A Region in Urokinase Plasminogen Receptor Domain III Controlling a Functional Association with α5β1 Integrin and Tumor Growth*

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Highly expressed urokinase plasminogen activator receptor (uPAR) can interact with α5β1 integrin leading to persistent ERK activation and tumorigenicity. Disrupting this interaction reduces ERK activity, forcing cancer cells into dormancy. We identified a site in uPAR domain III that is indispensable for these effects. A 9-mer peptide derived from a sequence in domain III (residues 240–248) binds purified α5β1 integrin. Substituting a single amino acid (S245A) in this peptide, or in full-length soluble uPAR, impairs binding of the purified integrin. In the recently solved crystal structure of uPAR the Ser–245 is confined to the large external surface of the receptor, a location that is well separated from the central urokinase plasminogen binding cavity. The impact of this site on α5β1 integrin-dependent cell functions was examined by comparing cells induced to express uPAR‡4 or the uPARS245A mutant. Transfecting uPAR‡4 into cells with low endogenous levels of uPAR, inactive integrin, low ERK activity, and a dormant phenotype in vivo restores these functions and reinstates growth in vivo. In contrast, transfection of the same cells with uPARS245A elicits only very small changes. Incubation of highly malignant cells with the wild-type, but not the S245A mutant peptide, disrupts the uPAR integrin interaction leading to down-regulation of ERK activity. The relevance of this binding site, and of the lateral uPAR–α5β1 integrin interaction, to ERK pathway activation and tumor growth implicates it as a possible specific target for cancer therapy.

In a large proportion of patients that undergo surgical removal of primary cancer and who, at the time of surgery, show no detectable disseminated disease, the cancer recurs, sometimes more than decades later. This means that small numbers of cancer cells, undetected by currently available techniques, can persist in a dormant state. The mechanisms that allow them to remain dormant and alive, as well as those causing them to initiate proliferation and form overt metastases, are largely unknown.

We previously reported on a model of human head and neck carcinoma, which retained its partial dependence on extracellular matrix for proliferative signals in vivo (1, 2). These cells express very high levels of both the urokinase plasminogen activator (uPA)§4 and its receptor (uPAR), which cause the activation of the α5β1 integrin, and by recruiting epidermal growth factor receptor, initiate a signaling cascade that leads to persistently high level of extracellular signal-regulated kinase (ERK) and tumorigenicity (3). We have shown that reduction of uPAR levels through stable uPAR-antisense expression forces cells into a state of prolonged dormancy (1, 4–6).

Although uPAR is linked through a glycosylphosphatidylinositol anchor to the surface of cells (7), it has been shown to possess signaling properties (8–10). Because uPAR has no cytoplasmic domain, it became obvious that it must be signaling by partnering with other “competent” receptors. Several such proteins, belonging to different families, such as internalization receptors (low density receptor-related protein and uPAR-associated protein, also called uPARAP/Endo) (11, 12) or growth factor receptors (3, 13) have been identified. Among the interacting partners, integrins belonging to at least three families, β1, β2, and β3 (14–16), have been identified as potential signaling uPAR partners. These latter interactions have been shown to alter a variety of functions, including phagocytosis, adhesion, migration, protease secretion (8–10), and, of specific relevance to the current work, proliferation.

Until recently, the uPAR and integrin interactions have been gleaned from co-immunoprecipitation experiments, fluorescence resonance energy transfer analysis, and co-localization by immunocytochemistry. The integrin (αvβ3) structure has been solved recently (17), facilitating the mapping of the interaction sites with uPAR on both the α and β subunits of several integrins (18–20). Equivalently reliable data for the site on uPAR involved in integrin interaction were missing, in part because until very recently (21, 22) the structure of uPAR has not been solved.

We have previously shown that treatment of cancer cells with a monoclonal antibody (R2), directed to an epitope located in domain III of uPAR, blocked activation of α5β1 integrin by uPAR and strongly reduced signaling to ERK (5). This suggested domain III as a plausible site for interaction with integrin.

Because uPAR is overexpressed in many malignant tumors (23, 24),

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4 The abbreviations used are: uPA, urokinase plasminogen activator; uPAR, uPA receptor; ERK, extracellular signal-regulated kinase; ECL, enhanced chemiluminescence; DOC, deoxycholate; HRP, horseradish peroxidase; FACS, fluorescence-activated cell sorting; FN, fibronectin; CAM, choriocallantoic membrane; GFP, green fluorescent protein; HEp3, human epidermoid carcinoma 3; HEK, human embryonic kidney; suPAR, soluble recombinant uPAR; DIMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; FBS, fetal bovine serum; NHS, N’-hydroxysuccinimide.
and because we showed that the activating event between uPAR and integrin occurs only in cancer cells that express high uPAR levels, we consider this interaction to predominate in cancer and thus to constitute a potentially unique target for cancer therapy. We further argued that a successful intervention to disrupt this interaction might induce tumor dormancy.

In this report we describe a series of experiments that include a test of binding interactions between purified α5β1 integrin and short synthetic uPAR-derived peptides, as well as full-length uPAR, with individual amino acids substitutions. These experiments allowed us to identify a sequence within domain III of uPAR that is crucial for interaction with the integrin. Substituting one of these residues (Ser-245) with alanine impaired the ability of uPAR to enter into functional interactions with the integrin, inhibiting signaling and growth in vivo.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Bovine serum albumin, Me2SO, Triton X-100, sodium orthovanadate, sodium fluoride, and human fibronectin were purchased from Sigma. Aprotinin and trypsin were from ICN Bio- 
medicals, Inc. (Aurora, OH). DMEM, Opti-MEM medium, glutamine, antibiotics, and Lipofectin were from Invitrogen. Fetal bovine serum was from JRH Biosciences (Lenexa, KS); COFAL-negative embryonated eggs were from specific Pathogen-Free Avian Supply (North Franklin, CT); protein G-agarose beads were from Roche Molecular Systems Inc. (Branchburg, NJ); polyvinylidene difluoride membranes and enhanced chemiluminescence (ECL) detection reagents were from Amersham Biosciences; protran nitrocellulose 0.2 μm (Schleicher and Schuell), was from PerkinElmer Life Sciences. The plasmid pIREs-EGFP was from Clontech (Paolo Alto, CA). Anti-phospho-ERK1/2 (anti-phospho-tyr-
204, clone E4) was from Santa Cruz Biotechnology (Santa Cruz, CA), and because we showed that the activating event between uPAR and integrin occurs only in cancer cells that express high uPAR levels, we...

**Cell Lines**—Tumorigenic human epidermoid carcinoma HeP3 (T-HeP3) cells were serially passaged on chorioallantoic membranes (CAMs) of chick embryos as described previously (30). To obtain dormant HeP3 (D-HeP3) cells, the T-HeP3 cells were passaged in culture for 120–170 passages as described (30). D-HeP3 cells express ~20% of uPAR found in T-HeP3 cells (2). HEK293 cells were obtained from ATCC (Manassas, VA). Cells were cultured in DMEM with 10% heat-inactivated FBS, penicillin (500 units/ml), and streptomycin (200 μg/ml).

**Site-directed Mutagenesis and Transfection**—A construct expressing full-length uPAR-cDNA in HindIII site of pcDNA3.1-Hyg from Invitrogen was as described (3). Site-directed mutagenesis (Ser-245 to alanine substitution in domain III of uPAR) was carried out according to manufacturer’s instructions (Stratagene) using pcDNA3.1-uPAR as template and the following two primers: 5'-GCTGTGCAACGGCTCTAATGTGCAA-
CATG-3' (forward) and 5'-CATGTGGCAACATTTTGCGTGGCA-
CACG-3' (reverse). The mutation was validated by DNA sequencing using oligonucleotides 5’-GAGACCTTCTCTGTTC-3’ (forward) and 5’-AATTGAGCAATGCCCT-3’ (reverse) for sequencing. D-HeP3 and HEK293 cells were transiently transfected with empty vector (pcDNA3.1), or uPAR⁵⁵⁴⁵⁴, or uPAR⁵⁵⁴⁵⁴, using FuGENE reagent (3 μl FuGENE/μg of DNA).

**Surface Biotinylation with Sulfo-NHS-Biotin and Cell Lysis**—48 h after transfection, subconfluent monolayers were washed three times with cold PBS, incubated with 5 ml of 0.5 mg/ml sulfo-NHS-biotin from Pierce on ice for 20 min, washed twice with ice-cold PBS, scraped into 1 ml of pre-chilled PBS containing mixture of protease inhibitors, and briefly spun at 4 °C. The pellets were lyzed for 30 min on ice in integral lysis buffer (1% Triton X-100, 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 1 mM orthovanadate, 1 mM sodium fluoride) containing a mixture of proteinase inhibitors, the cell lysates were spun down at 14,000 rpm for 10 min at 4 °C, the supernatants were collected and subjected to immunoprecipitation, SDS-PAGE, and biotinylated protein detection.

**Co-immunoprecipitation of α5β1 Integrin and uPAR**—Cell lysates (0.8 mg of protein) were pre-cleared with protein G-agarose beads pre-incubated with isotype-matched IgG for 45 min at 4 °C on a rolling...
platform, and the supernatants were incubated for 3 h at 4 °C with protein G-agarose beads to which 5 µg of anti-α5β1 (HA5), R3 antibodies, or isotype-matched IgG was bound. The protein G-beads were washed twice with PBS and protease inhibitors and once with 0.1% Nonidet P-40, resuspended in 2X Laemmli sample buffer, heated, separated on SDS-PAGE, transferred onto a polyvinylidene difluoride membrane, probed either with rabbit anti-uPAR polyclonal antibody or streptavidin-HRP, washed, and developed using ECL and scanned with NIH Image. To disrupt the preformed α5β1 integrin-uPAR complex, the pre-cleared cell lysates were incubated for 20 min at 4 °C with either the 240–248 or the mutant S245A peptides (5 and 20 µM), followed by pull-down with anti-α5β1 (HA5) antibody. The rest of the procedures were as in co-immunoprecipitation.

Detection of ERK1/2 and p38 Activation and the Effect of Peptides—To analyze ERK activity, D-HEp3 and HEK293 cells were transiently transfected with pcDNA3.1, uPARwt, and uPARS245A and, 48 h after transfection, the cells were lysed in RIPA buffer (1% Triton X-100, 0.1% SDS, 10 mM Tris, pH 8.0, 140 mM NaCl) for 30 min on ice, the lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatants were analyzed by Western blotting using anti-P-ERK and anti-ERK antibodies. The level of uPAR expression was also tested in the same lysates by Western blotting using R2 antibody. The bands produced by anti-P-ERK and ERK antibodies were scanned by NIH Image, and the ratios of P-ERK to ERK were calculated.

To test the effect of uPAR integrin-inhibiting peptides on ERK activity, T-HEp3 cells transfected with two plasmids that report through GFP level on the state of ERK activation and designated T-ELK (5) were plated in 48-well plates, serum-starved overnight, and treated, in duplicates, with 5 and 25 µM uPAR-derived synthetic peptides (240–248) or 25 µM (17–24) in DMEM for 42 h. Untreated cells served as positive control, and cells not expressing GFP as negative control. The cells were detached and analyzed for GFP by FACS analysis using FACS Canto (BD Biosciences) and FACSDiva software. Alternatively, T-HEp3 cells, serum-starved overnight were incubated in serum-free medium with 5, 20, and 40 µM uPAR-derived synthetic peptide 240–248, 17–24, or S245A for 1 h or with 25 µM peptide 240–248 and 17–24 for 10 min, 45 min, and 3 h at 37 °C. The cells were lysed in radioimmune precipitation assay buffer and processed as described above. Concentration dependence of P-ERK inhibition was analyzed by scanning P-ERK and ERK bands with NIH Image and expressing their ratios as the percentage of the ratio in untreated control (T-HEp3) cells.

Adhesion Assay—HEK293 cells were transiently transfected with pcDNA3.1, uPARwt, or uPARS245A. After 40 h the cells were detached with 4 mM EDTA, suspended in DMEM, and inoculated (1.5 × 10⁶ per well) in a 48-well plate, pre-coated overnight at 4 °C with 4 µg/ml fibronectin, and blocked with 0.1% bovine serum albumin for 1 h at 37 °C. Following 15- and 30-min incubation at 37 °C, the cells were washed twice with PBS with CaCl₂ and MgCl₂, fixed with 1% glutaraldehyde, stained with 1% crystal violet for 10 min, washed, dried, and destained with 10% methanol and 5% acetic acid. Then the optical density of the extracted dye was measured in triplicate using an ELISA microplate reader, ELX800 from Bio-Tek Instruments, Inc. (Shelton, CT) at 570 nm. To analyze the uPA-induced adhesion to fibronectin, uPARwt- and uPARS245A-transfected HEK293 cells were incubated prior to adhesion assay in suspension in DMEM with pro-uPA (10 nM), either in the presence or absence of anti-uPAR antibody R2 (10 µg/ml) for 10 min, washed with DMEM, and plated as above. To test the effect of peptides on adhesion to FN, T-HEp3 and HEK293 cells (the latter transiently transfected with uPARwt) were incubated with DMEM with RGD or RAD peptide (500 µM), as a positive and negative control of the assay) or 240–248 or 17–24 peptide (20 and 200 µM) for 15 min at room temperature, and plated as above.

Detection of Active-β1 Integrin by FACS Analysis—HEK293 cells were transiently transfected with pcDNA3.1, uPARwt, and uPARS245A. After 48 h the cells were detached with 2 mM EDTA in PBS and resuspended in RPMI and aprotinin (20 µg/ml) at 5 × 10⁵ cells/100 µl. Vector-transfected cells were incubated with, or without, MnCl₂ followed by HUTS-4 antibody (1.0 µg). uPARwt- and uPARS245A-transfected cells were incubated with HUTS-4 (1.0 µg) or isotype-matched IgG2b, or R2 (2.0 µg), or isotype-matched IgG1, at 37 °C for 20 min, washed, and incubated with rabbit anti-mouse Alexa 488-coupled IgG (1:400) at 4 °C for 25 min. Finally, the cells were washed and suspended in 400 µl of FACS buffer and analyzed in FACS Canto using FACSDiva software. Cells incubated with the isotype-matched Ig were used to gate the HUTS-4 and R2, respectively.

Detection of Cell-associated Fibronectin by Immunofluorescence Microscopy—HEK293 cells were transiently transfected with uPARwt and uPARS245A and plated on coverslips 5 h after transfection. After 24 h in serum-containing medium, the medium was replaced with medium with 10% FBS-depleted serum supplemented with human FN (30 µg/ml) with or without 20 µg/ml α5β1-blocking antibody (BIIG2). In another set, pro-uPA (15 nM) was added to medium with FBS from which plasminogen was removed and human FN (30 µg/ml) was added. 48 h after transfection the cells were stained with rabbit anti-human FN antibody (Sigma) followed by goat anti-rabbit antibody coupled to Alexa 488, and the nuclei were stained with 4',6-diamidino-2-phenylindole. The images were observed in a fluorescence Nikon Eclipse E600 microscope and photographed with a SPOT-RT™ camera (Spot Diagnostic Instruments, Sterling Heights, MI).

Deoxycytolase-insoluble FN—Cells were prepared as in Fig. 3 except that they were not passaged onto coverslips and nor treated with anti-α5β1 antibody. The methods used to extract FN were as previously described (1), except that no β-mercaptoethanol was added to the sample buffer.

Growth of uPARwt- and uPARS245A-transfected D-HEp3 Cells in Vivo (CAM)—Semi-confluent D-HEp3 cells were transfected with vector alone or with uPARwt or uPARS245A mutant-expressing plasmids, and 36 h later the cells were detached with EDTA, counted and inoculated at 7 × 10⁵ cells per CAM on 4 eggs each, as previously described (1). One dish of each transfec tant was lysed and used to determine uPAR expression by Western blotting using R2 antibody. Four days after inoculation the CAMs were excised and dissociated into single cells with collagenase (1), and the tumor cells, which are larger than the chick embryo cells, were counted. We previously established that only 50% of the cells are recovered from the CAM 24 h after inoculation.

Statistics—Paired t test was used for statistical analysis of data in Fig. 4 (adhesion) and Fig. 6 (growth on CAM).

RESULTS

We have previously shown the uPAR-α5β1 integrin interaction to be crucial for ERK signal activation and in vivo growth of tumor cells. We now present the first identification of a site in uPAR domain III that is indispensable for this function.

A Synthetic uPAR-derived Peptide Binds the α5β1 Integrin—To establish parameters for peptide testing we first examined whether the two full-length-purified proteins, uPAR and α5β1 integrin, interact directly, in a modified solid-phase Dot-ELISA protocol (see “Experimental Procedures”). Increasing concentrations of soluble recombinant uPAR (suPAR) were immobilized on a nitrocellulose membrane and incubated with a constant amount of purified α5β1 integrin. The results...
FIGURE 1. Interaction between α5β1 integrin and immobilized uPAR and uPAR-derived synthetic peptides. A–F, solid-phase Dot-ELISA. A, binding of α5β1 integrin to suPAR and FN. Purified suPAR, human fibronectin, or bovine serum albumin (as a negative control) were immobilized on nitrocellulose at the indicated concentrations as described under “Experimental Procedures” and incubated with purified α5β1 integrin (750 ng/ml). Bound integrin was detected with anti-α5 integrin antibody followed by goat anti-rabbit IgG (H+L) HRP-conjugated secondary antibody and developed with ECL. B, binding of integrin to suPAR is saturable. suPAR (1.5 μg) was immobilized on nitrocellulose membrane in triplicates, incubated with 0, 0.1, 0.5, 1, 2, and 4 μg/ml α5β1 integrin and tested for integrin binding as in A. The dots were scanned with NIH Image. Data are mean ± S.E. C, binding of α5β1 integrin to suPAR peptides. Peptides representing regions located in each of the three uPAR-domains were immobilized, incubated with α5β1 integrin, and analyzed as in A. Only peptide 240–248 binds α5β1 integrin. D, peptide 240–248 inhibits integrin binding to suPAR. The α5β1 integrin (750 ng/ml) was preincubated for 15 min at room temperature with 100-fold molar excess of 240–248 or 17–24 peptides, or with no peptide (Control), and the mixtures were applied to immobilized suPAR (1.5 μg), two dots per mixture, and developed as in A. The dots were scanned with NIH Image. The results (bars) show percentage of control, and the lines show range. E, binding of α5β1 and α3β1 integrin to suPAR and peptide 240–248. Dot-ELISA was performed in duplicate as in A, except that purified α5β1 integrin and α3β1 integrin (750 ng/ml) were used. Bound integrins were detected with the appropriate, previously titrated antibodies followed by goat anti-rabbit IgG (H+L) HRP-conjugated secondary antibody and detection with ECL. F, single amino acid substitution (S245A) in peptide 240–248 eliminates integrin binding. Peptide 240–248 (domain III), 17–24 (domain I), and the S245A mutant of peptide 240–248 were immobilized and tested for integrin binding as in A. G, crystal structure of human uPAR shown as a surface representation. The three individual domains of uPAR are indicated by a color code (DI: wheat; DII: green; DIII: white). The front view illustrates the deep central cavity that constitutes the uPA-binding site, and the back view shows the partial exposure of the residues corresponding to the peptide 240–248 (in blue). The location of the C terminus in uPAR domain III harboring the glycosylphosphatidylinositol anchor is indicated by the white arrow. This figure was created by the program PyMol (DeLano Scientific) using the coordinates 1YWH.
(Fig. 1A) show a dose-dependent increase in band intensity of the bound integrin. As expected, the integrin bound to immobilized fibronectin while there was only background binding to bovine serum albumin (Fig. 1A). A control in which the incubation step with α5β1 was omitted, yielded no detectable binding to either of the immobilized ligands (data not shown). Binding of increasing concentrations of α5β1 integrin to a constant amount of uPAR reached saturation between 1.0 and 2.0 μg/ml integrin (Fig. 1B).

To assess which part of the suPAR protein might be involved in the integrin binding, we took advantage of a collection of synthetic peptides corresponding to regions in human uPAR, which previously were tested for binding to uPAR and vitronectin (i.e. amino acids 17–24: CALGQDLC; 66–74: LTEVVGGLD of domain I; 84–95: AVTYSRSRYLEC; 108–118: GRHSYLCRSP of domain II; and 240–248: GCATASMCQ of domain III) (26). This peptide ensemble was screened individually for their integrin binding capacity. We found that only one of the peptides tested, peptide 240–248, bound α5β1 integrin in a dose-dependent fashion (Fig. 1C). The remaining tested peptides showed no binding (Fig. 1C). To test whether peptide 240–248 can compete for integrin binding to suPAR, the purified α5β1 integrin was preincubated with excess of peptide prior to analysis in a Dot-ELISA. As shown in Fig. 1D, this treatment reduced the integral binding to immobilized uPAR by >70%, whereas peptide in domain I of uPAR (residue 17–24) had only a negligible effect, suggesting that peptide 240–248 represents the integrin interacting site on suPAR. The fact that a sequence in domain III was capable of binding the integrin was not entirely surprising in view of our previous findings showing that a monoclonal anti-uPAR antibody (R2) was able to block the functional outcome of uPAR-integrin interaction in tumor cells (5). Moreover, this antibody recognizes an epitope on an isolated domain III of uPAR as judged by Western blotting and surface plasmon resonance studies.5 This observation has been exploited for immunoaffinity purification of recombinant proteins tagged with uPAR domain III (31). To further probe the specificity of the newly identified interaction, we tested another integrin of the β1-family, the α3β1. A set of control experiments revealed a similar band intensity for α3β1 integrin bound to immobilized laminin-5 and for α5β1 bound to immobilized FN (results not shown), when tested with antibodies titered to produce bands of equal intensity on membrane-immobilized α3β1 or α5β1 integrins. We found that, compared with α5β1 integrin, the purified α3β1 integrin produced dots of much lesser intensity when incubated with increasing concentrations of suPAR or peptide 240–248 (Fig. 1E).

Sequence alignment revealed that the integrin binding synthetic peptide, representing residues 240–248 in human uPAR, contains four positions that are conserved among human, horse, bovine, mouse, and rat uPAR (i.e. Gly-240, Cys-241, Ser-245, and Cys-247). A synthetic peptide with both cysteines replaced by alanines exhibited a reduction in uPAR binding activity (results not shown), but even a more pronounced reduction was found when a single amino acid (Ser-245) was replaced by alanine (Fig. 1F). The difference in integrin binding between peptide 240–248 and S245A shown in Fig. 1F was not due to unequal immobilization of the peptides to the membrane, which bound with the same efficiency, as determined using biotinylated peptides (results not shown). We have taken precaution to assure that the peptides remain in linear form by using only freshly solubilized aliquots and carrying the experiments at room temperature, to slow down the oxidation reaction.

Importantly, as evident from the crystal structure of uPAR (21, 22) and the consensus for the Ly-6/uPAR/α-neurotoxin (32), these two cysteines do not form a disulfide bond in the natural uPAR protein. The integrin-binding peptide sequence we have identified is partly confined to the outer surface of the newly solved crystal structure of uPAR (Fig. 1G). In particular, Ser-245 and Gln-248 occupy a position that is distant from the uPA binding cavity (21).

The Effect of S245A Mutation in the uPAR Protein on Integrin Binding—Because short synthetic peptides, such as 240–248, rarely adopt the three-dimensional structure found in the intact protein from which they are derived, we examined the impact on integrin binding of the S245A mutation in the full-length, purified suPAR. This and other single-site uPAR mutants were generated by site-directed mutagenesis as previously described (27). Using the solid-phase Dot-ELISA assay, we thus compared the binding of purified α5β1 integrin to purified suPARw and suPARS245A. Several additional single-site amino acid uPAR-mutants were also generated and tested. In accordance with the data obtained for the synthetic peptides, suPARS245A also lost the ability to bind α5β1 integrin as assessed by the Dot-ELISA assay (Fig. 2). Moreover, several additional single-site amino acid mutations (M246A, Q248A, and H249A) located in proximity to Ser-245 also affected integrin binding (Fig. 2), while a mutation introduced distant to this site in domain I (suPARR33A) did not cause an impairment of the integrin binding (Fig. 2, left and right panels).

Impairment of integrin binding to these particular suPAR mutants was not the result of a gross misfolding of the proteins, because the rate constants determined by plasmon resonance using immobilized pro-uPA and purified wt or mutant uPAR (Table 1) were very similar. A single site mutant (L66A), previously shown to affect the uPA-uPAR interaction (28), and used here as a control, showed a >5-fold increase in the koff rate compared with uPARw and a corresponding change in free energy of ΔG of 1.35 kcal/mol. In contrast, the single site mutant that did not bind integrin (S245A, Fig. 2A) had only minimal change in koff and free energy as compared with uPARw (Table 1). Moreover,

5 M. Ploug, unpublished observations.
TABLE 1

| Rate constants for the interaction between immobilized pro-uPA and uPAR as measured by surface plasmon resonance |
|---------------------------------------------------------------|
| uPAR mutants | $k_{on}$ | $k_{off}$ | $K_d$ | $\Delta G$ |
|----------------|---------|---------|-------|---------|
| wt             | 3.98 $\pm$ 0.98 | 1.77 $\pm$ 0.31 | 0.46 $\pm$ 0.11 | kcal/mol |
| E33A           | 2.52     | 2.19     | 0.87   | 0.37     |
| L66A           | 2.26     | 10.6     | 4.70   | 1.35     |
| R239A          | 2.33     | 1.48     | 0.64   | 0.19     |
| S245A          | 5.28     | 2.14     | 0.41   | $-0.08$  |
| H249A          | 3.84     | 1.92     | 0.50   | 0.05     |

Several monoclonal anti-uPAR antibodies recognizing conformational-dependent epitopes on uPAR bound equally well to the wt and S245A mutant uPAR (results not shown). Thus, the identification of the sequence, which showed integrin-binding activity in vitro, allowed us to proceed with biochemical and biological experiments in cells and in vivo. We further investigated only the functional role of the S245A mutation.

Glycosylphosphatidylinositol-anchored uPAR has Impaired Ability to Activate α5β1 Integrin—Our published results showed that re-expression of uPAR in cells that also express α5β1 integrin leads to activation of the integrin (1). We therefore tested whether the impaired binding observed for suPAR5245A to purified α5β1 integrin in vitro translates into a loss of its ability to activate α5β1 integrin when expressed in cells. A glycolipid-anchored uPAR5245A was generated by site-directed mutagenesis (see “Experimental Procedures”) and HEK293 cells, which express α5β1 integrin (mean fluorescence 17.3, results not shown) were transfected with a plasmid (pcDNA3.1) encoding either uPARwt or uPAR5245A. Expression levels of uPAR5245A and uPARwt, examined by FACS analysis (Fig. 3A, bottom panel), or Western blot (results not shown), were very similar. The activation state of the endogenous β1 integrin in these cells was assessed by FACS analysis using a conformation sensitive HUTS-4 antibody, which recognizes the active state of β1 integrin. In vector-transfected HEK293 cells, half (50.9%) of the population had fluorescence intensity above the median (Fig. 3A, left panel). Treatment of these cells with Mn$^{2+}$, an established activator of β1 integrins (33), increased the population with fluorescence above the median to 86.2% (second panel from the left). In HEK293 cells expressing uPARwt, 74.4% of the population was above the median (third panel), a value similar to that of the Mn$^{2+}$-treated vector transfected cells, while the uPAR5245A-expressing cells had even fewer cells with active β1 integrins (44.6%) than the vector control (Fig. 3A, right panel). A similar difference in β1 activation produced by uPARwt and uPAR5245A was found in uPARwt- and uPAR5245A-transfected D-HEP3 cells (results not shown).

To examine whether the activated β1 integrin subunit formed an active heterodimer specifically with the α5, and thus produced activated α5β1 integrin, we examined FN binding to the uPAR-transfected cells using immunofluorescence. 24 h after transfection the cells were plated on coverslips, incubated overnight with medium with 10% FBS and then, for the next 24 h, with medium with FN-depleted FBS, supplemented with 30 μg/ml human FN with, or without, 10 μg/ml α5β1 integrin blocking antibody. In addition, because we previously showed that pro-uPA binding increased the signaling capacity of uPAR (2), coverslips with uPARwt- or uPAR5245A-transfected cells were incubated for 24 h in plasminogen-depleted FBS with 15 nM pro-uPA. Cell-associated FN was detected by immunofluorescence, and cell nuclei were identified by 4’,6-diamidino-2-phenylindole staining. Fig. 3B shows that uPARwt-transfected HEK293 cells had much more surface-bound FN than uPAR5245A-transfected cells (top left panel); anti-α5β1-blocking antibodies reduced the fluorescence to a barely detectable level (top middle panel), indicating a specific binding through the α5β1 integrin. Incubation of cells with pro-uPA, which we and others (2, 16, 34, 35) showed to increase uPAR interaction with the integrin, strongly increased the level of cell-bound FN in uPARwt cells (top right panel). In contrast, uPAR5245A-transfected cells had on their surface barely detectable levels of FN (lower left panel), and the integrin-blocking antibodies or pro-uPA had very small impact on the binding, suggesting that the observed binding may not be α5β1-specific. In ~10% of uPARwt-transfected and pro-uPA-treated cells FN was organized into fibrils (results not shown). The difference in FN binding was not due to a difference in FN production, because both uPARwt- and uPAR5245A-transfected cells produced similar levels of FN in Western blot analysis (results not shown) and the medium was supplemented with exogenous FN. Using similar incubation conditions we also compared the level of deoxycholate (DOC)-insoluble FN-fibers in cells transfected either with uPARwt or uPAR5245A. As shown in Fig. 3C, cells transfected with uPARwt had easily detectable level of DOC-insoluble FN, and treatment of cells with pro-uPA increased the level by ~3-fold. In contrast, cells transfected with uPAR5245A produced a very small amount of DOC-insoluble FN that was unaffected by pro-uPA treatment of the cells. These results corroborate the immunofluorescence findings that, under similar conditions, show a reduced ability of uPAR5245A to activate α5β1 integrin, bind fibronectin, and form fibrils (Fig. 3B).

Another indication of α5β1 integrin activation is the enhanced ability of cells to adhere to FN. To compare the effect of uPARwt and uPAR5245A expression on cell adhesion to FN, HEK293 cells were transiently transfected with pcDNA3.1 vector alone, or with a plasmid coding for either uPARwt or uPAR5245A, and the cells were tested for adhesion to FN. As evident from Fig. 4A, compared with vector-transfected cells, the adhesion of cells transfected with uPARwt was 1.9-fold greater at 15 min of incubation and 2.3-fold greater at 30 min. The increase in adhesion for cells transfected with uPAR5245A over vector-transfected cells was only 1.2-fold at both 15 and 30 min.

We and others have previously showed that pro-uPA binding to uPAR strengthens the signaling cascade leading to ERK activation (2, 8) suggesting that it may increase uPAR-integrin interaction. Because the binding affinity of uPA for suPAR is comparable (Table 1), it allowed us to directly compare the effect of pro-uPA on adhesion to FN. HEK293 cells transfected with uPARwt or uPAR5245A were thus preincubated with 10 nM pro-uPA and inoculated on FN-coated wells. Although this treatment significantly ($p = 0.00$) increased adhesion of cells expressing uPARwt, it had no significant effect on the adhesion of uPAR5245A—expressing cells ($p = 0.14$) despite their similar uPAR expression levels (Fig. 4B, inset). Furthermore, cells transfected with uPARwt and treated with pro-uPA spread rapidly (within 15 min) when plated on FN, producing multiple lamellipodia, a phenomenon that was not observed for uPAR5245A—transfected cells (results not shown). Importantly, treatment of the pro-uPA-pretransfected uPARwt—expressing cells with anti-uPAR antibodies reduced adhesion by ~80%, whereas a reduction of only 35% was observed for identically treated, uPAR5245A—expressing cells. This shows that complex formation with uPA enhances the uPAR-dependent cell adhesion. The functional impairment of integrin activation introduced by the uPAR5245A muta-
tion is not neutralized by the complex formation with uPA, arguing that the enhanced adhesion to FN is governed by the uPAR-integrin interplay per se. This conclusion was further confirmed by an experiment in which the effect of disruption of uPAR-integrin interaction by the 240–248 peptide on the adhesion to FN was tested. This was done both in T-HEp3 cells, which constitutively express high uPAR and adhere avidly to FN (2), and in uPARwt-transfected HEK293 cells. As a control for blocking of adhesion, we used an RGD peptide, known for its ability to interfere with integrin interaction with its FN ligand (36); an inactive RAD peptide was used for comparison. As shown in Fig. 4C, 500 μM of the RGD peptide blocked adhesion of both T-HEp3 and HEK293-uPARwt cells to FN. Incubation of both cell types with either 20 or 200 μM of peptide 240–248 reduced adhesion to FN while peptide 17–24 had no effect.

The loss of integrin activation suggests that a single amino acid mutation in domain III of uPAR may abrogate the physical association between uPAR and integrin, necessary for this functional interaction. To test this directly, we examined the effect of the S245A mutation on uPAR-integrin co-immunoprecipitation. D-HEp3 cells were used for this set of experiments because we showed previously that re-expression of the uPARwt returns these cells to a tumorigenic state (1). Cells were transiently transfected with the plasmid pcDNA3.1 (vector control), or plasmids encoding uPARwt or uPARS245A. (Transfection efficiency, determined using pIRES2-EGF plasmid was 30–40%.) Cells were surface-biotinylated, lysed, and immunoprecipitated with antibody to either α5β1 integrin or with anti-uPAR antibody to domain I (R3) (25), separated by PAGE, and blotted with streptavidin. Association of uPAR with the integrin was quantified by scanning the resulting bands and expressing the intensity of uPAR band immunoprecipitated with the α5β1 integrin as percentage of total uPAR precipitated by R3 anti-uPAR antibody. In the experiment shown in Fig. 5, 32% of the total uPARwt, and only 7% of uPARS245A, co-immunoprecipitated with the α5β1 integrin. Only 3% of co-immunoprecipitation was observed in vector-control cells, supporting the
notion that a certain threshold level of uPAR expression is required for uPAR-integrin interaction. We have repeated this experiment six times, using additional anti-uPAR and anti-α5β1 antibodies, and found that, although the percentage of uPARwt that associated with α5β1 varied (20–84%), the strong reduction in uPARS245A co-immunoprecipitation was always maintained (results not shown).

We also showed that the preformed uPAR-integrin complex could be disrupted by incubation of the HEK293-expressing uPARwt cell lysates with 20 μM 240–248, but not with the same concentration of the S245A mutant peptide (Fig. 5B).

**Cells Expressing uPARS245A Have Impaired ERK Activation and Lose Their Ability to Grow in Vivo**—One of the functional hallmarks of the uPAR-integrin interaction is the initiation of a signaling pathway that leads to high levels of phosphorylated ERK allowing cancer cells to form tumors (2, 5). We thus tested whether the compromised activation of α5β1 integrin by uPARS245A is accompanied by a loss of ERK activation. D-HEp3 and HEK293 cells were transiently transfected with either pcDNA3.1 vector alone, or vectors encoding uPARwt or uPARS245A, and analyzed for uPAR expression as well as total and P-ERK content. The expression of uPARwt increased the ratio of P-ERK to ERK 4.2-fold in HEK293 cells relative to vector control and 3.5-fold in D-HEp3 cells. Despite a comparable expression level, uPARS245A transfection caused only a 1.2- and 1.9-fold increase in P-ERK to ERK ratio, respectively, in the two cell lines (Fig. 6A).

We have previously shown that high ERK activity is required for in vivo growth of tumors (9). This implies that cells expressing uPARS245A with reduced ability to activate ERK should be restricted in their in vivo growth. To directly test this assumption we transiently transfected dormant D-HEp3 cells (30) with vector alone, uPARwt, or uPARS245A (transfection efficiency was 40% in all cases leading to very similar expression levels) (Inset, Fig. 6B). Thirty-six hours post transfection the cells were inoculated on CAMs, incubated for 4 days, the CAMs were excised and the tumor cells counted (see “Experimental Procedures”). In accordance with previous experiments (1), D-HEp3 cells transfected with uPARwt completed more than 2 divisions in 4 days on CAM (median 2.2, mean 2.7 divisions) compared with less than 1 division for the mock-transfected cells (median 0.6, mean 0.8). More, importantly uPARS245A-transfected cells exhibited no growth stim-

![Figure 4](image-url)

**FIGURE 4.** The uPARwt but not the uPARS245A mutant enhances adhesion of HEK293 cells to FN. A, uPARS245A does not stimulate cell adhesion to FN. HEK293 cells transiently transfected with pcDNA3.1, or expression constructs for uPARwt or uPARS245A were detached 40 h after transfection and 1.5 × 105 cells were seeded on FN (4 μg/ml in a 48-well plate) for 15 and 30 min at 37 °C. Cell adhesion was quantified as described under “Experimental Procedures.” The results shown are mean (±S.D.) of two individual experiments, four determinations for each cell type. Two additional experiments were performed with similar results. B, effect of uPA and anti-uPAR antibody (R2) on adhesion to FN. HEK293 cells transiently transfected with expression constructs for uPARwt and uPARS245A as in Fig. 4A were incubated in suspension without or with pro-uPA (10 nM). The cells were divided into two aliquots, and one aliquot was incubated for 10 min with anti-uPAR (R2) antibody (10 μg/ml). Cells (1.2 × 105) were seeded onto wells of a 48-well plate, and the adhesion to FN was tested as in A. The results are mean (±S.D.) of six determinations. Pro-uPA treatment of uPARwt cells increased adhesion significantly (p = 0.00 by t test), whereas the increase of adhesion in uPA-treated uPARS245A cells was not significant (p = 0.14). Inset shows uPAR levels determined by Western blot. C, effect of peptides RAD and RGD (500 μM each), and 17–24 and 240–248 (20 and 200 μM for each). Cells were seeded onto 96-well plates, and adhesion to FN was tested as in A. The results are mean (±S.D.) of three determinations for each peptide. Experiment was repeated twice.
uPAR and Integrin

A

Transfection

uPAR wt

IP

α5β1

uPAR E324A

uPAR wt Vector

Anti-uPAR polyclonal

Streptavidin detection

B

uPAR pulled down with α5β1 (%)

C

5 μM

5 μM

5 μM

240–248

FIGURE 5. Physical interaction between uPAR and α5β1 integrin. A, effect of S245A mutation on integrin-uPAR co-immunoprecipitation. D-HEp3 cells were transfected with vector (pcDNA3.1) alone or with constructs expressing uPAR wt or uPAR E324A, and after 48 h the cells were surface biotinylated and lysed as described under “Experimental Procedures.” Half of each lysate (0.8 mg of protein) was immunoprecipitated with 5 μg of monoclonal anti-uPAR (R3) antibody and half with 5 μg of monoclonal anti-α5β1 integrin (HAS) antibody. Isotype-matched IgG served as negative control. The immunoprecipitates were separated on an SDS-PAGE, and the bands were detected with streptavidin-HRP conjugate and ECL. The left single panel shows uPAR wt-transfected cell lysates protein (0.8 mg) immunoprecipitated with anti-α5β1 integrin and probed with a rabbit polyclonal anti-uPAR antibody. The numbers represent uPAR precipitated by anti-α5β1 antibody as a percentage of total uPAR. The experiment was repeated five times using the same and additional anti-integrin and anti-uPAR antibodies with essentially similar results. B, disruption of uPAR and α5β1 integrin complex with peptides 240–248 and S245A. D-HEp3 cells were surface-biotinylated and lysed as described under “Experimental Procedures.” The lysate was precleared with isotype-matched IgG, and aliquots containing 0.8 mg of protein were incubated with peptides 240–248 and S245A (5 and 20 μM) or without peptides (control, C) for 20 min at 4 °C followed by immunoprecipitation with 5 μg of monoclonal anti-α5β1 integrin (HAS) antibody for 90 min. The immunoprecipitates were analyzed as in A and scanned with NIH Image. The bars show mean ± S.E.D. of uPAR pulled down by anti-α5β1 integrin antibody as percentage of control without peptide.

DISCUSSION

We report here that functional interaction between uPAR and α5β1 integrin depends on a stretch of amino acid residues located within domain III of uPAR, and the side chain of Ser-245 in particular is indispensable for these interactions. The identified sequence is located on the large outer surface in the 3-domain crystal structure of uPAR (21, 22) distinct from the central uPA-binding cavity. The engagement of uPAR in lateral interactions with integrins on the cell surface is well established (9, 10), and the binding site for uPAR has been identified on several integrins (37). However, the site on uPAR that binds α5β1 integrin has not been identified. We have previously shown that uPAR-α5β1 integrin interaction initiates a signaling cascade that leads to ERK activation crucial for cancer cells growth in vitro (1). Importantly, we consider this interaction to be a potential target site for anticancer therapy, because disruption of the uPAR-α5β1 integrin interaction forces cancer cells into a state of dormancy.

Although a complete assignment of the functional or structural interface between two interacting proteins is a daunting task, we believe that the convergence of the experimental results obtained by several different approaches provides strong support to the conclusion that the 240–248 stretch of amino acids in uPAR is indeed representing at least a part of the functional binding site for the α5β1 integrin. In the Dot-ELISA only 1 (peptide 240–248) out of 5 peptides tested showed binding activity. This peptide bound only weakly another purified integrin of the β1-family, α3β1, suggesting specificity of the newly discovered site of interaction. However, the remarkable observation that a single amino acid substitution (S245A) in this synthetic peptide 240–248 completely eliminated its ability to bind the integrin lends credibility to its specific role in integrin interaction. Nonetheless, short peptides rarely adopt defined tertiary structures, and very high concentrations generally are needed to obtain these biological effects, calling for precaution in the interpretation of such data. For that reason we also tested full-length, recombinant uPAR proteins with single amino acid mutations within the stretch defined as the site of interaction by the synthetic peptide. We found that several mutations within the 240–248 region, including mutation of S245A, disabled the integrin-binding ability of uPAR. A mutation in domain I of uPAR (E33A) had no effect. We concluded that the loss of integrin binding is not caused by gross aberrant folding of the recombinant uPAR E324A, because this mutant binds uPA with similar affinity as uPAR wt (Table 1) and furthermore binds several monoclonal anti-uPAR antibodies that recognize different conformation-dependent epitopes, with comparable kinetic rate constants as determined for uPAR wt.5 This, and our published result showing that an anti-uPAR antibody that recognizes an epitope in domain III of uPAR (25, 31) disrupts the uPAR-integrin interaction and reduces the signal to ERK (5), fueled further inquiry into the functional relevance of the newly identified sequence.

To probe the biological relevance of the identified interaction site, cells with low or no endogenous uPAR were transfected with uPAR E324A-expressing plasmid. The loss of in vitro binding of the puri-
fied α5β1 integrin to uPAR<sup>S245A</sup> would predict that the two receptors also loose their ability to interact in vivo, when present on the surface of cells. Indeed, we found that anti-α5β1 antibody pulled down 32% of the total cell uPAR<sup>wt</sup> while only a very small fraction (7% or less) of the total uPAR<sup>S245A</sup> was pulled down by anti-α5β1 antibody (Fig. 5 and results not shown). Because we showed that the expression levels of wild-type and mutated uPAR are similar, and that the mutated receptor is properly localized to the cells surface (Fig. 3A), the loss of its association with

**FIGURE 6. ERK activation and growth in vivo.** A, transfected uPAR<sup>wt</sup> produces stronger activation of ERK than uPAR<sup>S245A</sup> (top and middle panels); HEK293 and D-HEp3 cells, transiently transfected with pcDNA3.1 vector, or with an expression construct for uPAR<sup>wt</sup> or uPAR<sup>S245A</sup> were lysed 48 h later and analyzed by Western blotting for phospho-ERK (top panels) and ERK1, -2 (middle panels) with specific antibodies. The bands were scanned with NIH Image. The ratio of phospho-ERK to ERK was calculated and expressed as a fraction of the ratio of pcDNA3.1-transfected cells. Bottom panels, uPAR<sup>wt</sup> and uPAR<sup>S245A</sup> expression determined by Western blotting. HEK293 cells do not express uPAR. B, uPAR<sup>S245A</sup> does not provide a proliferative stimulus in vivo. D-HEp3 cells transfected with vector, or uPAR<sup>wt</sup> or uPAR<sup>S245A</sup> were detached with EDTA 36 h after transfection, and 7 × 10<sup>5</sup> cells in 30 μl of PBS was inoculated onto CAM of 10-day-old chick embryos. Each sample was inoculated onto four CAMs. The CAMs were excised 4 days later, and the number of tumor cells was determined as under “Experimental Procedures.” The bars show mean ± S.D. Shown are paired t test vector/uPAR<sup>S245A</sup> (p = 0.76), vector/uPAR<sup>wt</sup> (p = 0.0005) and uPAR<sup>S245A</sup>/uPAR<sup>wt</sup> (p = 0.0002). Insert: uPAR expression detected by Western blot with R2 antibody. C, peptide 240–248 but not peptide 17–24 down-regulates P-ERK level. T-HEp3 cells were serum-starved and treated in serum-free medium with 25 μM of peptides 240–248 and 17–24 for 45 min and 3 h. The cells were lysed, and the lysates were analyzed for P-ERK (upper panel) and total ERK (lower panel) by Western blotting. D, S245A mutant peptide has no effect on ERK activation. T-HEp3 cells were treated with 5, 20, and 40 μM peptides 240–248 and 17–24, and S245A for 1 h. The cells were processed as in C, except that the bands were scanned by NIH Image, and the P-ERK/ERK ratio was calculated. The results show mean ± S.E. of three determinations. E, effect of peptide 240–248 on ERK activation in T-ELK GFP cells. Sub-confluent T-ELK:GFP cells grown in 48-well plate, serum-starved overnight were incubated with the uPAR peptides 240–248 at 5 and 25 μM and with 17–24 peptide at 25 μM for 40 h at 37 °C in serum-free medium. The cells were detached and analyzed for GFP in FACS Canto using FACSDiva software. The numbers represent GFP-positive cells as percentage of total. The dose response for peptide 240–248 and highest concentration of peptides 17–24 and S245A were repeated two additional times with similar results.
the integrin can be directly ascribed to the S245A and, consequently, Ser-245 must participate in the in vivo interaction of the two proteins.

We previously established that, in cells expressing high levels of uPAR, such as cancer cells, the uPAR α5β1 integrin interaction is responsible for integrin activation (1) as determined by uPAR-induced FN-fibrillogenesis and increased cell adhesion to FN (2). The downstream effect of this interaction is activation of ERK, which is further enhanced by pro-uPA binding to uPAR. To directly compare the ability of uPAR<sup>wt</sup> and uPAR<sup>S245A</sup> to activate integrin, we chose a conformation-sensitive "reporter" antibody HUTS-4, which recognizes an epitope in the hybrid domain (residue 355–425) of the β1-subunit (33). This epitope is unmasked by an "outside in" activation of β1 integrins by Mn<sup>2+</sup> or stimulatory antibodies (33), that cause a downward movement of α7 helix region of the βA domain and "swing-out" of the hybrid region of the β1-subunit (38). We postulated that a putative interaction between uPAR and the surface loop in the α-subunit of integrin might expose the HUTS-4 epitope. Indeed, as shown (Fig. 3A), expression of uPAR<sup>wt</sup>, but not uPAR<sup>S245A</sup>, increased HUTS-4 binding with ~86% of the efficacy of the MnCl<sub>2</sub> treatment, suggesting that uPAR may induce similar conformational change.

A more definitive indication of functional activation of α5β1 integrin was derived from testing of uPAR<sup>wt</sup>-induced FN binding to the cell surface and its enhancement by pro-uPA, which not only increased binding but induced fibrillogenesis in ~10% of cells. Binding of FN was almost completely prevented when cells were treated with blocking antibodies or α5-blocking antibodies. To directly compare the ability of uPAR<sup>wt</sup> and pro-uPA, which not only increased fibrillogenesis, a sign of active integrin, we measured the adhesion induced FN fibrillogenesis, a sign of active integrin activation (1), demonstrating the possibility of a functional interaction "in the trans configuration." Alternatively, it is possible that, in addition to an extended active conformation, a "primed" partially bent state, in which the integrins are capable of ligand binding, also exists (41). Recently (39), investigators using transmission electron microscope have shown that a stable complex of Mn<sup>2+</sup>-bound extracellular domain of αβ3 integrin with FN-type III domains 7–10 displayed compact triangular shape, indicative of bent conformation. Others have earlier suggested that straightening is not required to render integrin competent to bind physiological ligands (42, 43). It is premature to draw any definitive conclusions regarding the mechanism of integrin activation by uPAR but with the identification of an integrin binding site on uPAR and the solving of its structure such analyses may now be possible.

While this manuscript was being prepared, another group has identified a αβ3 integrin binding sequence on domain II of uPAR (44), which was shown to harbor a short chemotactic sequence (GEEG). This peptide could induce migration in a αβ3 integrin and uPAR-dependent manner at 1 pM. Another "chemokine" residing in the linker region between domain I and II of uPAR has previously been described (45). Unlike the sequence in domain III described in this report, whereby a single mutation of S245A produces simultaneous drastic reduction of the interaction with the α5β1 integrin and its activation as well as a reduction in signal transduction to ERK, the sequence in domain II of uPAR was shown to contain distinct interacting and chemotactic sequences (45). Binding of physiological integrin ligands is of relatively low affinity (K<sub>e</sub> in micromolar) (46), presumably to protect integrins from unintended activity. At present it appears that the newly identified sequence in domain II of uPAR might belong to a new category of integrin activators different from the known physiological ligands of integrins.

The uPAR sequence we have identified has a direct impact on our ability to design compounds that will disrupt the uPAR-integrin interaction and will also facilitate a more thorough mapping of the fine details of the uPAR integrin interaction interface by a comprehensive alanine scanning mutagenesis. We have now definitively shown that uPAR and α5β1 integrin interact directly and that a mutation (S245A) that blocks integrin activation renders uPAR<sup>S245A</sup> incapable of efficient ERK activation and, renders tumor cells that express it, incapable of in vivo growth. Moreover, we provided proof of principle indicating that effective targeting of this site of interaction reduces ERK activation and may force malignant cells into dormancy.

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<sup>7</sup>P. Chaurasia, J. A. Aguirre-Ghiso, O. D. Liang, H. Gardsvoll, M. Ploug, and L. Ossowski, unpublished results.
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