spectroscopy—Far UV circular dichroism (CD) spectra were recorded in a 1-mm quartz cuvette using a Jasco J-810 equipped with a PTC-4233 Peltier temperature controller. The spectra were acquired at 20 °C from 250 to 190 nm with a scan speed of 20 nm/min and a response time of 1 s averaging 40 scans for each measurement. All measurements were performed in 20 mM sodium phosphate buffer, pH 7.2, 50 mM Na$_2$SO$_4$, and a protein concentration of 3 μM. Protein samples were purified and monomer status was ensured by gel filtration prior to CD analysis.

ANS Fluorescence Measurements—8-Anilino-1-naphthalene sulfonate (ANS) fluorescence was recorded using a PerkinElmer LS50B fluorimeter using a 5-mm square cuvette. The fluorophore was excited at 370 nm, and the fluorescence emission was measured from 400 to 600 nm using slit widths of 5 nm. Measurements were performed in the same buffer as CD analysis using a protein concentration of 5 μM and an ANS concentration of 10 μM. GFD was added from a concentrated stock to a final concentration of 15 μM, resulting in a 2.5% dilution.

Surface Plasmon Resonance Studies—Real-time interaction studies were carried out on Biacore 3000 TM or T200 TM instruments (Biacore, Uppsala, Sweden) using 10 mM HEPES, 150 mM

MATERIALS AND METHODS

Chemicals and Protein Reagents—Human pro-uPA$^{1–411}$ and ATF$^{1–144}$ were expressed by Drosophila melanogaster S2 cells and affinity purified as described previously (19). The growth factor-like domain of human uPA (GFD$^{1–48}$) was a kind gift from S. Rosenberg (30). A His-tagged SMB$^{1–47}$ domain from human vitronectin was expressed in Pichia pastoris and purified as described (31). TLCK-treated chymotrypsin was from Worthington (Freehold, NJ) and was stored at −80 °C as 1 mg/ml stock solutions in 1 mM HCl. Monoclonal anti-uPAR antibodies were produced in-house (R2, R3, R5, R8, R9, R21, and mR1), purchased from Acris Antibodies, Herford Cambridge, UK (VIM-5), or kindly supplied by Dr. U. Weidle, Roche Applied Science (H2).

Expression, Purification, and Chymotrypsin Cleavage of Soluble Human uPAR Mutants—Soluble forms of human uPAR$^{1–283}$ were expressed by stably transfected D. melanogaster S2 cells as described previously (32). The secreted proteins were purified from the culture medium by a peptide-based, one-step affinity chromatography for uPARwt (33) and by immunoaffinity chromatography using the monoclonal anti-uPAR antibody R2 for the new uPAR$^{1447C–N259C}$ and uPAR$^{50C–D254C}$ mutants.

Chymotrypsin treatment of the purified recombinant uPAR variants was performed essentially as described (34) using conditions delimiting the cleavage to the single peptide bond between Tyr$^{87}$–Ser$^{88}$. This specificity was accomplished by incubating purified uPAR with chymotrypsin at a ratio of 5000:1 (w/w) for 12 h at room temperature.

Size Exclusion Chromatography—Recombinant and affinity-purified uPAR$^{1447C–N259C}$, uPAR$^{1447C–N259C}$/chym, and uPAR$^{1447C–N259C}$/chym were subjected to size exclusion chromatography using a Superdex$^{TM}$ 200 (10/300) column operated at 1 ml/min by an Äktapurifier$^{TM}$ HPLC system. The column was equilibrated with 20 mM sodium phosphate buffer, pH 7.2, 50 mM Na$_2$SO$_4$, and calibrated with the appropriate molecular mass markers (ribonuclease (13,700 Da), carbonic anhydrase (29,000 Da), ovalbumin (43,000 Da), and conalbumin (75,000 Da)). Sample volumes of 200 μl containing 3 mg/ml of the respective recombinant uPAR mutants were applied and analyzed at room temperature.

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NaCl, 3 mM EDTA, supplemented with either 0.005 or 0.05% (v/v) surfactant P-20 at pH 7.4 as running buffer. For kinetic evaluations, soluble uPAR (0.1 µg/ml) was immobilized covalently on a carboxymethylated dextran matrix (CM5 sensor chip) using N-hydroxysuccinimide/N-ethyl-N’-(3-diethylamino)propyl)-carbodiimide yielding coupling levels of ~200 RU, ~6 fmol/mm². To obtain comparable measurements for uPAR<sup>wt</sup>, uPAR<sup>H47C-N259C</sup>, and uPAR<sup>H47C-N259C/chym</sup>, they were immobilized to similar densities in parallel flow cells. Kinetic rate constants for these uPA-uPAR interactions were determined from the analyses of serial 2-fold dilutions covering 0.1–50 nM of the various uPA derivatives (GFD, ATF, and pro-uPA) at a flow rate of 50 µl/min at 20 °C. After each run the sensor chip was regenerated by two consecutive injections of 0.1 M acetic acid in 0.5 M NaCl. The kinetic rate constants, <i>k</i><sub>on</sub> and <i>k</i><sub>off</sub>, were derived from these real-time interaction analyses by fitting the association and dissociation phases to a bimolecular interaction model using BIACore evaluation 4.1 software (Biacore, Uppsala, Sweden), as described in detail previously (19). Thermodynamics of the uPA-uPAR interaction were assessed by coupling pro-uPA onto the sensor chip and measuring the interaction with 2-fold dilution series covering 3–50 nM uPAR<sup>wt</sup>, uPAR<sup>H47C-N259C</sup>, and uPAR<sup>H47C-N259C/chym</sup> at 6 different temperatures covering 5–37 °C at 50 µl/min by a Biacore T200 instrument. Entropy (∆S°), enthalpy (∆H°), and heat capacity (∆C<sub>p</sub>) changes were derived from nonlinear van’t Hoff plots using the evaluation software (version 1.0) supplied with the equipment (supplemental Fig. S1).

**Inducible Expression of Glycolipid-anchored uPAR Mutants in HEK293 Cells**—The uPAR mutants were stably expressed in HEK293 cells by the Flp-In T-Rex system from Invitrogen: uPAR<sup>wt</sup>, uPAR<sup>H47C-N259C</sup>, and uPAR<sup>H47C-N259C/chym</sup>. Construction of the expression vector pcDNA5/FRT/TO/ uPAR encoding full-length uPAR<sup>wt</sup> has previously been described (18). Mutant constructs were generated by introducing the corresponding mutations into this vector with the QuikChange Multi Site-directed Mutagenesis kit (Stratagene, AH Diagnostics, Aarhus). The parental Flp-In T-Rex-293 cell line was grown in minimal essential medium supplemented with Earle’s and GlutaMAX I (Invitrogen 41090–028) plus nonessential amino acids, 10% (v/v) FCS, 100 units/ml of penicillin, 100 µg/ml of streptomycin with the addition of 15 µg/ml of blasticidin S and 100 µg/ml of Zeocin at 37 °C in a 5% CO<sub>2</sub> humidified incubator. Stable, tetracycline-inducible cell lines were generated by co-transfection with pcDNA5/FRT/TO expression vectors and pOG44 in a 1:10 ratio using Lipofectamine 2000 as transfection agent followed by selection in medium as above omitting Zeocin, but adding 150 µg/ml of hygromycin B. Expression of uPAR was induced by adding 1 µg/ml of tetracycline to the medium.

**Determining uPAR Expression Levels and Affinities of Transfected HEK293 Cells**—To compare the uPAR expression levels in the various transfected HEK293 cell lines, these were cultured in the presence of 1 µg/ml of tetracycline. Cells were harvested using a rubber policeman, washed in PBS, and counted in a NucleoCounter<sup>TM</sup>. The cell pellet was collected by centrifugation and lysed in 500 µl of 1.5% (v/v) Triton X-100, 0.1 mM Tris, pH 8.1, 10 mM EDTA, 10 µg/ml of Trasylol, and 1 mM phenylmethylsulfonyl fluoride at a cell density of 1–2 × 10<sup>7</sup> cells/ml. The uPAR contents in these lysates were determined by a specific ELISA (35).

To assess the uPA-binding capacity and affinity of the transfected HEK293 cell lines, these were seeded (6 × 10<sup>5</sup> cells/well) in 24-well plates and allowed to adhere for 2–3 days in the presence of 1 µg/ml of tetracycline, reaching confluence before being subjected to a solid-phase uPA binding assay. In brief, the adherent cells were incubated with 3-fold dilution series of Eu<sup>3+</sup>-labeled pro-uPA (0.05–300 nM pro-uPA with a labeling density of 2.0 Eu/uPA) in 400 µl of minimal essential medium with 10% (v/v) FCS for 24 h at 4 °C. After extensive washings, the number of adherent cells in representative wells was counted in a NucleoCounter after detachment by trypsin/
EDTA treatment for 1–2 min at 37 °C. For quantification of pro-uPA binding, the adherent cells were lysed for 5 min in 400 μl of PBS containing 0.125% (v/v) Triton X-100. After centrifugation for 10 min at 4 °C (10,000 × g), the supernatants were diluted 5 times in DELFIA™ enhancement solution, and the bound uPA was quantified by time-resolved fluorescence using a Fluostar Optima fluorometer (PerkinElmer Life Sciences) with excitation set at 340 nm and reading emission at 615 nm with a 400-μs delay and a 400-μs acquisition window. Nonspecific binding was assessed by a parallel measurement of Eu-labeled pro-uPA binding to the corresponding cell lines without prior tetraacycline induction of uPAR expression.

**Lamellipodia Formation on Vitronectin-coated Coverslips**—The functional impacts of the above mentioned mutations in glycolipid-anchored uPAR on cellular responses upon vitronectin exposure were measured as described previously (16, 24). In brief, transfected HEK293 cells were seeded on vitronectin-coated glass coverslips for 24 h allowing their attachment to the matrix, and this was followed by an additional incubation for 24 h after exchange to fresh medium, which in some cases contained additives such as mAbs or uPA derivatives. To facilitate examination of lamellipodia formation, the adherent cells were fixed on the coverslips, permeabilized, and stained with Alexa 488-conjugated phalloidin to visualize actin filaments. Finally, the extent of lamellipodia formation was evaluated using the previously described quantitative protrusion index (24), providing a score ranging from 0 to 24 for each determination.

**Miscellaneous**—All graphic artwork on protein structures were generated by PyMol (Schrödinger, Portland, OR). Protein masses were determined by matrix-assisted laser desorption mass spectrometry (Autoflex II™ MALDI-TOF/TOF, Bruker Daltonics GmbH, Bremen, Germany) after deposition in sinapinic acid.

**RESULTS**

**Rational Design of an Interdomain Disulfide Cross-link in Human uPAR**—The formation of a functional high-affinity binding cavity for the growth factor-like domain of uPA requires the modular assembly of all three LU domains in the glycolipid-anchored uPAR (36). Nonetheless, we have recently proposed a model for the role of uPAR in vitronectin-dependent cell adhesion and migration, where unoccupied uPAR predominantly exists in an open inactive conformation (24). According to this proposition, the mere occupancy of the hydrophobic cavity in uPAR by the β-hairpin in GFD induces the formation of a more closed and compact receptor conformation, which exhibits an increased activity in inducing lamellipodia on cells plated on vitronectin-rich matrices. One important ramification of this model, which emphasizes the importance of the conformational dynamics of uPAR for its function, is the prediction that constraining the interdomain mobility in uPAR by introduction of a covalent cross-link in the interdomain interface could potentially create a constitutively active receptor. Inspired by this intriguing possibility, we searched the interface between DI and DIII for optimal distances and geometries, which could permit the formation of a stable disulfide bond, i.e. with an optimal distance between the Cα-atoms of 5.6 Å (37). Search in three different crystal structures of uPAR-ATF complexes (Protein Data Bank codes 2FD6, 219B, and 3BT1) identified two potential candidates, i.e.
His\textsuperscript{47}-Asn\textsuperscript{259} and Lys\textsuperscript{50}-Asp\textsuperscript{254}, both of which are involved in the formation of interdomain hydrogen bonding and having their C\textsubscript{\alpha}-atoms positioned 4.4–4.9 and 8.1–8.8 \textAA apart, respectively. The location of the more promising of these positions is illustrated in Fig. 1.

Engineering a Covalent Cross-link between DI and DIII in uPAR—To explore the aptness of these hydrogen-bonded residues as foci for new covalent interdomain cross-links, we introduced their pairwise replacement by cysteine residues and expressed the corresponding proteins uPAR\textsuperscript{H47C-N259C} and uPAR\textsuperscript{K50C-D254C} in Drosophila S2 cells. Although both mutant proteins are expressed well by this system and both produce high yields of pure protein after immunoaffinity chromatography, uPAR\textsuperscript{H47C-N259C}, nonetheless, proved far superior in its ability to oxidize the free thiol groups and thus form the desired interdomain covalent cross-link. This property is clearly demonstrated in Fig. 2, where the liberation of uPAR DI from the rest of the molecule requires cleavage of the linker region at Tyr\textsuperscript{87} by chymotrypsin as well as subsequent reduction of the protein disulfide bonds (panel B, lanes 3 and 4). This is in sharp contrast to uPAR\textsuperscript{wt}, where the mere cleavage of the linker region releases DI (34) without reduction of disulfide bonds, as illustrated in panel A, lanes 3 and 4. In all subsequent studies, we therefore focused on uPAR\textsuperscript{H47C-N259C}, as this mutant exhibits a remarkable inherent efficacy in forming this non-natural interdomain disulfide bond.

Physicochemical Properties of Purified uPAR\textsuperscript{H47C-N259C}—To further characterize the consequences of this interdomain cross-link on the physicochemical properties of uPAR, we subjected the purified uPAR\textsuperscript{wt}, uPAR\textsuperscript{H47C-N259C}, and uPAR\textsuperscript{H47C-N259C/chym} produced in Drosophila S2 cells to size exclusion chromatography in 20 mM phosphate buffer, pH 7.2, including 150 mM NaCl (data not shown). The most likely explanation for the differences in contrast to uPAR\textsuperscript{wt}, where the mere cleavage of the linker volume of uPAR\textsuperscript{H47C-N259C} is also observed after chymotrypsin treatment was repeated in 20 mM phosphate buffer, pH 7.2, including 50 mM Na\textsubscript{2}SO\textsubscript{4} (compatible with subsequent CD analysis). As illustrated by the chromatograms shown in Fig. 3A, this experiment reveals a surprisingly large difference in the hydrodynamic volume of uPAR\textsuperscript{wt} (eluting at 15.8 ml) compared with uPAR\textsuperscript{H47C-N259C} (eluting at 16.2 ml). Identical elution profiles displaying similar differences in retention times were obtained if the experiment was repeated in 20 mM phosphate buffer, pH 7.2, including 150 mM NaCl (data not shown). The notable difference in elution profiles cannot be ascribed to dissimilar molecular masses of the two recombinant proteins caused by micro- and/or macroheterogeneity in the post-translational processing of N-linked glycosylation sites as the average masses recorded by MALDI-MS are almost identical, i.e. uPAR\textsuperscript{wt} \sim 35.051 Da and uPAR\textsuperscript{H47C-N259C} \sim 35.064 Da (data not shown). The most likely explanation for the differences in the hydrodynamic volumes is that uPAR\textsuperscript{wt} adopts a more open and extended conformation with a larger Stokes radius compared with a more closed and compact conformation stabilized by the disulfide constraint in uPAR\textsuperscript{H47C-N259C}.

A distinct, but much smaller increase in the hydrodynamic volume of uPAR\textsuperscript{H47C-N259C} is also observed after chymotrypsin cleavage of the Tyr\textsuperscript{87}-Ser\textsuperscript{88} peptide bond (eluting at 16.1 ml), which is located in the linker region connecting DI and DII (Fig. 3A). This experiment thus clearly shows that DI remains attached to DII/DIII in the cleaved uPAR\textsuperscript{H47C-N259C} mutant, as opposed to uPAR\textsuperscript{wt} treated identically (34), and that this cleav-
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**TABLE 1**

| Ligand         | Immobilized reactant   | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_{d}$ | $R_{\text{max}}$ |
|---------------|------------------------|----------------|----------------|--------|----------------|
| Pro-uPAR$^{336\text{A}}$ | uPAR$^{\text{wt}}$     | $7.89 \pm 1.52$ | $1.10 \pm 0.13$ | $0.14$ | $2.4$          |
| Pro-uPAR$^{336\text{A}}$ | uPAR$^{H47\text{C}-N259\text{C}}$ | $7.49 \pm 1.34$ | $0.67 \pm 0.09$ | $0.09$ | $2.1$          |
| Pro-uPAR$^{336\text{A}}$ | uPAR$^{H47\text{C}-N259\text{C}/\text{chym}}$ | $7.25 \pm 1.08$ | $0.56 \pm 0.04$ | $0.08$ | $3.1$          |
| ATF$^{1-143}$ | uPAR$^{\text{wt}}$    | $10.2 \pm 2.18$ | $1.06 \pm 0.16$ | $0.11$ | $2.3$          |
| ATF$^{1-143}$ | uPAR$^{H47\text{C}-N259\text{C}}$ | $9.91 \pm 2.18$ | $1.06 \pm 0.19$ | $0.12$ | $2.2$          |
| ATF$^{1-143}$ | uPAR$^{H47\text{C}-N259\text{C}/\text{chym}}$ | $9.86 \pm 1.37$ | $0.63 \pm 0.23$ | $0.07$ | $3.2$          |
| GFD$^{3-48}$ | uPAR$^{\text{wt}}$    | $4.23 \pm 1.40$ | $1.76 \pm 0.25$ | $0.48$ | $2.2$          |
| GFD$^{3-48}$ | uPAR$^{H47\text{C}-N259\text{C}}$ | $4.59 \pm 1.68$ | $1.65 \pm 0.34$ | $0.43$ | $2.2$          |
| GFD$^{3-48}$ | uPAR$^{H47\text{C}-N259\text{C}/\text{chym}}$ | $4.38 \pm 1.86$ | $1.02 \pm 0.34$ | $0.36$ | $2.6$          |

* $k_{\text{on}}$, $k_{\text{off}}$, and $K_{d}$ values were calculated from the Langmuir fitting assuming that 1 RU $= 1$ pg/mm$^2$. This shows that uPAR$^{H47\text{C}-N259\text{C}}$ retains a higher specific binding activity (40%) compared to uPAR$^{\text{wt}}$ (24%) after covalent immobilization, which presumably reflects the increased protein stability of the constrained uPAR mutant.

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**Kinetic Rate Constants for the Interaction with uPA**

**Is Not Stimulated by uPA Occupancy**—We have previously demonstrated that the affinity of the interaction between the SMB domain of vitronectin and uPAR$^{\text{wt}}$ is increased ∼4-fold upon receptor occupancy with GFD, ATF, and pro-uPA (17). To test if a similar uPA dependence in SMB binding also applies to uPAR$^{H47\text{C}-N259\text{C}}$, we immobi...
by nonlinear curve fitting to the respective binding isotherms (Fig. 4, B and C). The stimulatory effect of occupying the hydrophobic ligand-binding cavity in uPAR wt with receptor-binding derivatives of uPA is, reassuringly, recapitulated in this experimental setting, where the affinity for SMB binding is increased 3-fold after binding to GFD or ATF (Table 2). Notably, unoccupied uPAR H47C-N259C already adopts the moderately higher affinity state for SMB, which normally requires saturation by uPA, and no additional increase in this affinity is recorded on binding GFD or ATF. This suggests that the conformational constraint induced by the covalent cross-link between DI and DII bypasses the regulatory role of uPA binding that is generally modulating the affinity for SMB (17). This raises the intriguing possibility that the conformational constraint introduced by the distant interdomain cross-link has rendered the receptor "constitutively active" with respect to vitronectin binding.

Although the chymotrypsin cleavage of the Tyr 87-Ser 88 peptide bond situated in the linker region between DI and DII does not directly involve residues confined to the structural and functional epitope on uPAR for SMB binding (17, 22), this cleavage is, nonetheless, accompanied by a ~10-fold increase in the $K_d$ for this interaction (Table 2). This impact on SMB binding is most likely caused by a local distortion of the adjacent SMB binding site, which could involve displacement of the hot spot residue Arg 91. Occupancy by ATF or GFD did not increase the affinity of uPAR H47C-N259C/chym for SMB (Table 2).

**Lamellipodia-inducing Potential in HEK293 Cells Expressing uPAR H47C-N259C**—To measure the impact of locking uPAR in the closed conformation on its ability to induce lamellipodia in cells plated on vitronectin, we stably transfected HEK293 cells with 8 different uPAR mutants. These were designed to compare the constrained versus unconstrained states of uPAR, but they also harbor various additional mutations to superimpose a gradual impairment on their inherent vitronectin-binding properties (17). Based on our functional and structural data (17, 22), we thus introduced two mutations (W32A and R91D) in the vitronectin-binding interface of uPAR to obtain a differential attenuation of this interaction. All transfected HEK293 cell lines express comparable levels of both uPAR protein and functional high-affinity binding sites for uPA as assessed by ELISA and time-resolved fluorescence assay for uPA binding (supplemental Table S1 and Fig. S3). As reported previously (16, 18, 24), we find that HEK293 cells first acquire lamellipodia in response to exposure to vitronectin-coated surfaces when they are transfected with uPAR wt. This ability is, however, not acquired if they are transfected with uPAR mutants having reduced affinity for vitronectin, i.e. uPAR W32A, uPAR R91D, or uPAR W32A-R91D (Fig. 5A). As noted previously, the functional deficiency of uPAR W32A can, nevertheless, be restored by receptor occupancy with uPA or its minimal receptor-binding module GFD (16, 24). Further substantiating this finding, we now report that the level of rescue appears correlated to the severity of the impairment in vitronectin binding, i.e. with a decreasing efficacy from uPAR W32A → uPAR R91D → uPAR W32A-R91D (Fig. 5B). This relationship emphasizes the importance of the interplay between uPAR and vitronectin for lamellipodia formation under these experimental conditions.
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Turning to the constrained uPAR^{H47C-N259C}, we now demonstrate that stabilizing the interface between DI and DIII via a covalent cross-link endows uPAR with a constitutive lamellipodia-inducing activity, which then circumvents the regulatory role of uPA binding (Fig. 5, compare A and B versus C). Importantly, HEK293 cells expressing uPAR^{H47C-N259C/W32A} mount an uncompromised induction of lamellipodia on vitronectin (Fig. 5C), which is in clear contrast to the impaired responsiveness displayed by the correspondingly unconstrained uPAR^{W32A} (Fig. 5A). We have thus managed to create a molecular mimicry of the regulatory gain-of-function, which under normal conditions is elicited by receptor occupancy with uPA, as illustrated for uPAR^{W32A}. GFD complexes in Fig. 5B. The magnitude of the functional rescue obtained by the engineered disulfide parallels the potency of the GFD-bound receptor, as both partly restore the deficiency of uPAR^{R91D}, whereas neither can rescue the more severely compromised uPAR^{W32A-R91D} (Fig. 5, A–C). The observed correlation between the lamellipodia-inducing efficacy of both uPAR^{wt}, GFD and uPAR^{H47C-N259C} with the severity of the mutational impact on vitronectin binding thus further emphasizes the importance of this interaction for both receptors.

We have previously shown that several monoclonal anti-uPAR antibodies having their epitopes located in DI close to the interdomain interface with DIII (epitope bin 2, e.g. mAbs R5, R9, and mR1) inhibit the ability of uPAR to stimulate lamellipodia formation (24). This effect is presumably instigated by trapping an open uPAR conformation incapable of promoting lamellipodia formation. In the present study, we have now mapped the functional epitope for an additional anti-uPAR mAb designated H2, which effectively inhibits uPA and vitronectin binding, and in so doing attenuates, e.g. angiogenesis (39). As illustrated in Fig. 6A, the dominating hot spot for H2 is Asn^{259} in uPAR DIII, which is one of two anchoring sites for the covalent cross-link introduced in uPAR^{H47C-N259C}. In the crystal structure of uPAR-ATF complexes, Asn^{259} is repositioned more than 10 Å within the domain interface of DI and DIII compared with the corresponding uPAR-peptide antagonist complex (Fig. 6A). We therefore investigated the impact of H2 as well as previously characterized anti-uPAR mAbs representing epitope bins 1 and 2 (24) on lamellipodia formation in HEK293 cells transfected with uPAR^{wt} and uPAR^{H47C-N259C}.

As shown in Fig. 6B, anti-uPAR mAbs belonging to epitope bins 1 and 2 in uPAR DI all inhibit lamellipodia formation in HEK293 cells expressing uPAR^{wt} (24), as does the newly characterized mAB H2. Remarkably, none of these mAbs inhibit the lamellipodia induced by uPAR^{H47C-N259C}. The topographic location of these epitope bins in the uPAR-ATF complex are illustrated in Fig. 6C, emphasizing the pairwise proximity of epitope bin 1 to the SMB binding site and epitope bin 2 to the engineered cross-linking site. The resilience of lamellipodia in uPAR^{H47C-N259C}-transfected HEK293 cells to inhibition by H2 and mAbs from epitope bin 2 may thus be anticipated due to the proximity of their epitopes to the introduced interdomain cross-link. To our surprise, mAbs recognizing epitope bin 1 are, however, also incapable of preventing lamellipodia formation in the uPAR^{H47C-N259C}-transfected HEK293 cells, despite their epitopes being located adjacent to the vitronectin binding site and distant to the introduced disulfide (Fig. 6C). This conundrum is most likely related to the same allosteric changes that increase the affinity for SMB in uPAR^{H47C-N259C}. We thus hypothesize that although these changes are beneficial for the formation of an optimal SMB binding site, they do, nevertheless, distort epitopes recognized by mAbs belonging to bin 1. This proposition is further substantiated experimentally by the reduced affinities of these mAbs toward uPAR^{wt} as measured by surface plasmon resonance. The predominant effect, which we observe primarily on the koff values, argues for a suboptimal spatial arrangement of these epitopes, which slows down the formation of a productive bimolecular encounter complex (Table 3).

**DISCUSSION**

With the advent of a steadily growing number of experimentally determined crystal structures for uPAR in complex with various natural and synthetic ligands (5, 21, 22, 25), our perception of the structural basis underlying uPAR function is becoming progressively more coherent (13). In recent years, it has

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**Table 2**

| Immobilized reactant* | Preformed complex* | K_d (SMB)* | R_max (SMB)* | R_max (GFD or ATF)* |
|------------------------|--------------------|------------|--------------|---------------------|
| uPAR^{wt}              | None               | 7.0 ± 0.7  | 14.5 ± 0.9   | 31.5                |
| uPAR^{wt}              | GFD                | 2.2 ± 0.4  | 17.1 ± 1.1   | 26.5                |
| uPAR^{wt}              | ATF                | 2.5 ± 0.3  | 15.9 ± 0.8   | 67.7                |
| uPAR^{H47C-N259C}      | None               | 2.1 ± 0.2  | 28.6 ± 0.8   | 6.7                |
| uPAR^{H47C-N259C}      | GFD                | 1.6 ± 0.2  | 28.1 ± 1.1   | 56.5                |
| uPAR^{H47C-N259C}      | ATF                | 1.8 ± 0.2  | 26.5 ± 1.1   | 56.5                |
| uPAR^{H47C-N259C}      | None               | 23 ± 6     | 28.6 ± 6.0   | 77.8                |
| uPAR^{H47C-N259C}      | GFD                | 16 ± 2     | 29.1 ± 2.6   | 63.7                |
| uPAR^{H47C-N259C}      | ATF                | 18 ± 3     | 26.3 ± 3.4   | 63.7                |

* Relatively high levels of uPAR were immobilized by amine chemistry (5 μg/ml) in 3 consecutive flow cells of a CM5 sensor chip: uPAR^{wt} (2632 RU), uPAR^{H47C-N259C} (2665 RU), and uPAR^{H47C-N259C/chym} (2671 RU). These levels are equivalent to a surface density of approximately 76 fmol/mm².

* Two-fold dilution series of purified SMB were consecutively injected at a flow rate of 20 μl/min at 20 °C covering the concentration range from 0.14 to 9 μl. No regeneration was needed due to the very fast dissociation rates. The equilibrium binding constants K_d and R_max for these weak interactions were calculated from the corresponding equilibrium binding isotherms by nonlinear curve fitting assuming saturation of a single binding site: K_d = (R_max(SMB))/(R_max + (SMB)), where R_max is the binding level at equilibrium, and R_max is the binding capacity of the chip.

* R_max values for the first interaction with the respective pro-uPA derivatives, which were injected at high concentration (100 nM), were measured directly from the recorded sensograms just before the first SMB injection (see Fig. 4A). The following average molecular masses were used for these calculations: GFD (104 kDa) and ATF (114 kDa).
Non-constrained H47C-N259C

Non-constrained H47C-N259C

FIGURE 5. Glycolipid-anchored uPARH47C-N259C in HEK293 cells mimics GFD-bound uPAR in the induction of lamellipodia. The ability to form lamellipodia on vitronectin-coated surfaces was measured for HEK293 cells after stable transfection with 8 different uPAR constructs. Representative micrographs are shown in the upper panels for uPARwt (A1 and B1), uPAR (A2 and B2), uPARH47C-N259C (C1), and uPARH47C-N259C/W32A (C2). A graphic representation summarizing the results is provided in the lower panel. Non-constrained uPARwt (lane 1) and mutants with impaired vitronectin binding properties, i.e. uPARW32A (lane 2), uPARR91D (lane 3), and uPARH47C-N259C/W32A (lane 4), were probed in their unoccupied state in cluster A. The impact of uPA ligation is probed in cluster B for the same set of transfected HEK293 cells by incubation with 100 nM GFD during cell seeding and incubation on vitronectin. The impact of locking uPAR in the closed conformation per se is tested in cluster C for HEK293 cells transfected with uPARH47C-N259C (lane 1) combined with the same mutations as in cluster A, i.e. uPARH47C-N259C/W32A (lane 2), uPARH47C-N259C/R91D (lane 3), and uPARH47C-N259C/W32A-R91D (lane 4). The impact of GFD saturation is measured for these constrained uPAR mutants in cluster D.

Design of a Constitutively Active uPAR

Become increasingly clear that the so-called “nonproteolytic role” of uPAR in cell adhesion and migration in many cases is tightly controlled by uPA occupancy (17, 18, 40). Although many reports on uPAR-vitronectin-mediated cell adhesion and migration employ transfected cell lines expressing high levels of uPAR (16, 18, 24, 41, 42), the regulatory role of uPA occupancy is, nevertheless, allegedly more noticeable in cells either expressing moderate to low levels of uPAR (40, 43, 44) or alternatively, as in this study, in cells expressing high levels of uPAR mutants with an attenuated vitronectin binding. The proposition of such a regulatory role of uPA binding is quite conceivable from a physiological point of view, taking into consideration that its affinity for uPAR ($K_d \sim 0.5$ nM) nicely complements the levels of pro-uPA present under normal homeostatic conditions, i.e. 20 pm in plasma (45), thus setting the stage for an efficient responsiveness to increased local concentrations of pro-uPA. We have launched a new molecular model for the structure-function relationships in the uPA-uPAR interaction, which emphasizes the importance of the conformational flexibility in the modular assembly of uPAR (24). Importantly, this model enables uPA occupancy per se to act as the driving force for the allosteric regulation of uPAR-assisted lamellipodia formation, as is observed in HEK293 cells (summarized in Fig. 7). One of the important ramifications of this model is the prediction that in theory, it should be possible to engineer a constitutively active uPAR by stabilizing the closed conformation of uPAR. In the present study, we have verified this prediction experimentally by introducing a covalent cross-link (disulfide bond) between DI (position 47) and DIII (position 259). The remarkable efficacy in oxidation of the introduced pair of non-natural cysteines yields a homogenous ensemble of uPAR molecules with a uniform interdomain tethering under normal expression conditions, which renders this mutant optimally suited for studies both in vitro and in vivo.

The restricted conformational space explored by our constrained uPARH47C-N259C mutant does at first sight not affect the real-time binding kinetics for uPA. Scrutinizing the thermodynamic parameters of the uPA-uPAR interaction reveals, nevertheless, different contributions from enthalpy and entropy (supplemental Fig. S1). Although some precaution should always be exercised when translating thermodynamic properties into simplified structural models, it is tempting to suggest that this difference pertains to the pre-assembly of an active hydrophobic ligand-binding cavity in the uPARH47C-N259C mutant.

Another relevant observation is the finding that enzymatic cleavage in the linker region between DI and DII of uPARH47C-N259C does not reduce uPA binding as is the case for uPARwt. It should therefore be interesting to test how this interdomain stabilization affects functions proposed to be regulated by the cleavage of the linker region in uPARwt, e.g. FPR-like-1 receptor-mediated chemotaxis (28, 46) or LRP-mediated internalization (27).

The present demonstration of functional mimicry of the vitronectin binding properties of uPA-uPAR complexes by uPARH47C-N259C has wide implications for our understanding of the regulatory mechanisms for uPAR-assisted lamellipodia formation in cells responding to matrix-embedded vitronectin. In cases where uPA-independent functions of uPAR are reported in genetic studies, e.g. in the development of focal segmental glomerulosclerosis (47, 48), it is reasonable to consider the open form of uPAR as a putative causative agent. In other cases, however, where uPA-dependent functions of uPAR are reported to be independent of plasminogen activation, e.g. in motor neuron remodeling (49) or in adhesion and migration on vitronectin (17, 18, 40), it is reasonable to consider the closed form of uPAR as the active substance. Being an almost perfect surrogate for the “nonproteolytic functions” elicited by the bimolecular uPA-uPAR complexes in adhesion on vitronectin, our uPARH47C-N259C mutant can thus conveniently be used as a stable, mono-component bait in ligand experiments to isolate potential interaction partners involved in the latter processes. A particularly elegant genetic setup to further challenge the nonproteolytic functions of uPA-uPAR complexes in vivo.
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would be to replace the Plaur gene with one encoding an equivalently constrained mouse uPAR and test this transgenic mouse strain after crossing it into a uPA−/− background.

In future attempts to develop selective inhibitors targeting active uPAR, the stable conformation of the constrained uPARH47C-N259C may also be superior: 1) as bait in drug screening programs using fragment-based or small molecule libraries, or 2) as an antigen for developing mAbs recognizing this particularly active conformation of uPAR. As a first challenge, we have used this mutant to successfully solve the first crystal structure of an unoccupied uPAR,3 which has proven elusive for many years. In line with the ANS data reported here, this structure reveals that the stabilized unoccupied uPARH47C-N259C manages to assemble a bona fide

FIGURE 6. Several anti-uPAR mAbs targeting domain interfaces abrogate lamellipodia in uPARwt, but not in uPARH47C-N259C-expressing HEK293 cells. The location of the dominating hot spot for mAb H2 in intact uPAR1–283 is illustrated in panel A. The interaction between immobilized mAb H2 and a serial 2-fold dilution series of purified human recombinant uPAR mutants (range 6–200 nM) was measured by surface plasmon resonance. The determined dissociation rate constants ($k_{\text{off}}$) are shown ($n = 6$) as a function of the positions in the primary sequence of human uPAR DIII192–277 that were individually mutated to alanine (omitting positions occupied by cysteines). Secondary structure elements of uPAR DIII are shown in the upper section following a previously established nomenclature (25), whereas the primary sequence of DIII is shown at the bottom along with the disulfide connectivity and the sequence conservation relative to mouse uPAR (asterisks represent identical residues). Two molecular models are shown to visualize the locations of His$^{47}$ in DI (blue) and Asn$^{259}$ in DIII (green) in the crystal structures solved for uPAR in complex with a peptide antagonist (left) and the amino-terminal fragment of uPA (right). The bound peptide antagonist or ATF are both shown as ribbon diagrams, whereas uPAR is shown in a surface representation with DI, DII, and DIII colored yellow, light blue, and red. The impact of various anti-uPAR mAbs on lamellipodia formation in HEK293 cells transfected with either uPARwt (open bars) or uPARH47C-N259C (filled bars) are shown in panel B. An overview of the topographic location of epitope bins 1 and 2 (blue) relative to the introduced disulfide constraint (His$^{47}$ and Asn$^{259}$ in green) and the SMB binding site is illustrated in panel C using PDB 3BT1 coordinates for the ternary ATF–uPAR–SMB complex.
large, but basically empty, hydrophobic ligand-binding cavity, despite its exposure to the solvent (26). It should, nonetheless, be emphasized that this structure represents the closed conformation of uPAR and that the structure of the unoccupied open conformation of uPAR still remains to be solved. We are currently attempting to define the conformational space explored by unoccupied uPAR<sup>WT</sup> using small angle x-ray scattering (50).

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