Up-regulation of urokinase receptors is common during tumor progression and thought to promote invasion and metastasis. Urokinase receptors bind urokinase and a set of β1 integrins, but it remains unclear to what degree urokinase receptor/integrin binding is important to β1 integrin signaling. Using site-directed mutagenesis, single amino acid mutants of the urokinase receptor were identified that fail to associate with either α5β1 (D262A) or α5β1 (H249A) but associate normally with urokinase. To study the effects of these mutations on β1 integrin function, endogenous urokinase receptors were first stably silenced in tumor cell lines HT1080 and H1299, and then wild type or mutant receptors were expressed. Knockdown of urokinase receptors resulted in markedly reduced fibronectin and α5β1-dependent ERK activation and metalloproteinase MMP-9 expression. Re-expression of wild type or D262A mutant receptors but not the α5β1 binding-deficient H249A mutant reconstituted fibronectin responses. Because urokinase receptor-α5β1 complexes bind in the fibronectin heparin-binding domain (Type III 12–14) whereas α5β1 primarily binds in the RGD-containing domain (Type III 7–10), signaling pathways leading to ERK and MMP-9 responses were dissected. Binding to III 7–10 led to Src/focal adhesion kinase activation, whereas binding to III 7–14 caused Rac 1 activation. Tumor cells engaging fibronectin required both Type III 7–10 and 12–14-initiated signals to activate ERK and up-regulate MMP-9. Thus urokinase receptor binding to α5β1 is required for maximal responses to fibronectin and tumor cell invasion, and this operates through an enhanced Src/Rac/ERK signaling pathway.

The urokinase receptor (uPAR), a glycosylphosphatidylinositol-anchored membrane protein, has been shown to initiate signal transduction and regulate cell proliferation, adhesion, migration, and invasion (1–4). The expression of uPAR on tumor cells strongly correlates with their migratory and invasive phenotype (5–8). Down-regulation of uPAR expression by antisense or RNAi strategies inhibits tumor invasion and metastasis of various cancer types (8–12). But because uPAR has multiple functions, the mechanisms underlying its influence on tumor cell invasion remain incompletely defined.

One mechanism by which uPAR is reported to influence cellular behavior is by associating with signaling molecules and initiating signal transduction (3, 13–15). As uPAR lacks both transmembrane and cytoplasmic domains, uPAR-mediated signaling is thought to require transmembrane partners, particularly integrins (3, 16, 17) and tyrosine kinase receptors such as platelet-derived growth factor receptor and EGFR (18–21). uPAR has been shown to associate with β1, β2, β3, and β5 integrins (17, 22–24). The uPAR binding sites on β1 integrins have been identified (25–27); both domains II and III of uPAR are implicated in the integrin interaction (28, 29). Recently we have shown that uPAR directly binds integrin α5β1 and regulates its conformation and function (25); however, the exact signaling pathway(s) initiated by uPAR/α5β1 integrin interactions remains unclear. Ossowski and co-workers (30, 31) have found that high levels of uPAR expression and its interaction with α5β1 enhanced the basal level of activated ERK favoring tumor growth in vivo, possibly through FAK and Src signaling pathways downstream of the integrin as well as EGFR (19, 32).

Classical studies by Werb et al. (33) established that signaling through the fibronectin receptor induces up-regulation of several MMPs. More recently, signaling mechanisms that underlie these effects and a role for the urokinase-type plasminogen activator (uPA) system in MMP expression have begun to emerge. Cell adhesion on fibronectin (Fn) induces MMP-9 expression, and this induction requires α5β1 integrin (34–36). Src and Src/FAK interactions, as well as Rac activation, have been implicated in integrin-mediated MMP-9 secretion (37, 38). It is also reported that MEK-1-MAPK is required for the Fn-dependent activation of MMP-9 secretion (39), and Rac activation could enhance the association of ERK2 with MEK1, promoting MAPK activity (40). The uPA system and MMP-9 are both overexpressed in malignant tumors and highly correlated with cancer metastasis (41–43). uPA/uPAR is not only important in binding domain of fibronectin; EGFR, epidermal growth factor receptor; WT, wild type; mut, mutant; mAb, monoclonal antibody; pAb, polyclonal antibody; RNAi, RNA interference; FACS, fluorescence-activated cell sorting; HRP, horseradish peroxidase; GFP, green fluorescent protein; siRNA, short interfering RNA; shRNA, short hairpin RNA.
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MMP-9 activation through the uPA-plasmin-MMP-3 cascade (44) but also appears to be involved in regulating MMP-9 production. Suppression of uPAR by an antisense approach in a colon cancer cell line or disruption of uPAR/β1 integrin complex by a uPAR binding peptide, P25, resulted in complete inhibition of pro-MMP-9 secretion and decrease of basal or uPA-induced ERK activation (10). Although uPAR expression is reported to activate Rac and promote cell motility (45), the role of uPAR or uPAR/α5β1 in Fn-induced MMP-9 production has not been explored, and mechanisms underlying Rac activation via uPAR are unknown.

Fn has been shown to regulate multiple cellular functions including gene expression, survival, and cytoskeleton organization through interaction with its principal cell surface receptor, α5β1 (46). Fn contains NH2-, gelatin-, cell-, and COOH-terminal heparin-binding domains. The central cell-binding domain of fibronectin (CBD) has an RGD sequence in domain III 10 recognized by α5β1 integrin, and several sites in the COOH-terminal heparin-binding domain of fibronectin (HepII) also interact with the cell surface with varying affinities (47–49). Our recent data indicate that direct binding of uPAR with α5β1 forms an additional binding site within the HepII that is RGD-independent (25). It has been documented that signaling events are often mediated by two different but adjacent sites within Fn. Fibroblasts plated on the HepII induced formation of filopodia and lamellipodia, whereas cells plated on the CBD require additional signals from HepII to form focal adhesions and stress fibers (50, 51). In addition, in some systems both CBD and HepII cooperatively regulate p125FAK activity, affecting cell survival (52). These observations invite the hypothesis that the second binding site on Fn created by uPAR/α5β1 association is critical for Fn signaling and enhanced protease expression and raise the possibility that invasive tumor cells are addicted to this pathway for their malignant phenotype.

In this study, we tested these hypotheses by generating stable knockdown of uPAR in invasive tumor cell lines of different origins and then reintroducing point mutants of uPAR with selective defects in α5β1 binding. This allowed us to dissociate the capacity of uPAR to bind uPA from its direct binding of α5β1 and to dissect the signaling pathways initiated by direct binding of uPAR to the integrin. We demonstrate that suppression of uPAR expression in tumor cells reduces α5β1/Fn-dependent induction of ERK activation and MMP-9 secretion. Reconstitution of signaling requires expression of uPAR capable of α5β1 binding. Because uPAR/α5β1 binds Fn HepII (III 12–14) whereas α5β1 primarily binds the RGD sequence in Fn fragment III 7–10, we tested whether ERK and MMP-9 responses are binding site-dependent. Our results demonstrate that Fn adhesion-mediated ERK activation and MMP-9 up-regulation require cell engagement with both RGD and HepII sites on Fn or a single fusion protein or as separate polypeptides. For the first time, we show that uPAR is required for maximal α5β1-dependent responses to Fn that promote tumor cell invasion, and this operates through integration of two signals initiated by cell engagement to the HepII and RGD sites on Fn by uPAR-bound and unbound α5β1.
Expression of Wild Type and Mutant uPARs in uPAR Knockdown Cells—To express wild type (WT) or mutant (mut) uPARs in cells already expressing uPAR RNAi, we introduced three silent mutations in the siRNA uPAR-targeting region and used this as the template uPAR cDNA for Alc point mutations. Preliminary experiments varying the number of silent bp substitutions determined that 3-bp substitutions in the targeting region produced optimal expression in the presence of uPAR RNAi. The point mutations on uPAR were generated by site-directed mutagenesis using a PCR megaprimer procedure using Pfu polymerase (Stratagene) and pcDNA-uPAR as a template as described previously (53). All the mutated DNAs containing the desired mutation(s) were introduced into pCEP4 for expression. The PCR-generated sequences of all constructs were verified by DNA sequencing. The uPAR knockdown cells expressing WT or mut (H249A or D262A) uPARs were stably selected and sorted. Protein expression was verified by FACS analysis and/or Western blot.

Adhesion Assay—Cells were seeded onto Fn or Vn (5 μg/ml)-coated plates and incubated in Dulbecco’s modified Eagle’s medium, 0.1% bovine serum albumin with or without peptides for 1 h at 37 °C. After washing, attached cells were fixed and stained with Giemsa. The data were quantified by measuring absorbance at 550 nm as described previously (26).

TagMan Quantitative PCR—Verification of transcript quantity in several selected cDNAs was performed using TaqMan real time PCR. The primer pairs and probe for each cDNA were designed using Primer Express software (Applied Biosystems). The quantification was performed using the standard protocol of ABI PRISM 7700 (Applied Biosystems).

Gelatin Zymography—Cells were starved and seeded on Fn or Fn fragment- (5 μg/ml), Vn- (5 μg/ml), collagen I- (5 μg/ml), or polylysine (50 μg/ml)-coated wells in Dulbecco’s modified Eagle’s medium, 0.01% BSA for 24 h. The conditioned media were collected, centrifuged, and frozen until use. 10 μl of conditioned medium were separated by 10% SDS-PAGE gel containing 1% gelatin (Invitrogen) under non-reducing conditions. The gels were rinsed in renaturing buffer (2.5% Triton X-100); developed in buffer containing 50 mM Tris-HCl (pH 7.7), 5 mM CaCl₂, and 0.02% NaN₃; stained with Coomassie Brilliant Blue to indicate MMP-9 clear bands; and imaged. Recombinant MMP-9 was used as positive control. In some cases, cells were treated with different inhibitors during a 24-h incubation: MEK1 inhibitor PD98059 (10 μM), EGF inhibitor AG1478 (1 μM), and peptide B1P1 or its scrambled control scB1P1 (0.4 μM).

Flow Cytometry—Stable clones expressing siRNA uPAR and WT or mut uPAR were stained with primary antibody to uPAR and secondary alkaline phosphacycin-conjugated anti-mouse IgG (Sigma) and analyzed on a flow cytometer (FACSCalibur®, BD Biosciences). Isolation of various uPAR-expressing cell lines was done by high throughput cell sorting (MoFlo, Dako).

uPAR Ligand Binding Assay—All the procedures were done in triplicate at 4 °C. Control cells, cells with uPAR silencing, and uPAR knockdown cells reconstituted with WT or mut uPAR were plated to form a monolayer, acid-washed, and incubated with NH₂-terminal fragment of uPA 1–48-Fc fusion protein (100 nM). The cells were then incubated with anti-Fc-HRP in Dulbecco’s modified Eagle’s medium, 0.02% BSA for 1 h. After washing, the bound 1–48-Fc was detected by HRP substrates and quantified by measuring A₄₉₀nm.

Western Blot—Control cells and cells with uPAR silencing or uPAR knockdown cells reconstituted with WT or mut uPAR were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS supplemented with protease inhibitors and 1 mM phenylmethylsulfonyl fluoride). Equal amounts of protein were loaded per lane and separated by SDS-PAGE. The protein was transferred to nitrocellulose membrane and blotted for uPAR using primary anti-uPAR mAb and secondary anti-mouse HRP-conjugated antibody. The same membrane was blotted for β-actin as loading control.

Kinase and Rac Activity Assays—Cells were serum-starved for 4 h and seeded on Fn-, Vn-, or polylysine-coated 6-well plates for 20 min. After incubating with or without treatment, cells were lysed in RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Lysates were immunoblotted for phospho-ERK or FAK and total ERK or FAK. For Rac pulldown assays, cells were lysed in cold Rac assay buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1% Triton X-100, 0.5% sodium deoxycholate plus protease inhibitors). Lysates were then incubated with purified glutathione S-transferase-p21-activated kinase 1 protein-p21-binding domain (GST-PAK-PBD) beads (54) for 30 min and washed three times with Rac assay buffer. The bead-bound active Rac and total Rac in the lysates were analyzed by Western blotting with anti-Rac mAb.

Immunoprecipitation—Cells were lysed in Triton lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, and 1% Triton X-100) supplemented with protease inhibitors and 1 mM phenylmethylsulfonyl fluoride. Clarified lysates were immunoprecipitated with antibody to integrin α5 (P1D6) or α3 (P1B5). The immunoprecipitates were blotted for uPAR or integrins (pAb).

Immunofluorescence Microscopy—Cells plated on Fn-coated chambered slides were fixed in 3.7% parafomaldehyde, permeabilized with 0.5% Nonidet P-40, and blocked with 10% horse serum and 1% bovine serum albumin in phosphate-buffered saline. The slides were stained with primary anti-paxillin antibody and Cy3-conjugated secondary anti-mouse IgG antibody or phalloidin-Texas Red. Slides were incubated with 4′,6-diamidino-2-phenylindole before mounting in Prolong (Molecular Probes).

RESULTS
duPAR has been shown previously to regulate α5β1 conformation and binding to Fn (25). In addition, most malignant tumor cells already express high levels of uPAR (55). Thus, we sought to test the role of uPAR on the regulation of α5β1/Fn-mediated signaling in tumor cells expressing uPAR. The expression of endogenous uPAR was knocked down using RNA interference with short hairpin (sh) RNA in HT1080 and H1299 cells by transfection with pSicoR-GFP-shRNA uPAR (uPAR knockdown) or control vector pSicoR-GFP (control). A pool of GFP-positive and low uPAR-expressing cells were sorted, and uPAR expression was inspected by Western blot (Fig. 1A) or FACS (see Fig. 4C). Because both HT1080 and H1299 cells attach to Fn via α5β1 (Fig. 1C), we tested the effect of uPAR
suppression on Fn adhesion. Although uPAR expression does not affect overall Fn adhesion on high concentrations of Fn, reductions in uPAR protein levels resulted in a conversion from RGD-resistant to RGD-sensitive adhesion to Fn (Fig. 1B). These data are consistent with prior observations in tumor cells transiently transfected with synthetic siRNA uPAR (25). Conversely overexpression of wild type uPAR in uPAR knockdown cells restored RGD-resistant Fn adhesion (see Fig. 5B).

It is well known that α5β1-mediated cell adhesion to Fn induces MMP-9 expression (34–36). To assess the impact of uPAR on α5β1-mediated MMP-9 expression in tumor cells, we first compared MMP-9 activation in uPAR knockdown cells. Indeed gelatin zymography revealed uPAR knockdown HT1080 cells failed to induce MMP-9 secretion in conditioned media from uPAR knockdown HT1080 cells (shu) or control cells (ctl) on different matrix proteins. Cells were serum-starved and cultured on polylysine (PL), type I collagen (Col), Vn, or Fn for 24 h in serum-free Dulbecco’s modified Eagle’s medium. The conditioned media were analyzed by gelatin zymography. Clear zones of degradation are seen at 86 kDa as also indicated in the positive control lane using recombinant active MMP-9 (rMMP-9). C, effects of inhibitors on Fn-induced MMP-9 secretion. HT1080 cells were cultured on Fn in the presence of MEK1 inhibitor PD98059 (PD) (10 μM), EGFR inhibitor AG1478 (AG) (1 μM), or peptide β1P1 or its scrambled control, scβ1P1 (0.4 mM). Conditioned media were collected, and gelatin zymography was performed. The following changes were consistently seen in three experiments: Fn-induced MMP-9 secretion was reduced by PD98059 and peptide β1P1 but not AG1478 or scβ1P1. For both C and D, the relative protein in cell lysates was used to normalize the loading, and the data shown are representatives of three independent experiments with similar results.

It is reported that MAPK is required for the Fn-dependent activation of MMP-9 secretion (39). In our MAPK assays of control or uPAR knockdown HT1080 cells on Fn, phospho-ERK was observed to be at least 2-fold higher in control HT1080 compared with uPAR knockdown cells, whereas there was no change in total ERK (Fig. 3A). There were no significant differences in phospho-ERK levels between control and uPAR knockdown cells when they were plated on polylysine or Vn. To further evaluate the potential role of uPAR/α5β1 in the regulation of MMP-9, we examined the expression of MMP-9 and ERK phosphorylation from HT1080 cells plated on Fn in the presence of various inhibitors. EGFR has been reported to co-signal with uPAR/α5β1 complexes to activate ERK in

**FIGURE 1. Stable knockdown of uPAR in human fibrosarcoma HT1080 and lung carcinoma H1299 cells.** A, GFP tagging and Western blot for uPAR. HT1080 and H1299 cells were co-transfected with pcDNA3.1-zeocin and control vector pSicoR-GFP (ctl) or pSicoR-GFP-shRNA uPAR (shu), selected, and sorted for GFP expression (see green cells in upper panel) and uPAR expression. Sorted cells were lysed in RIPA buffer and blotted with uPAR mAb (R2). B, HT1080 and H1299 adhesion to Fn. Cells with silenced uPAR (shu) or control cells (ctl) were seeded onto Fn-coated 96-well plates without or with RGD or RAD peptides (B) or blocking antibodies to integrin α3, α5, or αvβ3 (C). After incubation for 1 h at 37 °C, plates with triplicate determinations were washed, and attached cells were fixed and stained with Giemsa. All the above experiments were performed at least three times with similar results.

**FIGURE 2. Knockdown of uPAR in tumor cells blocks Fn-induced, uPAR/α5β1-dependent MMP-9 production.** A, uPAR and MMP-9 transcription on Fn. uPAR knockdown HT1080 cells (shu) or control cells (ctl) were serum-starved and grown on an Fn-coated surface for 24 h. uPAR and MMP-9 transcripts were analyzed using TaqMan quantitative PCR. Relative copy numbers are expressed as fold change from base-line uPAR knockdown cells (relative copy number = 1). Results shown represent a mean (± S.D.) of three independent experiments. B, MMP-9 secretion in conditioned media from uPAR knockdown HT1080 cells (shu) or control cells (ctl) on different matrix proteins. C, HT1080 Cells

**FIGURE 3.** Western blot analysis of MAPK phosphorylation from HT1080 cells plated on Fn in the presence of various inhibitors. 

**FIGURE 4.** Schematic diagram of uPAR-mediated signaling in tumor cells.
human epidermoid carcinoma HEp3 (T-HEp3) cells (19, 32). However, the EGFR inhibitor AG1478 did not affect either MMP-9 induction or ERK phosphorylation on Fn (Figs. 2C and 3B), indicating that EGFR is not involved in MMP-9 induction in these cells. In contrast, a peptide (β1P1) previously reported to disrupt uPAR\(\alpha_5\)β1 complexes (25) blocked both Fn-induced MMP-9 secretion (Fig. 2C) and ERK activation (Fig. 3B), confirming that the uPAR/α5β1 complex is critical for this event. As expected, the MEK1 inhibitor PD98059 inhibited ERK phosphorylation as well as MMP-9 production, indicating that the ERK pathway is upstream of MMP-9 (Fig. 3B). Collectively these data demonstrate that Fn-induced ERK activation leads to MMP-9 secretion, and this requires uPAR/α5β1 association but not EGFR activation. Completely parallel results were obtained with human lung carcinoma H1299 cells (not shown).

To dissect further the mechanism by which uPAR influences \(\alpha_5\)β1 signaling we screened uPAR point mutants expressed initially in human embryonic kidney 293 cells for uPA and integrin binding in an attempt to find a uPAR mutant capable of binding uPA but not integrins. We took a targeted approach to designing potential uPAR mutants defective in β1 integrin interaction by capitalizing on prior observations that histidines are key residues in a strong uPAR binding peptide (peptide 25; Ref. 17) and in the uPAR binding sequences within integrin α3 or β1 chains as well as on the recent three-dimensional organization of the uPAR crystal structure. We have shown previously that charged residues His-245 and Arg-244 on α3 integrin subunit (56) and Ser-227 on β1 integrin subunit (25) are critical for uPAR binding. We therefore mutated 18 charged groups or serine residues on uPAR that are also located away from the uPA binding site, mostly in domains II and III. Fig. 4A shows a ribbon diagram of uPAR structure using the Protein Data Bank code 1YWH (57) with domain I in blue, domain II in gray, domain III in yellow, and the uPAR binding peptide in red. All of the selected residues were mutated to alanine, and representative uPAR mutants in domains II and III that are expressible are shown in the three-dimensional structure. Human embryonic kidney 293 cells were transfected with a plasmid (pCEP4) encoding either uPAR WT or point mutants generated by site-directed mutagenesis, and uPAR/β1 association was assessed by co-immunoprecipitation of uPAR and α3β1 or α5β1 and ligand binding. Of the mutants generated, only H249A and D262A mutants had clear effects on uPAR/β1 association. Notably the H249A mutation on uPAR almost completely disrupted α5β1 association but not α3β1 association, whereas the D262A mutation only blocked uPAR/α3β1 interaction but not uPAR/α5β1 interaction when expressed in 293 cells. uPA binding in both mutants did not differ from that of WT uPAR as judged by enzyme-linked immunosorbent assays (data not shown). We therefore used the H249A and D262A mutants in all subsequent experiments involving human tumor cells.

To express WT and mutant uPARs in tumor cells with knockdown of endogenous uPAR, three silent mutations were introduced into the siRNA targeting region of WT uPAR in domain II (aa 133–138) (Fig. 4B), and this cDNA was used as a template for the site-directed mutagenesis of uPAR. We then reconstituted uPAR knockdown HT1080 and H1299 cells with WT, H249A, or D262A mut uPARs. Indeed the WT or mut uPARs containing silent mutations in the siRNA targeting region were expressible in uPAR knockdown cells. Cells with similar uPAR levels were sorted, and uPAR expression was further examined by both FACS analysis (Fig. 4C) and Western blot (not shown). To test whether the mut uPARs maintain their ligand binding capacity, we performed uPA binding assays using an amino-terminal fragment of uPA 1–48 Fc fusion protein on control GFP cells, cells with stable uPAR knockdown, and uPAR knockdown cells reconstituted with WT uPAR or H249A or D262A mut uPARs. As shown in Fig. 4D, uPAR knockdown H1299 cells reconstituted with WT or mut uPARs (H249A and D262A) have levels of uPA binding that is slightly higher than or similar to that of control cells consistent with a recent report that these two point mutations do not affect uPA binding to these mutants (58), whereas uPAR knockdown cells showed substantially weaker binding of 1–48-Fc. Similar results were observed in HT1080 cells (data not shown).

To confirm that the H249A mutation on uPAR affects its association with α5β1 in tumor cells, we assessed the uPAR/β1 integrin physical association by a co-immunoprecipitation approach using antibodies to α3 or α5. Consistent with what we had observed in 293 cells (which do not express endogenous uPAR), the H249A mutation on uPAR almost completely disrupted uPAR/α5β1 but not uPAR/α3β1 association in HT1080 cells, whereas the D262A mutation on uPAR only blocked the formation of uPAR/α3β1 complex but did not affect uPAR/α5β1 association (Fig. 5A). Both uPAR/α3β1 and uPAR/α5β1 complexes were detected in uPAR knockdown HT1080 cells reconstituted with WT uPAR.

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### Figure 3. Knockdown of uPAR in tumor cells blocks Fn-induced, uPAR/α5β1-dependent ERK activation

(A) ERK activation on different matrix proteins. uPAR knockdown HT1080 cells (shu) or control cells (ctl) were serum-starved and seeded on polylysine (PL), Vn, or Fn and incubated for 20 min. Cell lysates were then made and immunoblotted with anti-phospho-ERK and anti-ERK antibodies, respectively. B: effects of inhibitors on Fn-induced ERK phosphorylation. Serum-starved HT1080 cells were cultured on Fn for 20 min in the presence of MEK1 inhibitor PD98059 (PD) (10 μM), EGFR inhibitor AG1478 (AG) (1 μM), or peptide β1P1 or its scrambled control scβ1P1 (0.4 μM). Cell lysates were immunoblotted with anti-phospho-ERK and anti-ERK antibodies, respectively. The above experiments were performed at least three times with similar results. P-ERK, phospho-ERK; T-ERK, total ERK.
We have demonstrated that tumor cells such as HT1080 and H1299 cells attach to Fn through α5β1 (Fig. 1C) (25) and to Vn through α3β1 (not shown), both of which require uPAR to bind in a RGD-resistant manner. Therefore, we tested whether disrupting uPAR interactions with α5β1 and α3β1 would have a similar effect on cell adhesion as knocking down uPAR. Indeed...
WT HT1080 cells showed RGD-resistant adhesion to both Fn and Vn (Fig. 5B), and in both cases adhesion was inhibited by a uPAR/β1 integrin blocking peptide, β1P1. On the other hand, H249A or D262A mutants showed RGD-sensitive adhesion to Fn or Vn, respectively. These results indicate that H249A or D262A mutation on uPAR affects tumor cell adhesion specifically by interfering with the formation of uPAR-α5β1 or uPAR-α5β1 complexes, respectively, and not through a uPA-dependent mechanism. The fact that expression of either mutant in HT1080 cells or H1299 cells (not shown) led to complete disruption of an integrin partner in co-immunoprecipitations and altered adhesion in the expected manner confirms that expression of the mutant cDNAs did not lead to a loss of endogenous uPAR suppression by the RNAi.

To evaluate the potential effect of the uPAR H249A mutation on Fn-induced MMP-9 production, we examined MMP-9 secretion in the conditioned medium from uPAR knockdown HT1080 cells reconstituted with WT uPAR or mut uPARs H249A or D262A (Fig. 5C). Gelatin zymography on 24-h conditioned medium showed low expression of MMP-9 in all cells on Vn. Although WT and D262A cells showed high levels of Fn-induced MMP-9 secretion, the induction of MMP-9 was absent in H249A cells (Fig. 5C), further confirming that the uPAR-α5β1 complex is crucial to this process. We also tested the effect of H249A mutation on Fn-induced ERK and FAK phosphorylation. Serum-starved WT or mutant (H249A and D262A) HT1080 cells were cultured on Fn for 20 min, and the cell lysates were immunoblotted with anti-phospho-ERK and anti-ERK pAbs or anti-phospho-FAK and anti-FAK mAbs, respectively. WT and D262A cells showed induction of both phospho-ERK and phospho-FAK when cells were plated on Fn versus on Vn, whereas no induction of ERK activation was detected in H249A cells on Fn (Fig. 5D). Importantly although the H249A mutation showed complete suppression of ERK phosphorylation, FAK phosphorylation was indistinguishable from WT (Fig. 5D), consistent with the known capacity of RGD-mediated ligation of α5β1 by Fn to activate FAK. Phospho-FAK induction in Fn in uPAR knockdown cells was not lower than WT (not shown), further supporting the idea that FAK activation alone does not appear capable of mediating ERK activation or MMP-9 expression. All of the above experiments were also performed in H1299 cells, and the same pattern of results was obtained. Collectively these data demonstrate that uPAR-α5β1 interactions are required for elevated expression of MMP-9 induced by Fn through an ERK-dependent pathway.

Fn fragments (Fig. 6A) including those containing the HepII have been reported to induce MMP production (59–62). Therefore, multiple Fn fragments were tested with HT1080 and H1299 cells for the induction of MMP-9. Serum-starved HT1080 cells were cultured on Fn or Fn fragments (10 μg/ml) at 37 °C for 24 h, and conditioned media were collected. Gelatin zymography revealed that only the Fn fragment III 7–14 that contains both the RGD and HepII induced MMP-9 secretion in HT1080 cells. None of the other fragments we tested including the 70-kDa amino-terminal fragment of Fn, III 7–10 containing only the RGD site, or III 12–14 containing only the HepII site stimulated MMP-9 production (Fig. 6B). In contrast, a mixture of III 7–10 and III 12–14 fragments robustly induced MMP-9 secretion virtually as well as the single peptide containing III 7–14 (Fig. 6B). Increased phosphorylation of ERK was only observed in tumor cells plated on III 7–14 or on mixed III 7–10 and III 12–14 (Fig. 6C, upper panel), indicating that ERK activation also requires both RGD and HepII sites of Fn, consistent with the observed requirement of ERK activation for MMP-9 induction (Figs. 2 and 3).

As expected, FAK phosphorylation was increased in tumor cells plated on Fn III 7–14 or on mixed III 7–10 and III 12–14 (Fig. 6C, lower panel). In addition, the III 7–10 fragment alone, although only containing the RGD site, also largely augmented FAK phosphorylation (~90%), whereas ERK phosphorylation was not increased in cells on this fragment. Of note, tumor cells spread rapidly on the 70-kDa amino-terminal fragment, indicating that cell shape changes induced by spreading alone were insufficient to activate FAK or ERK. All the above experiments were also performed three times in H1299 cells, and similar results were obtained. These data indicate that in addition to a signaling pathway leading to FAK, and likely Src, activation, there must be one or more additional signal(s) generated to activate ERK and up-regulate MMP-9. We turned our attention to a strong candidate for such a second signal, Rac 1.

Rac 1 activation has been reported to be a downstream consequence of uPAR expression, although the signaling pathway underlying Rac 1 activation has not been defined (63). We
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A. H1299 Cells

| ctl | shu | WT |
|----|----|----|
| Actin | Paxillin |

B. HT1080 Cells

| 70kDa | 5-10 | 12-14 | 7-10/12-14 | 7-14 |
|------|------|------|-----------|------|
| A-Rac | T-Rac |

C. HT1080 Cells

| PL | Fn |
|----|----|
| A-Rac | T-Rac |

D. HT1080 Cells

| Fn |
|----|
| P-ERK | T-ERK |

FIGURE 7. Rac and Src/FAK are upstream of ERK mitogen-activated protein kinase pathway that leads to Fn-induced MMP-9 secretion. A, F-actin organization and paxillin localization in H1299 cells. Control GFP cells (ctl), cells with stable uPAR knockdown (shu), and uPAR knockdown cells reconstituted with WT uPAR were cultured on Fn and stained with phalloidin-Texas Red (upper panel) to visualize F-actin or immunostained for paxillin (lower panel) to show focal adhesions. Secondary antibodies were conjugated with Cy3. Cells were imaged using a ×10 oil immersion lens. B, Rac activation is dependent on uPAR/α5β1 interaction and requires both RGD and HepII sites of Fn. Serum-starved HT1080 cells were seeded on Fn or Fn fragments, and uPAR knockdown cells reconstituted with WT uPAR or H249A (H) or D262A (D) mut uPARs were seeded on Fn for 20 min. Rac-GTP (A-Rac) level was determined by using a pulldown assay as described under “Experimental Procedures.” Total Rac (T-Rac) was detected by Western blot using total cell lysates. Representative Western blots from three independent experiments are included. The same experiments were also performed in H1299 cells, and similar results were obtained. C, Rac activity is required for Fn-induced ERK phosphorylation. HT1080 or H1299 cells were transfected with dominant negative Rac (dn) or full-length Rac complexes mediate uPA-dependent Rac 1 activation requires both RGD-containing fragment of Fn (III 7–10) or if cells only expressed a mutant uPAR (H249A) incapable of physical association with α5β1. We interpret these data to indicate that uPAR-dependent Rac 1 activation requires both uPAR/α5β1 association and the engagement of the complex with the Fn heparin-binding domain (III 12–14). Rac 1 activation appears to be upstream of MMP-9 expression because Rac 1 and ERK activation (Fig. 7C) or MMP-9 production (not shown) on Fn are decreased by dominant negative Rac-N17 expression.

In contrast to FAK activation (Fig. 6), Rac 1 activation was not observed when uPAR-expressing cells were plated onto only the RGD-containing fragment of Fn (Fig. 7B). Conversely, plating cells on the Fn heparin-binding domain (III 12–14) alone led to neither FAK nor Rac 1 activation even though binding to III 12–14 proved critical to Rac 1 activation. These observations raised the possibility that Src/FAK activation, which does occur through binding to the RGD-containing fragment of Fn (III 7–10) alone, may be required for subsequent Rac 1 activation. To test whether Src activity is required for Fn-induced Rac 1 activation and MMP-9 secretion, we pretreated HT1080 cells with the Src kinase inhibitor PP2 (5 μM) before each assay. As expected, inhibition of Src activity by PP2 but not its inactive control PP3 almost totally blocked FAK, Rac 1, and ERK activation as well as MMP-9 production on full-length Fn (Fig. 7D). All the above experiments were also performed in H1299 cells with similar results. Altogether these findings indicate that uPAR/α5β1/Fn-dependent activation of both FAK/Src and Rac 1 signaling pathways are required for ERK activation that then leads to the induction of MMP-9 mRNA and active protein production.

DISCUSSION

Numerous studies have implicated uPAR as an important regulator of tumor cell invasion by affecting MMP activation through a protease cascade (44, 65–67). A recent study indicates that uPAR/β1 integrin complexes mediate uPA-induced ERK signaling that leads to an increase in pro-MMP-9 secretion and colon cancer cell invasion (10). However, independent of uPA, uPAR expression has major enhancing effects on cell motility and invasion (1), possibly by activating a Rac/ERK pathway, but with a poorly characterized mechanism (45, 63). We have taken advantage of distinct differences in the mechanism by which α5β1 complexed

noted that compared with WT HT1080 or HT1299 cells plated on Fn, uPAR knockdown cells showed excessive stress fibers. Knockdown cells reconstituted with WT uPAR showed no stress fibers and had altered localization of focal contacts (Fig. 7A), suggesting that uPAR expression affects the balance between active Rac 1 and RhoA and raising the possibility that, consistent with prior observations, uPAR expression leads to Rac 1 activation in these tumor cells. Active Rac 1 is known to suppress RhoA activity (64). Indeed preliminary experiments confirmed that suppression of uPAR expression by RNAi blocked Fn-dependent Rac 1 activation (not shown). Therefore, we explored further the conditions under which uPAR expression promoted Rac 1 activation. Increased GTP-Rac 1 was apparent within 20 min of attachment of HT1080 cells or HT1299 cells (not shown) to full-length Fn (Fig. 7B). However, enhanced GTP-Rac 1 did not develop if cells were plated only on the RGD-containing fragment of Fn (III 7–10) or if cells only expressed a mutant uPAR (H249A) incapable of physical association with α5β1. We interpret these data to indicate that uPAR-dependent Rac 1 activation requires both uPAR/α5β1 association and the engagement of the complex with theFn heparin-binding domain (III 12–14). Rac 1 activation appears to be upstream of MMP-9 expression because Rac 1 and ERK activation (Fig. 7C) or MMP-9 production (not shown) on Fn are decreased by dominant negative Rac-N17 expression.

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with uPAR and α5β1 alone interacts with Fn to reveal novel information about uPAR signaling and its impact on the tumor cell phenotype. Our data show that uPAR-bound and unbound α5β1 trigger two separate signals: one initiated by α5β1 binding to the RGD site and a second initiated by α5β1/uPAR binding to the HepI. Together these cooperate in activating downstream ERK and subsequent MMP-9 gene transcription and protease secretion (summarized as a model in Fig. 8). Binding of α5β1 to Fn III 7–10 activates a Src family kinase and FAK (Fig. 6) and leads to AKT activation within 20 min in either HT1080 or H1299 cells (data not shown). Fn-initiated AKT phosphorylation is independent of uPAR expression and likely dependent upon integrin-linked kinase (68). Under these conditions we could not detect Fn-initiated ERK activation or enhanced MMP-9 secretion (Fig. 2). However, concurrent binding of α5β1/uPAR to the Fn HepII led to a Src kinase-dependent activation of Rac 1 and downstream ERK activation and MMP-9 secretion (Fig. 7). Because α5β1/uPAR binding to HepII alone (III 12–14 in Fig. 6) did not appreciably activate FAK, we favor the view that the primary site of Src/FAK activation is the canonical pathway of α5β1/Fn signaling (as depicted in Fig. 8). Activated Src kinase(s) then activates a currently unknown Rac 1 GTP exchange factor leading to Rac 1 and subsequently ERK activation (69–71). Active Rac 1 has been demonstrated to promote ERK activation by stabilizing its upstream activator, MEK1 (40). The known suppressive effect of Rac 1 activation on RhoA activity may explain the emergence of stress fibers seen with uPAR knockdown in HT1080 and H1299 cells (Fig. 7) (64, 72). Exactly why α5β1/uPAR is required for Rac 1 activation is uncertain, but it may be important that Rac 1 effector function appears critically dependent on its membrane localization to cholesterol-rich microdomains to which the glycosylphosphatidylinositol-anchored uPAR also localizes (73). This point will require further study and clarification.

A second important aspect of our findings is that the signaling response to Fn by tumor cells appears critically dependent on the capacity of uPAR to physically associate with α5β1. This conclusion was revealed by reconstitution of stable uPAR knockdown in two very different tumor cell types, the human fibrosarcoma HT1080 and lung carcinoma H1299 cells, with a point mutant of uPAR showing defective physical association with α5β1. The critical importance of UPAR binding to α5β1 is confirmed by the complete lack of Fn-initiated Rac activation in cells expressing the H249A mutant. Identification of this point mutant (H249A) was achieved by screening a panel of point mutants in domains I, II, and III of uPAR based on clues from prior work as well as recent elucidation of the crystal structure of uPAR (57). While this work was in progress Chaurasia et al. (29) reported a separate point mutant of uPAR, S245A, that also displayed defective binding to α5β1. Interestingly when expressed in tumor cells selected for low levels of uPAR, the S245A mutant showed defective basal ERK activation in comparison with that of WT uPAR expression. Our findings confirm and extend the observations of Chaurasia et al. by validating this region of uPAR domain III as an α5β1 binding region and by defining a signaling pathway by which this binding regulates α5β1 function. Moreover by finding a fundamentally similar requirement for UPAR expression and acquisition of invasive properties in two additional tumor cell lines, these studies raise the possibility that the requirement of UPAR for maximal cellular responses to Fn may be common among invasive cancers.

Surprisingly although the 245–249 region of UPAR domain III appears critical to α5β1 binding, data reported here indicate that this region has no critical role in binding to another β1 integrin, α3β1 (Figs. 2 and 3). Instead we found a separate domain III uPAR point mutant, D262A, to be critically involved in α3β1 interactions but to have no influence on α5β1 association or function. These findings indicate that there is integrin binding specificity for at least two and possibly more integrin α chains in distinct regions of uPAR domain III, implying that integrin binding has evolved as a major function of uPAR. This point is reenforced by recent studies reporting that UPA-initiated signaling is dependent upon UPAR and α3β1 association. Mazzieri et al. (18) reported that UPA-initiated uPAR cleavage required physical association of uPAR with α3β1 and that cleavage led to ERK activation. Similarly Ghosh et al. (78)

**FIGURE 8. Model for induction of ERK activation and MMP-9 production by uPAR-α5β1-Fn complexes.** The model proposes two types of α5β1 signaling. 1) α5β1 engaging the RGD binding site of Fn promotes AKT activation that is likely integrin-linked kinase (ILK)-dependent (68) as well as Src family kinase activation. 2) In the presence of uPAR-α5β1 complexes engaging the heparin binding site (HepII) of Fn, Src activation triggers a Rac 1 pathway that leads to the induction of ERK activation and MMP-9 production. The model proposes that one function of the uPAR-α5β1 complex is to localize active Rac 1 to cholesterol-rich membrane microdomains (hatched area in the diagram). By connecting integrins to the actin cytoskeleton and modifying the relative levels of active RhoA and Rac 1, both pathways cooperate in changing cell shape. Possible pathways of further cross-talk between the Src/FAK and Rac 1 pathways of signaling that may favor higher levels of ERK activation likely operate but are not shown. GEF, GTP exchange factor.
reported that clustering of α3β1 by matrix ligands (or α3 antibodies) led to ERK activation and induction of uPA but only if uPAR was physically associated with α3β1, consistent with other reports that uPAR/integrin interactions regulate proteinase production in a colon cancer cell line. That matrix ligands regulate proteinase production of cells binding to matrix is well documented (33). Although data in this study address only integrin-dependent responses to Fn, collectively, the emerging principle appears to be, at least in transformed cells, that matrix ligands use specific uPAR/integrin interactions to mediate and regulate proteinase production as part of their invasive phenotype.

Prior studies have implicated dual signals from the Fn CBD and the HepII in cellular responses to Fn adhesion. Signals from both the CBD and HepII have been reported to be required for focal adhesion/stress fiber formation (50, 51) and cell survival (52). HepII of Fn has also been reported to induce MMP-1, -2, -9, and -13 when added in soluble form to cultured human cartilage (59). The cellular receptors implicated in binding and responding to the HepII are thought to be primarily the heparin-containing proteoglycans: CD44 and syndecans (51, 59). Data reported here extend this prior work by showing an additional receptor complex involved in HepII-initiated signals: α5β1/uPAR. In contrast with cells on full-length Fn, tumor cells engaging a Fn fragment containing only the RGD site (III 7–10) or only HepII (III 12–14) or those on an Fn fragment lacking both sites (70 kDa) failed to activate Rac 1 and ERK or stimulate MMP-9 expression (Fig. 6). Instead the key requirement for tumor cells expressing a high level of uPAR to respond to Fn appears to be two separate signals from adjacent regions within the III Type III repeats of Fn (Fig. 8). Whether the signal emerging from Fn HepII binding to a proteoglycan is the same or different from that observed here with α5β1/uPAR remains to be defined.

Although our current results do not specifically address the role of uPA and its major inhibitor, plasminogen activator 1, in influencing uPAR-dependent responses to Fn, this protease/protease inhibitor system very likely contributes to the response in other settings. Binding of uPA and plasminogen activator 1 to uPAR-integrin complexes has been shown previously to promote turnover of the complexes (25, 74), potentially leading to cell detachment from Fn and/or modifying the signaling response. Several recent studies indicate that uPA binding to uPAR can lead to ligand-independent tyrosine kinase receptor activity, although the exact mechanism remains uncertain (19, 21). In addition, as uPA is co-expressed with MMPs in HT1080 cells and plays a role in MMP activation (75), it is likely that pro-MMPs initially secreted into the conditioned medium are subsequently activated by the uPA-plasmin system. Similarly human lung carcinoma H1299 cells express high levels of uPA and uPAR (11, 76) and also produce active MMP-9 in the conditioned media on Fn (not shown). Thus there are multiple mechanisms, including direct signaling by uPA cleavage of uPAR (18), by which the uPA/plasmin system could promote tumor invasion in concert with uPAR. Elucidating which of these are most important in an in vivo context and defining to what degree invasion in vivo depends on uPAR remain a challenge of future studies.

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