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# Urokinase Receptors Promote β1 Integrin Function through Interactions with Integrin α3β1

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The urokinase receptor (uPAR) is linked to cellular migration through its capacity to promote pericellular proteolysis, regulate integrin function, and mediate cell signaling in response to urokinase (uPA) binding. The mechanisms for these activities remain incompletely defined, although uPAR was recently identified as a *cis*-acting ligand for the β2 integrin CD11b/CD18 (Mac-1). Here we show that a major β1 integrin partner for uPAR/uPA signaling is α3. In uPAR-transfected 293 cells uPAR complexed (>90%) with α3β1 and antibodies to α3 blocked uPAR-dependent vitronectin (Vn) adhesion. Soluble uPAR bound to recombinant α3β1 in a uPA-dependent manner ($K_d$ < 20 nM) and binding was blocked by a 17-mer α3β1 integrin peptide (α325) homologous to the CD11b uPAR-binding site. uPAR colocalized with α3β1 in MDA-MB-231 cells and uPA (1 nM) enhanced spreading and focal adhesion kinase phosphorylation on fibronectin (Fn) or collagen type I (Col) in a pertussis toxin- and α325-sensitive manner. A critical role of α3β1 in uPA signaling was verified by studies of epithelial cells from α3-deficient mice. Thus, uPAR preferentially complexes with α3β1, promoting direct (Vn) and indirect (Fn, Col) pathways of cell adhesion, the latter a heterotrimeric G protein-dependent mechanism of signaling between α3β1 and other β1 integrins.

## INTRODUCTION

The heterodimeric α and β subunits of the integrin family of adhesion proteins have no intrinsic signaling capacity. Therefore, transduction of information into cells, after engagement of ligands by integrins, is dependent on the dynamic assembly of signaling complexes around their transmembrane and cytoplasmic integrin tails (Diamond and Springer, 1994; Schwartz *et al.*, 1995; Burridge and Chrzanowska-Wodnicka, 1996). These dynamic aspects of integrin function are regulated in part by the interaction of integrins with neighboring nonintegrin membrane-associated proteins, including tetraspan-4-superfamily 1 members (CD9, CD81, CD151, and others) (Berditchevski *et al.*, 1996; Maecker *et al.*, 1997), integrin-associated protein (CD47) (Cooper *et al.*, 1995), caveolin (Wei *et al.*, 1999), and the glycosylphosphatidylinositol-anchored urokinase receptor (uPAR, CD87) (Wei *et al.*, 1996). In all cases reported to date integrin-associated proteins in some way promote integrin signaling, although there is considerable mechanistic diversity. CD47 associates preferentially with αvβ3, promoting signaling through a heterotrimeric G protein-coupled pathway (Frazier *et al.*, 1999). CD151, in contrast, preferentially associates with the β1 integrin partners α3 and α6 and promotes association of a cytoplasmic lipid kinase with these integrins (Berditchevski *et al.*, 1996). Caveolin also associates with a set of β1 integrins, promoting their association with Src family kinases, probably by concentrating cholesterol-rich membrane "rafts" containing these kinases around integrins (Wary *et al.*, 1998; Wei *et al.*, 1999).

The influence of uPAR on integrin function appears complex. In experimental models either high levels of expressed recombinant uPAR or soluble uPAR have been reported to impair ligand binding by integrins and their adhesive functions (Wei *et al.*, 1996). On the other hand, in most cells bearing endogenously expressed uPAR, uPAR, like other integrin-associated proteins, promotes integrin function. For example, we and others have recently reported evidence that signaling through the Fn receptor α5β1, and cell migration on Fn, was promoted by the association of this integrin with uPAR (Aguirre Ghiso *et al.*, 1999; Wei *et al.*, 1999). In

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Abbreviations used: Col, collagen type I; FAK, focal adhesion kinase; Fn, fibronectin; uPA, urokinase; uPAR, urokinase receptor; Vn, vitronectin;.

one study soluble uPAR was found to promote signaling through $\alpha5\beta1$ (Aguirre Ghiso *et al.*, 1999). This is consonant with abundant, more circumstantial observations linking the expression of uPAR with cell migration important to inflammation and tumor metastasis (Bianchi *et al.*, 1996; Andreasen *et al.*, 1997; Ferrero *et al.*, 2000; Huang *et al.*, 2000). Whether the association of uPAR and $\alpha5\beta1$ is direct or indirect is unclear because there has been no structural evidence to explain how uPAR might affect ligand engagement or signaling through integrins.

On the basis of homology with G protein-coupled receptors, Springer (1997) has proposed that the N-terminal region (~450 amino acids) of integrin $\alpha$ subunits folds into a seven-bladed $\beta$-propeller. In this model repeating units (W1-W7) of antiparallel $\beta$ sheets connected by surface loops (~60 aa/unit) arrange into a torus around a small central cavity. The upper surface loops are thought to contain the major ligand-binding sites, which synergize with binding sites on the $\beta$ chain to define the specificity and affinity of interactions of integrins with their ligands. We have recently identified a linear sequence within the $\alpha$ chain of CD11b (Mac-1) ($\alpha$M424–440) that is a critical site for direct interaction between Mac-1 and uPAR (Simon *et al.*, 2000). In the $\beta$-propeller model, this sequence comprises the entire upper loop sequence of the W4 repeat and extends into the third $\beta$ strand of this repeat, indicating that uPAR is an atypical integrin ligand, at least for CD11b. We now extend these findings to $\alpha$ chain partners of $\beta1$ integrins, identifying $\alpha3\beta1$ as a preferential uPAR-binding integrin.

## MATERIALS AND METHODS

### *Reagents*

Prourokinase was a kind gift of Dr. Jack Henkin (Abbott Laboratories, Abbott Park, IL). Human soluble uPAR with or without biotinylation and murine uPA were kindly provided by Dr. Steven Rosenberg (Chiron Corporation, Emeryville, CA). Integrin $\alpha3\beta1$ was purified as described (Eble *et al.*, 1998). Purified integrin $\alpha5\beta1$ was a gift from Dr. Sarah C. Bodary (Genentech, South San Francisco, CA). Human fibronectin (Fn), collagen type I (Col), pertussis toxin, and goat anti-mouse IgG secondary antibody were purchased from Sigma (St. Louis, MO) and vitronectin (Vn) was from BD Biosciences (San Jose, CA). Monoclonal antibodies to integrin $\alpha2$ (P1E6), $\alpha3$ (P1B5), $\alpha5$ (P1D6), and $\alpha5\beta1$ (HA5), and polyclonal anti-$\beta1$ (AB1937) were obtained from Chemicon (Temecula, CA). A monoclonal antibody (mAb) against integrin $\beta1$ (JB1A) was a kind gift from Dr. John Wilkins (University of Manitoba, Winnipeg, MB, Canada). The polyclonal antibody to a G$\alpha$i subunit of heterotrimeric G proteins (G$\alpha$i-3) and the polyclonal antibody to Src family kinases (Src2) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mAb to focal adhesion kinase (FAK) and the mAb to phospho-FAK were obtained from Transduction Laboratories (Lexington, KY). Rabbit anti-uPAR polyclonal antibody was purchased from American Diagnostica (Greenwich, CT). Purified mouse anti-human human leukocyte antigen-A,B,C mAb was obtained from BD PharMingen (San Diego, CA). Cy3 conjugated goat anti-rabbit IgG secondary antibody was from Zymed Laboratories (South San Francisco, CA). Monoclonal antibodies to integrin $\alpha2$ (A2IIE10) and $\alpha3$ (A3x8) and polyclonal antibody to integrin $\alpha3$ were raised in Dr. Martin E. Hemler's lab, and the first two were conjugated with fluorescein isothiocyanate (FITC) with the use of a kit from Molecular Probes (Madison, WI). Peptides $\alpha325$ (PRHRHMGAVFLLSQEAG), sc$\alpha325$ (HQLPGAHRGVEARFSML), $\alpha525$ (PKGNLTYGYVTILNGSD), $\alpha625$ (PRANHSGAVVLLKRDMK), $\alpha v25$ (PRAARTL GMVYIYDGKN), 25, and M25 were synthesized and purified by Quality Controlled Biochemicals (Framingham, MA).

### *Cell Lines and Culture Conditions*

Human embryonic kidney cell line 293 and human carcinoma cell line MDA-MB-231 were obtained from American Type Culture Collection (Rockville, MD). All these cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Urokinase receptor transfected 293 cells were cultured in DMEM complete medium containing 0.9 mg/ml geneticin (G418) (Invitrogen). Immortalized epithelial cells from $\alpha3\beta1$ integrin-deficient kidney (B12) and human $\alpha3$-transfected B12 cells (R10) were obtained and cultured as described (Wang *et al.*, 1999).

### *Adhesion and Spreading Assays*

Cells were seeded in fibronectin- (5 $\mu$g/ml), collagen type I- (5 $\mu$g/ml), or vitronectin (1 $\mu$g/ml)-coated 96-well tissue culture plates to assess adhesion or spreading to these matrix proteins. The cell adhesion assays were performed as previously described (Wei *et al.*, 1996). Briefly, $5 \times 10^4$/ml cells suspended in 100 $\mu$l of DMEM/0.1% bovine serum albumin (BSA) were seeded in triplicate on protein-coated 96-well plates and incubated for 1 h at 37°C, followed by three washes of phosphate-buffered saline (PBS). When performing inhibition assays, integrin $\alpha3$ mAb (P1B5) and $\alpha5$ mAb (P1D6) (5 $\mu$g/ml), pertussis toxin (100 ng/ml), or peptide $\alpha325$ or scrambled peptide $\alpha325$ (10–200 $\mu$M) were used. In some experiments, human pro-uPA (1 nM) was added to MDA-MB-231 cells or murine uPA (10 nM) to R10 and B12 cells. Cells attached to each plate were fixed with methanol and then stained with Giemsa. The data were quantified by measuring absorbance at a wavelength of 550 nm. When performing spreading assays, round and spread cells visualized by phase microscopy were counted from three different areas in each of triplicate wells after incubating with various peptides, antibodies, or urokinase.

### *Flow Cytometry*

Wild-type or uPAR-transfected 293 cells were detached and incubated with PBS containing 0.1% BSA and primary antibodies to integrins $\alpha3$ (P1B5) or $\alpha5\beta1$ (HA5) on ice for 30 min. After washing, cells were incubated with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) and analyzed on a flow cytometer (FACScan; BD Biosciences).

### *Immunoprecipitation and Blotting*

Cells ($5 \times 10^6$) expressing uPAR were lysed on ice for 30 min in 1.5 ml of RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 $\mu$g/ml leupeptin) or Triton lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100), supplemented with protease inhibitors. After preclearing with protein A agarose, lysates were incubated with antibodies to integrins $\beta1$ (JB1A), $\alpha3$ (P1B5), or $\alpha5\beta1$ (HA5) at 4°C overnight. In some experiments, 100 $\mu$M peptides $\alpha325$, sc$\alpha325$, or 25 was added to the lysates. The immunoprecipitates were blotted for uPAR (399R) or G$\alpha$i. In some cases, the membranes were stripped and reblotted for Src family kinases or $\beta1$ integrins. Initial experiments indicated that >95% of the total uPAR was solubilized by both 1% Triton and RIPA buffer. However, ~10% of the total cellular uPAR was coimmunoprecipitated with $\alpha3\beta1$ in either 1% Triton or RIPA buffer.

### *Purified Protein Binding Assay*

Nunc high-binding microtiter plates were coated with purified $\alpha3\beta1$ or $\alpha5\beta1$ (2 $\mu$g/ml) and blocked with 10 mg/ml BSA. Biotinylated

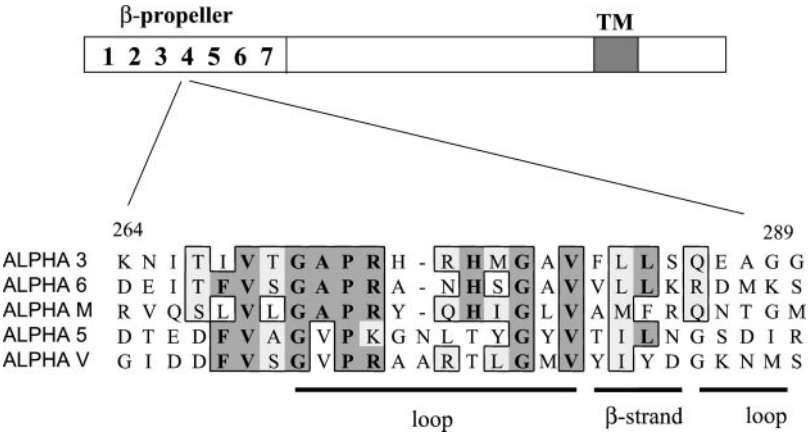
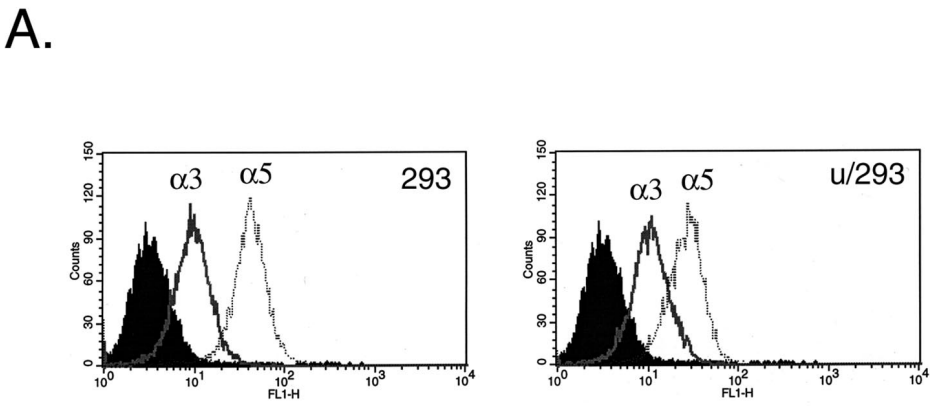



**Figure 1.** Sequence homologies between an $\alpha$M interaction site with uPAR and $\beta$1 integrin-coupled $\alpha$ chains. The N-terminal region (~450 amino acids) of integrin $\alpha$ subunits has been proposed to fold into a $\beta$-propeller (Springer, 1997). In this model repeating units (W1-W7) of antiparallel $\beta$ sheets connected by surface loops (~60 aa/unit) arrange into a torus. A peptide sequence of $\alpha$M spanning W4 was found to mediate binding of $\alpha$M to uPAR (Simon *et al.*, 2000). Homologous amino acid residues in the most similar $\alpha$ chains associating with $\beta$1 integrins are indicated in the figure.

soluble uPAR (suPAR) (1–200 nM) with or without equimolar amounts of pro-uPA was added to each well in PBS/1 mg/ml BSA, and the plates were incubated for 1 h at 25°C. After washing, bound suPAR was quantified with avidin-peroxidase as described (Wei *et al.*, 1994). To test specificity of binding, 100-fold molar excess non-biotinylated suPAR was added. Data were expressed as specific binding, i.e., total binding minus the binding observed in the presence of excess unlabeled suPAR, which accounted for <20% of the total. Binding to wells coated with BSA alone accounted for <10% of the total.



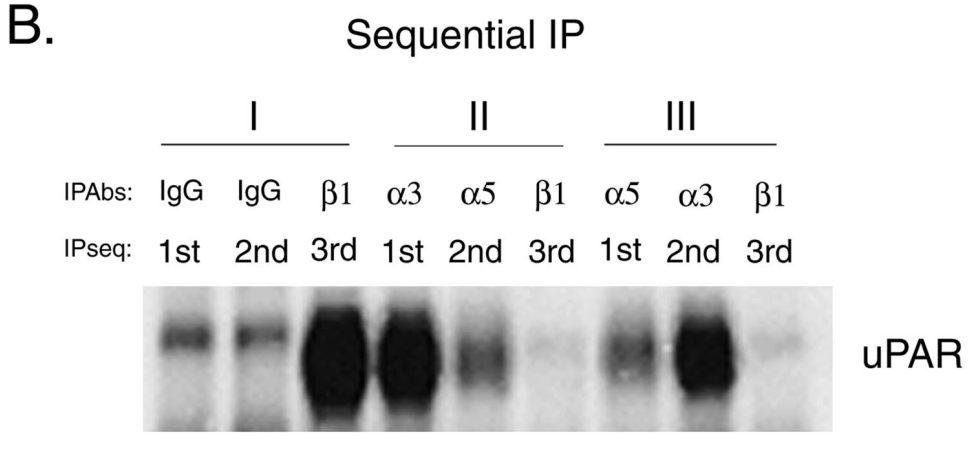




**Figure 2.** uPAR preferentially associates with $\alpha 3\beta 1$ in 293 cells. (A) FACS analysis of $\alpha 3$ and $\alpha 5\beta 1$ integrin expression on 293 and uPAR/293 cells. (B) Depletion of uPAR from $\beta 1$ integrin/uPAR complexes with antibodies to integrin $\alpha 3$ but not $\alpha 5$. uPAR/293 were lysed in RIPA buffer and sequentially immunoprecipitated with antibodies to integrins $\alpha 3$-$\alpha 5$-$\beta 1$, $\alpha 5$-$\alpha 3$-$\beta 1$, or nonimmune IgG-IgG-$\beta 1$. All precipitates were subjected to immunoblot analysis with antibodies to uPAR. Both experiments were performed three times with similar results.

The binding of biotinylated suPAR to peptide α325 was performed as described (Simon *et al.*, 2000). In brief, Nunc microtiter plates were coated with α325 (20 μg/ml) in PBS overnight at 37°C and blocked with 1% BSA. Biotinylated suPAR (100 nM) without or with α325, scα325, α525, or αv25 (1–50 μM) was then added to each well for 1.5 h at 25°C. After washing, avidin peroxidase was added and biotinylated suPAR was quantified as described above. Relative binding was calculated as the ratio of binding in the presence of peptide to binding in the absence of peptide.

### *Immunofluorescence and Confocal Fluorescence Microscopy*

To visualize integrin and uPAR clustering, human breast cancer cells (MDA-MB-231) were trypsinized, recovered in suspension at 37°C for 1 h to allow reexpression of surface proteins, washed with serum-free DMEM, and incubated with antibodies to α3 (P1B5) and control HLA or FITC-conjugated monoclonal antibodies to integrins α2 (A2IIE10) and α3 (A3x8) at 4°C for 30 min. After washing, cells in suspension were incubated without or with goat anti-mouse secondary antibodies for 1 h at 37°C, immobilized on 50 μg/ml polylysine-coated glass coverslips for 30 min, and then fixed 20 min in 3.7% paraformaldehyde. Fixed cells were blocked in 10% goat serum for 1 h and incubated with rabbit polyclonal antibody to uPAR for 1 h at room temperature then incubated with Cy3-conjugated secondary antibodies and coverslips mounted in Prolong (Molecular Probes). Fluorescence staining was analyzed by Zeiss microscope or confocal laser (model MRC1024; Bio-Rad Laboratories, Hercules, CA) attached to a Zeiss microscope (model Axiovert S100) with the use of separate filters for each fluorochrome. Ventral planes were imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) and processed.

### *FAK Kinase Assay*

To analyze FAK activity, cells were seeded on fibronectin- or collagen type I-coated 24-well plates. After incubating with peptides α325 or scα325 and antibodies to integrins α2 or α3, cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with protease inhibitors. Lysates were immunoblotted for phospho-FAK and total FAK.

## RESULTS

### *α3 and α6 Contain Sequences Most Homologous to M25*

The putative structural organization of integrin α chains is indicated in Figure 1. Although no crystal structure for an integrin α chain has been reported, several lines of evidence favor a seven-bladed β-propeller folding pattern for their amino-terminal, ligand-binding region (Irie *et al.*, 1997; Springer, 1997). The position and sequence of an uPAR/CD11b (αM) interaction site within the fourth blade (W4 repeat) of the β-propeller is also shown in Figure 1 (Simon *et al.*, 2000). Surprisingly, comparison of the Mac-1 sequence with sequences of all other integrin α chains in the GenBank database reveals the two integrin α chains with the closest homology to Mac-1 at this site are α3 and α6 (each 40% identical), two integrin chains not previously recognized to be physically associated with uPAR. Figure 1 shows the aligned sequences of the W4 repeat region of α3 and α6 along with the that of two integrins for which indirect evidence has favored a physical association with uPAR (Xue *et al.*, 1997; Aguirre Ghiso *et al.*, 1999). As is evident, the primary sequences of α5 and αv in this region are less homologous than either α3 or α6. Based on this information, we initiated a series of experiments to determine whether the α3 and α6 sequences, termed α325 and α625, respectively, are functionally analogous to the previously reported M25 and whether α3β1 is a major signaling partner of uPAR.

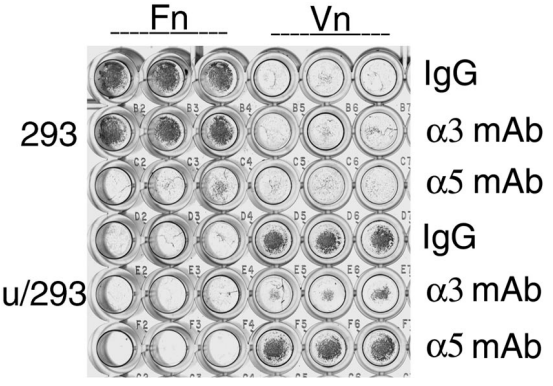



**Figure 3.** Association of integrin α3 subunit with uPAR is required for adhesion to vitronectin. Antibodies to integrin α3 (P1B5) completely inhibited uPAR-dependent adhesion to vitronectin, whereas antibodies to integrin α5 (P1D6) blocked fibronectin receptor-mediated adhesion of 293 cells to Fn. 293 and uPAR/293 cells were seeded to Fn- or Vn-coated wells with or without 2 μg/ml monoclonal antibodies to integrin α3 or α5, or nonimmune IgG. After 1-h incubation, cells were rinsed, and adherent cells were stained with Giemsa. The adhesion assay shown is representative of three independent experiments.

### *uPAR Preferentially Associates with α3β1 in 293 Cells*

To explore whether uPAR physically associates with α3β1 as predicted by sequence homologies (Figure 1), coprecipitation experiments were performed in uPAR-transfected 293 cells. Previous studies have shown that 293 cells only express uPAR after expression by transfection (Wei *et al.*, 1996). We verified that 293 cells express more α5β1 than α3β1 by fluorescence-activated cell sorter (FACS) analysis (Figure 2A). Lysates of uPAR/293 cells were immunoprecipitated sequentially with α3, α5, and β1 antibodies and the precipitates immunoblotted for uPAR. As is evident in Figure 2B, the bulk of β1-associated uPAR coprecipitates with α3, ~90% by densitometric analysis. A small but consistent fraction of uPAR (~10%) was not removed with α3 antibodies but was precipitated with α5 antibodies. After sequential α3 and α5 immunoprecipitations, antibodies to β1 integrin chains recovered little or no uPAR, indicating little uPAR associated with other β1 integrins. Reversing the order of sequential immunoprecipitations (α5 then α3) verified the finding that uPAR preferentially associates with α3β1 in these cells.

Consistent with prior studies, 293 cells were found to adhere avidly to Fn with the use of the classic Fn receptor α5β1. Antibodies to α5 but not α3 completely block adhesion (Figure 3). However, 293 cells expressing high levels of uPAR adhere poorly to fibronectin and instead adhere avidly to vitronectin, with the use of the vitronectin-binding site on uPAR. This adhesion is not blocked by EDTA or Arg-Gly-Asp (RGD) peptides (Wei *et al.*, 1994) or

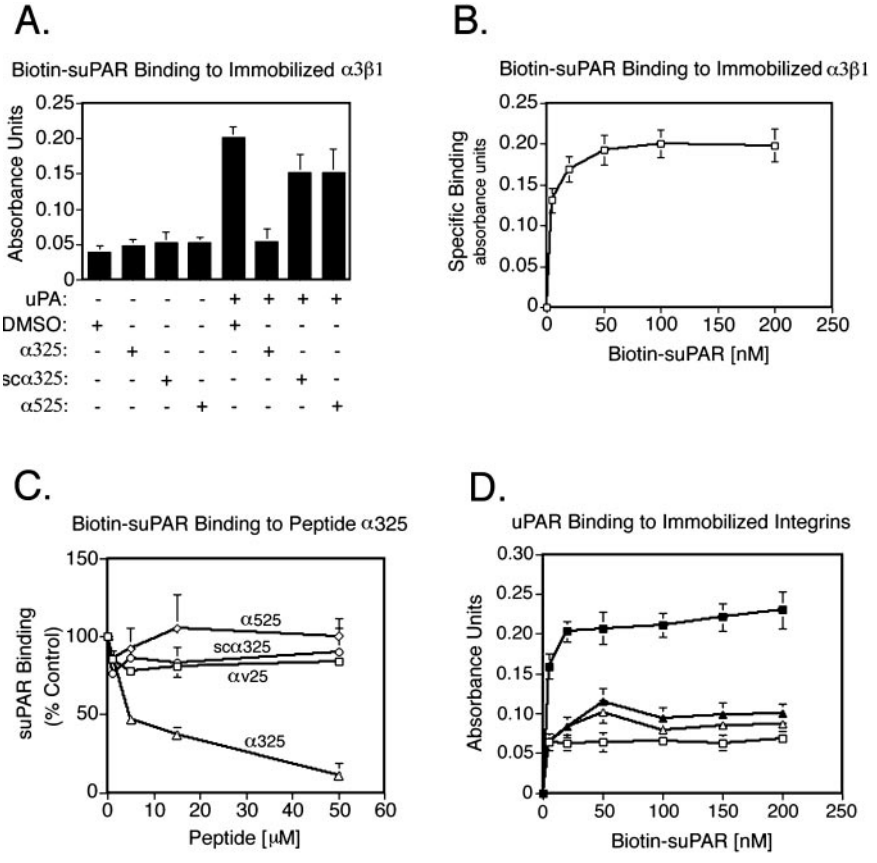



**Figure 4.** Soluble uPAR binds to immobilized, recombinant $\alpha3\beta1$ in a uPA-dependent manner. Binding is blocked by a peptide homologous to the CD11b site. (A) Binding of biotin-suPAR to immobilized $\alpha3\beta1$. $\alpha3\beta1$ (2 $\mu$g/ml)-coated wells were incubated with biotin-suPAR (20 nM) with or without pro-uPA (20 nM) in addition to 100 $\mu$M peptide $\alpha325$ or control peptides $\alpha525$ and sc$\alpha325$. Bound suPAR was detected with avidin-horseradish peroxidase and absorbance measured. Mean ± SD (n = 3). (B) Saturation binding of biotinylated suPAR to immobilized $\alpha3\beta1$ in the presence of pro-uPA. $\alpha3\beta1$-coated wells were incubated with increasing concentration of pro-uPA bound biotin-suPAR (0–200 nM) with or without 100-fold excess unlabeled suPAR to determine specific binding. After washing, bound suPAR was quantified as described above. Mean value of triplicate determinations are given from a representative experiment (n = 3). (C) Binding of biotin-suPAR to immobilized peptide $\alpha325$. Biotinylated suPAR (100 nM) preincubated without or with peptide $\alpha325$ (△), sc$\alpha325$ (○), $\alpha525$ (◇), or $\alpha$v25 (□) (1–50 $\mu$M) was added to microtiter wells coated with $\alpha325$ (20 $\mu$g/ml)). Binding of suPAR in the presence of peptides is expressed as percentage of suPAR binding in the absence of peptides (% control). Values represent averages of triplicate determinations of three separate experiments. (D) Biotin-suPAR binds integrin $\alpha3\beta1$ (squares) with higher affinity than integrin $\alpha5\beta1$ (triangles). $\alpha3\beta1$- or $\alpha5\beta1$-coated wells were incubated with pro-uPA bound biotin-suPAR (0–200 nM) (closed symbols). In some experiments peptide $\alpha325$ (100 $\mu$M) was added (open symbols). Bound suPAR was quantified as described above. Mean ± SD (n = 3).

enhanced by urokinase (Wei, unpublished observation). Given the finding that uPAR predominantly associates with $\alpha3\beta1$ in these cells, we asked whether uPAR-dependent adhesion was blocked by antibodies to $\alpha3$ (P1B5). These antibodies are reported to inhibit $\alpha3\beta1$ function, although they do not block association of uPAR with $\alpha3$ because P1B5 was used to coimmunoprecipitate uPAR and $\alpha3$ (Figure 2). Antibodies to $\alpha3$, but not $\alpha5$, completely blocked uPAR-dependent adhesion to vitronectin, consistent with the finding that uPAR requires an associated integrin to mediate adhesion and that in 293 cells at least this integrin is predominantly $\alpha3\beta1$.

### *uPAR Binds to Immobilized, Recombinant $\alpha3\beta1$*

Although a loop sequence in $\alpha3$ (Figure 1) is most homologous to the previously identified interaction site for uPAR in CD11b (M25), the $\alpha3$ sequence is not very homologous to the original phage display peptide sequence, peptide 25, used to identify M25 in the first place (Simon *et al.*, 2000). This raises questions as to whether the $\alpha3$ sequence, termed $\alpha325$, is really involved in uPAR/integrin interactions and whether the association of uPAR with $\alpha3\beta1$ (Figure 2) is even direct. To address these issues, we examined binding between purified, soluble $\alpha3\beta1$ and purified, suPAR under defined conditions in vitro. In this assay $\alpha3\beta1$ was immobilized on plastic and the binding of biotinylated, soluble uPAR was measured. As indicated in Figure 4A, suPAR binding to $\alpha3\beta1$ was dependent upon uPA. In the presence of uPA, suPAR bound to $\alpha3\beta1$ in a dose-dependent, saturable manner and with high affinity ($K_d < 20$ nM) (Figure 4B). The uPA/suPAR binding to $\alpha3\beta1$ was almost completely abrogated by $\alpha325$ but not by scrambled $\alpha325$ or homologous peptides from either $\alpha5$ (Figure 4A) or $\alpha$v. To determine whether $\alpha325$ itself affects uPA binding to uPAR we measured the capacity of $\alpha325$ and scrambled $\alpha325$ to inhibit binding of suPAR to immobilized uPA (residues 1–48, the receptor binding domain of uPA). In concentrations up to 50 $\mu$M, the highest concentration tested, neither $\alpha325$ nor scrambled $\alpha325$ affected binding of suPAR to uPA (Wei, unpublished observation).

We also tested whether suPAR interacts directly with the $\alpha325$ peptide. The $\alpha325$ peptide was immobilized on plastic and binding of suPAR to the peptide measured in the presence of increasing concentrations of $\alpha325$, scrambled $\alpha325$, or additional peptides as indicated in Figure 4C. suPAR binding to peptide was detectable and blocked only by $\alpha325$ ($IC_{50} = \sim5$ $\mu$M) and not the other peptides tested. Interestingly, unlike suPAR binding to intact $\alpha3\beta1$ (Figure 4A), the binding of suPAR to the $\alpha325$ peptide alone was not influenced by the presence or absence of uPA.

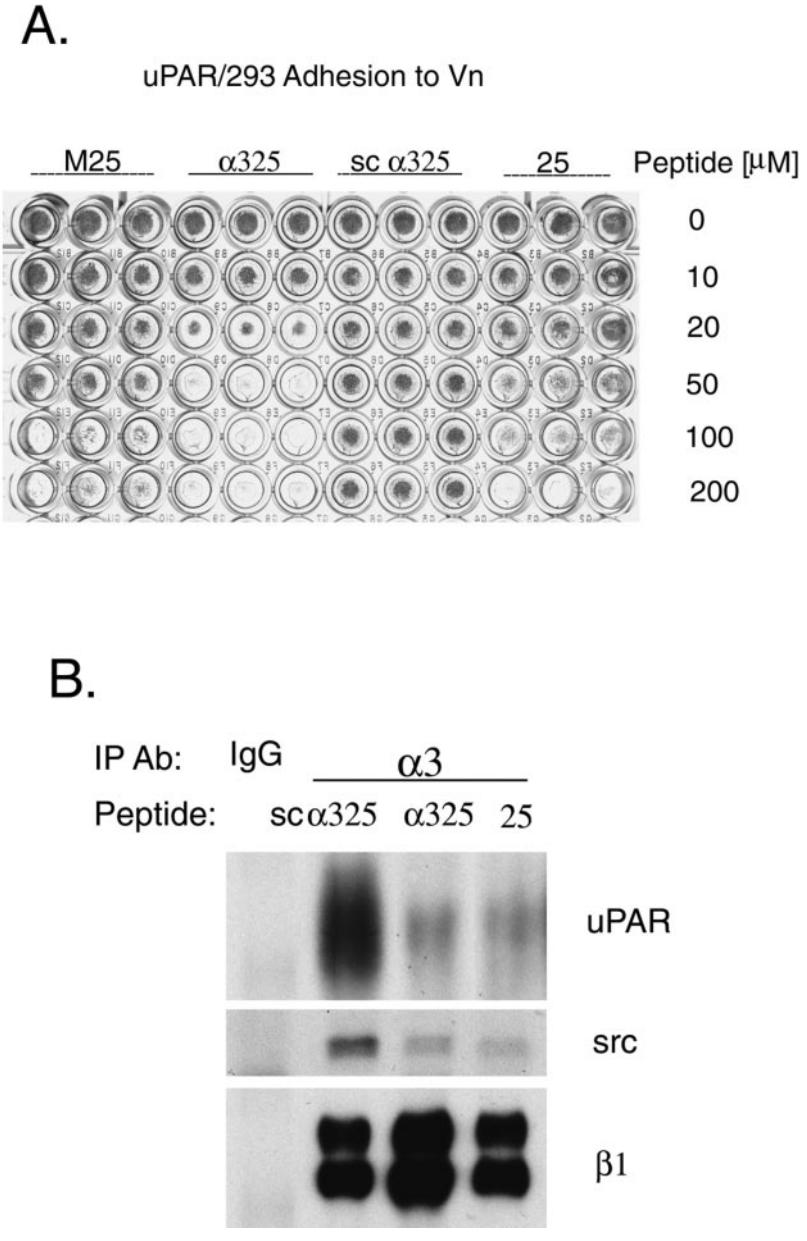



**Figure 5.** Peptides containing integrin $\alpha$ chain W4 loop sequences block uPAR-dependent adhesion to Vn and disrupt physical association between uPAR and $\beta$1 integrins. (A) Dose effect of $\alpha$ subunit peptides on uPAR-dependent adhesion to Vn. uPAR/293 cells were added to Vn-coated wells in the presence of various peptides (0–200 $\mu$M) and the adhesion was performed as described above. Peptides $\alpha$325 and M25 were from W4 loop of integrin subunits $\alpha$3 and $\alpha$M, respectively. Peptide sc $\alpha$325 is a scrambled version of $\alpha$325. Peptide 25 has been reported to disrupt uPAR/$\beta$1 integrin association (Wei *et al.*, 1996). (B) Effect of peptide $\alpha$325 on complex formation between uPAR and integrin $\alpha$3$\beta$1. uPAR/293 cells were lysed in RIPA buffer and incubated with anti-$\alpha$3 mAb (P1B5) in the presence or absence of peptide $\alpha$325, 25, or sc$\alpha$325 (100 $\mu$M). The immunoprecipitates were blotted with polyclonal anti-uPAR (399R), stripped, and reblotted for Src family kinases (Src2) and $\beta$1 (AB1937). Data shown are representative of three independent experiments.

To explore further the direct interaction of uPAR with $\beta$1 integrins, the binding of suPAR to immobilized $\alpha$3$\beta$1 was compared with $\alpha$5$\beta$1. suPAR/uPA binding was greater to immobilized $\alpha$3$\beta$1 than to $\alpha$5$\beta$1 (Figure 4D), and only binding to $\alpha$3$\beta$1 was blocked by $\alpha$325. A limitation of this analysis is that, although as judged by micro bicinchoninic acid protein assay (Pierce, Rockford, IL) equivalent integrins bound to the plastic, $\alpha$5$\beta$1 was purified from tissues, whereas purified $\alpha$3$\beta$1 had been expressed in soluble form. The folding on plastic of these two proteins could be different. Nonetheless, together these data indicate that uPAR directly binds $\alpha$3$\beta$1 in a uPA-dependent manner and that this binding, like that to CD11b, involves a W4 loop peptide ($\alpha$325) in the $\beta$-propeller region. The lack of effect of uPA on the direct interaction between uPAR and the $\alpha$325 peptide suggests that this loop sequence is only a part of the overall interaction between uPAR and $\alpha$3$\beta$1.

Consistent with these in vitro data, $\alpha$325 was found to have similar functional and biochemical properties to those previously described for 25 and M25, the homologous sequence in CD11b (Simon *et al.*, 2000). The 17-mer $\alpha$3 peptide ($\alpha$325) blocked uPAR-dependent adhesion to Vn in uPAR-transfected 293 cells in a dose-dependent manner, with and $IC_{50}$ value of ~25 $\mu$M (Figure 5A) and at 100 $\mu$M blocked coprecipitation of Src family kinases with $\alpha$3$\beta$1 integrins in these cells (Figure 5B). Neither peptides from $\alpha$5 or $\alpha$v nor scrambled versions of $\alpha$3 had any activities in these assays. Thus, the physical and

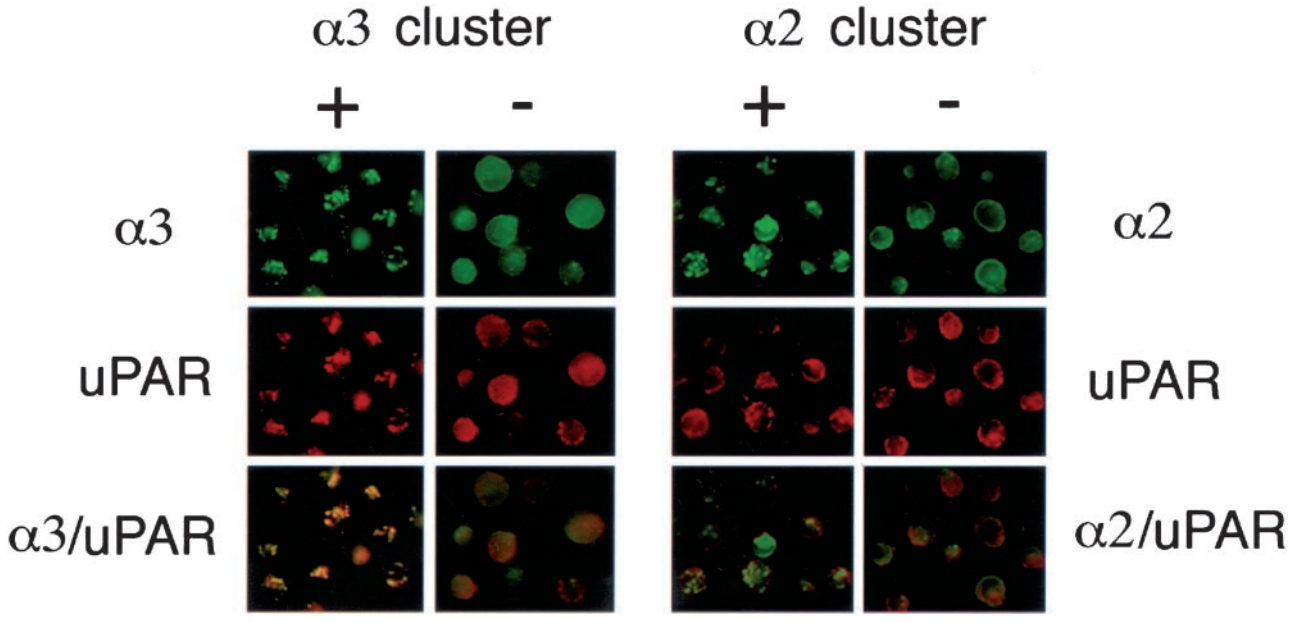



**Figure 6.** Integrin α3β1 and uPAR cocluster. Human breast cancer MDA-MB-231 cells were stained in suspension with FITC-A3x8 antibodies to integrin α3 or FITC-A2IIE10 antibodies to integrin α2. Cells were then clustered (+) with goat anti-mouse secondary antibodies or left nonclustered (−). After immobilization to polylysine-coated coverslips, cells were fixed and stained with polyclonal uPAR antibodies, and Cy3-conjugated anti-rabbit secondary antibodies were used to visualize uPAR. Substitution of FITC-conjugated control antibodies to α4 and rabbit IgG for primary antibodies resulted in much less or no immunostaining. Integrin (green), uPAR (red), and sites of colocalization (yellow).

functional effects of α325 appear nearly identical to that of M25 and peptide 25, confirming that the W4 repeat of α3 is probably an interaction site for uPAR paralleling that described for Mac-1.

### *MDA-MB-231 Cell Spreading on Fn and Col Is Regulated by α3β1 and uPA/uPAR*

We next asked whether uPAR/α3β1 interactions regulate integrin function in nontransfected cells. MDA-MB-231 cells are known to express uPAR (Solberg *et al.*, 1994) and a set of β1 integrins, including α2, α3, α5, α6, and αv (Morini *et al.*, 2000; Gui *et al.*, 1995; Lundstrom *et al.*, 1998; Meyer *et al.*, 1998). These cells migrate in vitro on the expected extracellular matrix proteins and grow and metastasize in vivo (Holst-Hansen *et al.*, 1999; Kruger *et al.*, 2000). Because the level of expression of uPAR in these cells is much lower than that of transfected 293 cells, we explored a possible interaction between uPAR and α3β1 functionally rather than biochemically. The α3β1 on MDA-MB-231 cells was clustered with secondary antibodies and then the distribution of uPAR determined by confocal microscopy. Clustering of α2β1 and MHC class I molecules served as controls. Clustering of α3β1 but not α2β1 resulted in dramatic coclustering of uPAR (Figure 6). Clustered HLA class I molecules also had no effect on uPAR distribution. These data confirm that uPAR associates preferentially with α3β1 in MDA-MB-231 cells.

The influence of uPAR and α3β1 on spreading of MDA-MB-231 cells on various extracellular matrix-coated surfaces was next tested. Although MDA-MB-231 cells attach and spread on Fn and Col with the use of α5β1 and α2β1, respectively, spreading on Fn occurs relatively slowly >2 h at 37°C. The addition of recombinant human urokinase (pro-urokinase), enhanced spreading of MDA-MB-231 cells on both Fn and Col (Figure 7A). uPA-stimulated spreading was blocked by α325 but not controls. As expected, antibodies to α5 and α2 caused cellular detachment from Fn and Col, respectively. When cells were plated on vitronectin, uPA did not enhance spreading. Remarkably, antibodies (P1B5) to α3 but not antibodies to α2 or α5 were also found to enhance the rate of spreading of MDA-MB-231 cells on Fn or Col (Figure 7A). The enhancing effect of α3 ligation with antibodies was again abrogated by the addition of α325 in a dose dependent manner. These functional effects of α325 were mirrored by biochemical effects on FAK phosphorylation (Figure 7B). The addition of uPA (Figure 7B) or α3 antibodies caused enhanced tyrosine phosphorylation of FAK as measured 30 or 15 min after addition of uPA or antibodies to MDA-MB-231 cells plated on either Fn or Col at 37°C. Again α325, but not scrambled α325, abrogated increased FAK phosphorylation on either surface, suggesting the enhancing effects of uPA or α3β1 ligation on Fn and Col spreading require association of uPAR and indicating that α3β1 regulates the spreading response to engagement of α5β1 and α2β1 by their cognate ligands. Consistent with these observations, MDA-MB-231 cells exposed to uPA in suspension, after plating on polylysine-coated surfaces, or after plating on vitronectin failed to increase FAK phosphorylation (Wei, observation). In MDA-MB-231 cells the major vitronectin adhesion receptor appears to be αvβ5 rather than a β1 integrin (Meyer *et al.*, 1998).

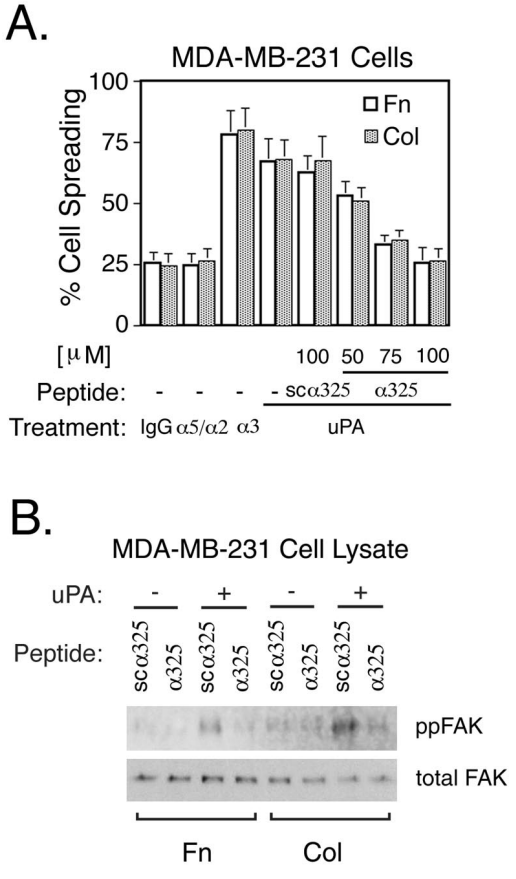



**Figure 7.** Urokinase promotes $\beta$1 integrin function of MDA-MB-231 cells. (A) Cell spreading. MDA-MB-231 cells were seeded into Fn- or Col-coated wells and human urokinase, various antibodies, and/or peptides (50–100 $\mu$M) added as indicated in the figure. Cells were incubated for 30 min on Fn and 15 min on Col and spread cells were then counted. Data are expressed as percentage of cells spread; mean ± SD, n = 3. (B) FAK phosphorylation. MDA-MB-231 cells were incubated without or with pro-uPA (1 nM) and peptides (100 $\mu$M) on Fn for 30 min or on Col for 15 min. Cell lysates were analyzed for FAK phosphorylation by monoclonal anti-phospho-FAK antibody (top). The same membrane was stripped and blotted for total FAK (bottom). This experiment has been conducted three times with similar results. P1B5-induced FAK phosphorylation was also blocked by peptide $\alpha$325.

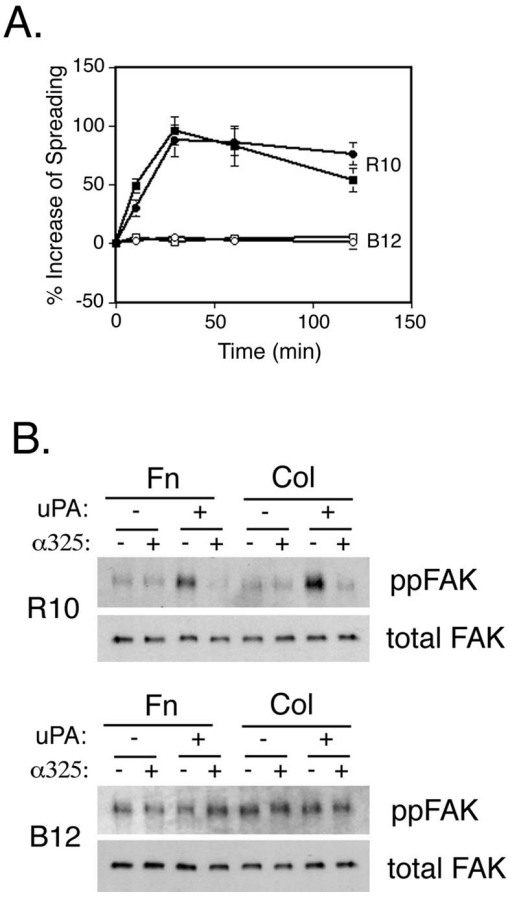



**Figure 8.** Urokinase-induced, $\beta$1 integrin-dependent spreading and FAK phosphorylation on Fn and Col requires $\alpha$3-reconstitution of $\alpha$3 knockout cells. (A) Cell spreading. Epithelial kidney cells derived from integrin $\alpha$3-deficient mouse (B12) and human $\alpha$3-transfected R10 cells were plated on Fn (5 $\mu$g/ml) (circles) or Col (5 $\mu$g/ml) (squares) in the presence or absence of murine uPA (10 nM). Spread cells were counted at different time point within 120 min. Data are expressed as percentage of increase of cell spreading by uPA. Value represent mean ± SD (n = 3). (B) FAK phosphorylation. B12 and R10 cells were incubated with or without murine uPA (10 nM) and peptides (100 $\mu$M) on Fn and Col for 2 h. Cell lysates were analyzed for phospho-FAK and total FAK. This experiment was performed three times with similar results.

### *Murine Kidney Epithelial Cell Spreading in Response to uPA Requires Presence of $\alpha$3*

To test further whether uPAR interactions with $\alpha3\beta1$ regulate the function of other $\beta$1 integrins, immortalized kidney epithelial cells derived from $\alpha$3-deficient mice were examined (Wang *et al.*, 1999). The influence of uPA on the Fn spreading response of both $\alpha3-/-$ cells (B12) and $\alpha3-/-$ cells reconstituted with human $\alpha$3 (R10) was tested. Of note, the baseline spreading response of the $\alpha3-/-$ cells to Fn was at least twofold greater than that of $\alpha$3-reconstituted cells, consistent with prior studies (Lichtner *et al.*, 1998). The addition of 10 nM uPA clearly induced spreading of the $\alpha3\beta1$-reconstituted cells, whereas uPA had no effect on spreading of $\alpha3-/-$ cells. Murine uPA enhanced spreading of the $\alpha3\beta1$-reconstituted cells 80–160% on Fn and 50–140% on Col within 120 min of plating (Figure 8A). Accordingly, uPA increased FAK phosphorylation >2-fold in R10 cells at 120 min and this effect was blocked by the $\alpha$325 peptide but not control (Figure 8B). The addition of uPA had no effect on the FAK phosphorylation state of B12 cells, which consistently had higher baseline phosphorylated FAK. Comparable amounts of uPAR were detected in B12 and R10 cells by semiquantitative PCR and by FACs analysis with the use of murine uPA-FITC.

### *Coprecipitation of G$\alpha$i and Src family Kinases with $\alpha3\beta1$ Is Blocked by $\alpha$325*

The enhancing effect of urokinase on Fn or Col spreading, but not basal adhesion, is completely blocked by the addition of pertussis toxin (Figure 9A). Spreading induced by

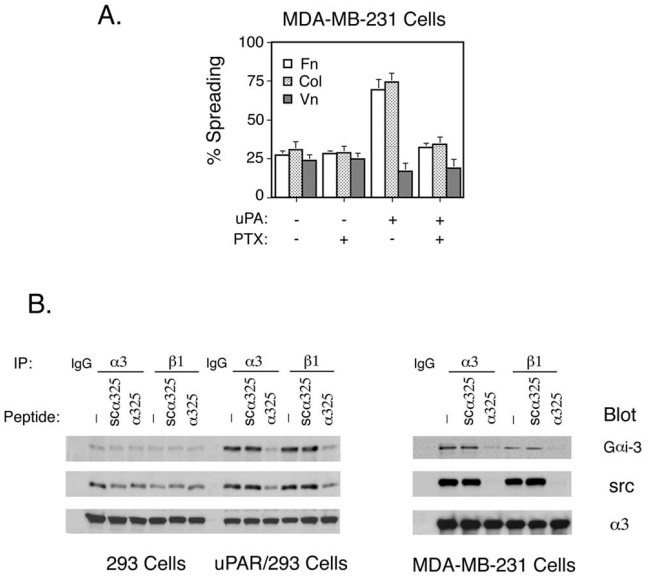



**Figure 9.** Heterotrimeric G protein is required for cross talk between $\alpha3\beta1$/uPAR and $\alpha5\beta1$ or $\alpha2\beta1$. (A) uPA-enhanced spreading of MDA-MB-231 cells on Fn or Col is blocked by the addition of pertussis toxin (PTX, 100 ng/ml). Cells were seeded on Fn- (5 $\mu$g/ml), Vn- (1 $\mu$g/ml), or Col (5 $\mu$g/ml)-coated wells with or without pro-uPA (1 nM) or pertussis toxin and spreading assessed as described in the text. Values represent mean $\pm$ SD, n = 3. (B) Nontransfected 293 cells, uPAR/293 cells or MDA-MB-231 cells were lysed in RIPA buffer. The lysates were incubated with $\alpha3$ mAb (P1B5) or $\beta1$ mAb (JB1A) in the presence or absence of peptide $\alpha325$ or sc$\alpha325$ (100 $\mu$M). The immunoprecipitates were blotted with polyclonal anti-G$\alpha$i-3, stripped, and reblotted for Src family kinases (Src2) and $\alpha3$. The experiment was performed three times with similar results. Intact cells treated with peptide $\alpha325$ before lysis gave identical results.

$\alpha3\beta1$ ligation was also pertussis toxin sensitive (Wei, observation). These results suggests that a heterotrimeric G protein is required for "cross talk" between $\alpha3\beta1$/uPAR complexes and $\alpha5\beta1$ or $\alpha2\beta1$. This is not completely unexpected because prior studies have indicated that signaling through $\beta1$ integrins is promoted by the presence of caveolin-1 (Wary *et al.*, 1998; Wei *et al.*, 1999). Caveolin-1 localizes to cholesterol-rich regions of cell membranes and has been demonstrated to associate with heterotrimeric G proteins such as G$\alpha$i. Gi proteins, like Src family kinases, are both myristylated and palmitoylated near their N terminus, providing a driving force for localization to cholesterol-rich membrane rafts (Li *et al.*, 1996; Harder and Simons, 1997). uPAR also localizes to membrane rafts via its glycolipid membrane anchor, the integrin/uPAR protein interaction then promoting accumulation of rafts around integrins (Wei *et al.*, 1999). To determine whether G$\alpha$i is in fact associated with $\alpha3\beta1$, coprecipitation experiments were again performed. In both uPAR/293 cells and MDA-MB-231 cells (Figure 9B), antibodies to $\alpha3\beta1$ precipitated not only the integrin but also both G$\alpha$i and Src family kinases. Similar results were obtained whether coimmunoprecipitation was done with cells lysed in either 1% Triton or RIPA buffer. The coprecipitation of both sets of these signaling molecules was blocked by the addition of 100 $\mu$M $\alpha325$ but not scrambled $\alpha325$ to the cell lysates. Treating intact cells with peptide $\alpha325$ before lysis gave similar results. Identical experiments in nontransfected 293 cells revealed detectable src family kinases coprecipitating with $\alpha3$ antibodies but little or no G$\alpha$i. In the absence of uPAR, the $\alpha325$ peptide had no effect on association of src family kinases with $\alpha3\beta1$ (Figure 9B). Similar expression of G$\alpha$i and Src family kinases in nontransfected and uPAR transfected 293 cells was detected by Western blotting of cell lysates. Although these results cannot be viewed as quantitative, the findings indicate that the presence of uPAR alters qualitatively the complement of signaling partners associated with $\alpha3\beta1$.

## DISCUSSION

Coupling of cellular adhesiveness with proteolytic cascades is an increasingly recognized paradigm for coordinating focal attachment and detachment important to cell migration (Werb, 1997). The uPAR is a prototypical example of this strategy (Chapman, 1997). By localizing with integrins and binding urokinase, uPAR focuses plasmin activation at or near sites of focal contact between the cell surface and extracellular matrix proteins (Blasi *et al.*, 1987). Plasmin activates cascades involving both matrix metalloproteases and growth factors in the pericellular milieu (Carmeliet *et al.*, 1997). Prior studies have also shown that the complexes uPAR forms with integrins are important to binding and adhesion of hematopoietic cells to matrix vitronectin, plasmin cleavage of vitronectin reversing this attachment (Wei *et al.*, 1996; Waltz *et al.*, 1997). Results reported here further develop this paradigm by elucidating the specificity of interaction between uPAR and $\beta1$ integrins. Our results indicate that uPAR preferentially interacts with $\alpha3\beta1$ and that this interaction has two important functional consequences: 1) uPAR/$\alpha3\beta1$ complexes enable a pathway of cellular adhesion to Vn, especially in cells with little or no $\alpha v\beta3$; and 2) these complexes initiate a signaling pathway promoting the function of $\alpha5\beta1$ and $\alpha2\beta1$. Both pathways of signaling and enhanced adhesion are activated by concurrent binding of urokinase to uPAR. The pathways are nonetheless distinct because pertussis toxin only blocks the cross talk between $\alpha3\beta1$/uPAR and other $\beta1$ integrins and not $\alpha3\beta1$/uPAR-dependent Vn adhesion. The observation that urokinase signals through uPAR/$\alpha3\beta1$ complex formation is consistent with a recent report that urokinase induces metalloproteinases in oral keratinocytes through an $\alpha3\beta1$-dependent mechanism (Ghosh *et al.*, 2000). Thus, the intricate connections between expression of proteases and function of the adhesive machinery of cells is epitomized by the reorganization of membrane partners induced by uPAR expression and its association with $\alpha3\beta1$.

Our current findings may help clarify previously reported, apparently contradictory observations regarding the influence of uPAR on the function of the Fn receptor $\alpha5\beta1$. In 293 cells, high levels of uPAR expression impair the function of Fn receptors (Wei *et al.*, 1996). Yet our data (Figure 2) indicate that the majority uPAR in these cells is associated with $\alpha3\beta1$ and not the Fn receptor. This finding suggests that the inhibition of Fn receptor function by uPAR is probably indirect. Because caveolin-1 is important to $\beta1$ integrin signaling and preferentially associates with uPAR/integrin complexes, and because 293 cells express relatively

low levels of caveolin-1, overexpression of uPAR may enrich $\alpha3\beta1$ complexes with caveolin and at the same time deplete Fn receptors of caveolin. This may explain why impaired Fn receptor function in uPAR-transfected 293 cells is reversed by overexpression of caveolin-1 (Wei *et al.*, 1999). In contrast, physiological levels of uPAR expression in most cells appear to promote rather than impair Fn receptor function. Our data suggest that this operates, at least in part, through signals derived from uPAR/$\alpha3\beta1$ complexes. Although Fn receptors do not require such signals for adhesion, the presence of these signals accelerates FAK phosphorylation and cell spreading on Fn, and therefore may promote Fn receptor-dependent cell migration. We have previously reported that peptides that disrupt uPAR/integrin association impairs smooth muscle cell migration on Fn (Wei *et al.*, 1999). We postulate that this may explain the recently observed requirement for uPAR expression in Fn receptor-dependent tumor invasion (Aguirre Ghiso *et al.*, 1999). This pathway may also underlie the requirement of uPAR for $\alpha v\beta5$-dependent migration of pancreatic carcinoma cells on vitronectin even though uPAR was not required for vitronectin adhesion of these cells (Yebra *et al.*, 1996).

A series of recent studies by Blasi and colleagues have defined a pathway of urokinase- and uPAR-mediated chemotaxis (Fazioli *et al.*, 1997; Degryse *et al.*, 1999). Urokinase stimulates chemotaxis of uPAR-bearing cells in a pathway involving Src kinase activation and sensitive to heterotrimeric G protein inactivation with pertussis toxin. The requirements for FAK and Src kinase activation for this migration favor integrin activation as a critical feature of urokinase-dependent chemotaxis. Data reported here may shed light on these observations. We find urokinase, by promoting uPAR/$\alpha3\beta1$ interactions, promotes FAK activation and spreading of MDA-MB-231 cells on either fibronectin or collagen in a G protein-dependent manner (Figures 7 and 9). This signaling is blocked by peptides that dissociate uPAR and G$\alpha$i-3 from $\alpha3\beta1$, increasing the possibility that urokinase is chemotactic for cells because urokinase enables ligand-dependent G protein activation through an integrin. It remains to be determined how conformational changes in uPAR or $\alpha3\beta1$ induced by urokinase could mediate G$\alpha$ or G$\beta\gamma$ activation. Although the mechanism is not defined, our observations are conceptually similar to recent reports that the integrin-associated protein CD47 promotes association of $\alpha v\beta3$ with heterotrimeric G proteins and that this is important to spreading mediated by this integrin (Frazier *et al.*, 1999; Green *et al.*, 1999). The finding of two distinct examples of coupling of integrins to heterotrimeric G proteins by integrin-associated proteins suggest this may be a common adaptive mechanism of cells to link matrix attachment to cell migration.

Prior studies have indicated that in addition to binding laminin-5, the integrin $\alpha3\beta1$ regulates the function of other $\beta1$ integrins (DiPersio *et al.*, 1995; Fukushima *et al.*, 1998; Hodivala-Dilke *et al.*, 1998). Antibodies (P1B5) to $\alpha3\beta1$ that block laminin-5 attachment promote spreading and migration of cells on Col and/or Fn (Kubota *et al.*, 1997; Lichtner *et al.*, 1998), consistent with findings reported here (Figure 7A). Furthermore, epithelial cells from mice deficient in $\alpha3$ show altered organization of integrin focal contacts and enhanced spreading on Fn, suggesting an inhibitory role for $\alpha3\beta1$ on Fn and Col integrin receptors (Lichtner *et al.*, 1998). Our finding that urokinase, mimicking P1B5, evokes $\alpha3\beta1$-dependent signals promoting activation of several $\beta1$ integrins indicates uPA-dependent association of uPAR with $\alpha3\beta1$ attenuates and even reverses the dominant negative function of $\alpha3\beta1$ on other $\beta1$ integrins. This observation raises the possibility that prior observations of "integrin activation" by soluble uPAR may operate through its association with $\alpha3\beta1$ (Aguirre Ghiso *et al.*, 1999). In addition, our observations may also shed some light on the possible molecular basis for such cross talk. The association of $\alpha3\beta1$ with uPAR appears to be required for coprecipitation of G$\alpha$ and Src family kinases with this integrin. Such complexes may complement the binding of the same integrin to CD151, a tetraspan family member that associates specifically with $\alpha3\beta1$ and that has been recently linked to signaling and migration of cells via this integrin (Yauch *et al.*, 1998; Berditchevski and Odinstova, 1999). Antibodies to CD151 coprecipitate uPAR in uPAR-transfected 293 cells. However, peptides $\alpha325$ and M25, which disrupt uPAR/integrin interactions, have no effect on $\alpha3\beta1$/CD151 complexes (Wei and Hemler, unpublished observations), consistent with the mapping of the interaction site between CD151 and $\alpha3\beta1$ to the membrane proximal region of the $\alpha$ chain and the mapping of the uPAR/integrin interaction site to the $\beta$-propeller (Simon *et al.*, 2000; Yauch *et al.*, 2000). We postulate that multimeric complexes involving CD151, uPA/uPAR, and $\alpha3\beta1$ have distinct signaling capacity promoting integrin signaling and migration on multiple matrix ligands for $\beta1$ integrins. How these complexes organize and whether other membrane adaptor proteins contribute importantly to their signaling function remains to be determined. It is important to reiterate that the discovery that uPAR associates preferentially with $\alpha3\beta1$ is based on sequence homology with a previously defined integrin interaction site on CD11b/CD18 (Mac-1). The $\alpha6$ amino acid sequence in the same region is also quite homologous and we show here that a $\alpha6$ peptide based on this sequence also disrupts uPAR/integrin coprecipitation and uPAR-dependent adhesion, whereas peptides of the identical region of $\alpha5$ (Figure 5A) or $\alpha v$ were inactive. The possible functional significance of uPAR/$\alpha6\beta1$ complexes in cells expressing both of these receptors remains to be defined.

Finally, our observations that uPAR associates preferentially with integrin $\alpha$ chains mediating laminin-5 binding may provide an explanation for prior findings that uPAR colocalizes with the distribution of laminin-5 in vivo at sites of tumor cell invasion (Pike *et al.*, 1995). Laminin-5 is a major basement membrane matrix protein that is breeched during the invasion of metastatic cells into or out of blood vessels. The finding that uPAR associates preferentially with the laminin-5 binding $\beta1$ integrins supports the hypothesis that invasive tumor cells have exploited the advantage of coordinate signaling of integrins, proteases, and protease receptors embodied by uPAR/integrin interactions to promote invasion and metastasis. This is also supported by studies correlating uPAR expression with metastatic capacity and poor prognosis of breast cancer patients (Solberg *et al.*, 1994). If so, our studies identifying a critical site for interaction between uPAR and laminin-5 binding integrins may be a site for intervention in the invasive process.

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