Urokinase-type Plasminogen Activator Receptor Regulates a Novel Pathway of Fibronectin Matrix Assembly Requiring Src-dependent Transactivation of Epidermal Growth Factor Receptor*

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Previous studies have indicated that the urokinase-type plasminogen activator receptor (uPAR) can functionally interact with integrins thereby modulating integrin activity. We have previously demonstrated that treatment of fibroblasts with the uPAR ligand, P25, results in an increase in the activation of the β1 integrin and a 35-fold increase in fibronectin matrix assembly (Monaghan, E., Gueorguiev, V., Wilkins-Port, C., and McKeown-Longo, P. J. (2004) J. Biol. Chem. 279, 1400–1407). Experiments were conducted to address the mechanism of uPAR regulation of matrix assembly. Treatment of fibroblasts with P25 led to an increase in the activation of the epidermal growth factor receptor (EGFR) and a colocalization of activated EGFR with β1 integrins in cell matrix contacts. The effects of P25 on matrix assembly and β1 integrin activation were inhibited by pretreatment with EGFR or Src kinase inhibitors, suggesting a role for both Src and EGFR in integrin activation by uPAR. Phosphorylation of EGFR in response to P25 occurred on Tyr-845, an Src-dependent phosphorylation site and was inhibited by PP2, the Src kinase inhibitor, consistent with Src kinase lying upstream of EGFR and integrin activation. Cells null for Src kinases also showed a loss of P25-induced matrix assembly, integrin activation, and EGFR phosphorylation. These P25-induced effects were restored following Src re-expression. The effects of P25 were specific for uPAR as enhanced matrix assembly by P25 was not seen in uPAR−/− cells, but was restored upon uPAR re-expression. These data provide evidence for a novel pathway of fibronectin matrix assembly through the uPAR-dependent sequential activation of Src kinase, EGFR, and β1 integrin.

Fibronectin is a dimeric glycoprotein that can be found in two forms; the soluble, protomeric molecule found in blood plasma, and the insoluble polymer, which forms an essential component of the extracellular matrix. Assembly of the fibronectin matrix is a dynamic process requiring the coordinate regulation of integrin receptor function, cytoskeletal organization, and intermolecular homophilic binding events between assembling fibronectin protomers (1). Fibronectin matrix, through its interaction with cell-surface integrin receptors, intersects with intracellular signaling cascades important in the regulation of cell spreading, cytoskeletal organization, cell cycle, and cell survival (2–4). Several studies have shown that the ongoing polymerization of soluble fibronectin into extracellular matrix, as well as appropriate fibronectin matrix architecture, can regulate cell-cycle progression as well as cell migration (5–10). In vivo kinetic studies have shown that the tissue fibronectin matrix undergoes constant assembly with the plasma fibronectin pool providing the fibronectin necessary for tissue fibronectin homeostasis (11–14). More recent studies have indicated that ongoing fibronectin polymerization plays an important role in the regulation of collagen deposition and in the stability of cell extracellular matrix contacts (15, 16). The initiating step in the assembly of exogenous fibronectin into matrix involves the binding of the amino-terminal domain of soluble fibronectin to matrix assembly sites on the cell surface in a saturable and reversible manner (17, 18). In subsequent steps, integrin-dependent unmasking of homophilic binding sites promotes polymerization of fibronectin into an insoluble fibrillar matrix (19, 20).

Fibronectin matrix assembly is primarily regulated by the α5β1 integrin receptor for fibronectin. α5β1 is thought to exist in multiple activation states, which likely impact its ability to support fibronectin fibrillogenesis (21–24). The differing ability of α5β1 to bind fibronectin is due to changes in the conformational state of the integrin, which can be monitored by changes in the binding of monoclonal antibodies to neoepitopes present in the integrin (25–27). The biological pathways that regulate the activation state of the α5β1 integrin are not well understood. Alterations in integrin activation that affect matrix assembly may arise from changes in signaling pathways (28), through the formation of complexes with cytoskeletal components (29), or cell-surface molecules such as the urokinase-type plasminogen activator receptor (uPAR)3 (30, 31) as well as growth factor receptors (32). Earlier studies have indicated that uPAR can modulate several activities of the α5β1 integrin, including adhesion (33), migration (34), and signal transduction (35–37). Modulation of α5β1 activity by uPAR is complex and may result in a gain or loss of function depending on cellular context (36, 38). Several peptides have been identified as uPAR ligands capable of regulating integrin function. Among them, peptide P25, identified by phage display, has been shown to bind uPAR directly and affect the functioning of the α5β1 integrin (38). Integrins have also been reported to complex with growth factor receptors, including the epidermal growth factor receptor (EGFR) (39). Integrin adhesion can trigger the Src-dependent but ligand-independent activation of EGFR resulting in the activation of ERK/mitogen-activated protein kinase and the promotion of cell growth and survival (39, 40). These complexes may also include uPAR,

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3 The abbreviations used are: uPAR, urokinase-type plasminogen activator receptor; uPA, urokinase-type plasminogen activator; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; PP2, amino-5-(4-chlorophenyl)-7-(4-butylpyrazolo)[4,3-d]pyrimidine; MEF, mouse embryo fibroblast; DME, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; FN, fibronectin.
because recent studies have proposed a tripartite complex of uPAR/EGFR-a5β1, which mediates uPA-initiated signal transduction leading to ERK activation (41, 42).

Previously we have shown that treatment of monolayers of human dermal fibroblasts with the uPAR ligand, P25, results in increased formation of fibronectin matrix through a pathway involving β1 integrin activation (43). In the present study, we address the molecular mechanism by which uPAR regulates fibronectin matrix assembly. Our results show that ligation of uPAR with P25 causes a Src-dependent transactivation of the EGFR and promotes the formation of EGFR-β1 complexes. Both Src kinase and EGFR are required for the uPAR-dependent increase in β1 integrin activation and fibronectin matrix assembly. These data support a model whereby uPAR ligation up-regulates fibronectin matrix assembly through a novel pathway involving sequential activation of Src kinase, EGFR, and β1 integrin.

**EXPERIMENTAL PROCEDURES**

Reagents and Antibodies—Unless otherwise stated, all chemicals were purchased from Sigma. Peptides P25, sequence AESTYHHL-SLGMYTTLN, and S25, sequence NYHYLESSMTALYTLGH, were synthesized by Cell Essentials (Boston, MA). The HUTS-4 antibody against the active conformation of the β1 integrin was purchased from Chemicon (Temecula, CA), and the fluorescein isothiocyanate-conjugated antibody against the β1 integrin was purchased from Immunotech (Marseille, France). SNAK-A51 monoclonal antibody to the activated form of the α5 integrin subunit was the generous gift of Dr. Martin Humphries (Wellcome Trust Center, Manchester, UK). Monoclonal antibody 74 against the activated EGFR and the total EGFR antibody were purchased from Cell Signaling. Secondary antibodies were obtained from BIOSOURCE (Camarillo, CA). PP2, the specific antibodies were purchased from Cell Signaling. Secondary antibodies, horseradish peroxidase-conjugated goat anti-mouse IgG, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from BioRad. Alexa Fluor 594-labeled goat anti-mouse antibody was obtained from Molecular Probes (Eugene, OR). Anti-Src PY418 and pan Src antibodies were obtained from BIOSOURCE (Camarillo, CA). PP2, the chemical inhibitor of Src family kinases, was purchased from BIOMOL (Plymouth Meeting, PA). PD168393, the inhibitor of EGFR kinase activity, was purchased from Calbiochem.

Cell Culture—Human foreskin fibroblasts (A1-F) were a gift from Dr. Lynn Allen-Hoffmann (University of Wisconsin, Madison, WI). Mouse embryo fibroblasts (MEF) cells were purchased from ATCC, and SYF and SYFwtSrc cells were a gift from Dr. Harold Singer (Albany Medical College, Albany, NY). The uPAR+/+ and uPAR−/− MEF cell lines, described previously (44), were provided by Dr. Steve Gionias (University of California-San Diego). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). A1-F cells were used at passages 6–12, and unless otherwise noted experiments were performed on monolayer cultures.

**Transient Transfection of uPAR in MEFs**—To re-express uPAR in uPAR−/− MEFs, cells were grown to confluency in 6-well plates and transfected with 4 μg of pcDNA 3.1+ plasmid containing the full-length cDNA for human uPAR (pcDNA-uPAR, a gift of Dr. Harold Chapman, University of California-San Francisco) and 12 μl of Lipofectamine, according to the manufacturer’s instructions (Invitrogen). Transfection efficiencies were >60%, as determined by counting green fluorescent cells in preparations transfected with pEGFP plasmid (Clontech). MEFs were transfected for 24 h before assays were performed.

**Purification and Derivatization of Proteins**—Human plasma fibronectin was purified from a fibronectin and fibrinogen-rich by product of Factor VIII production by ion exchange chromatography on DEAE-cellulose (Amersham Biosciences) as described previously (45) and further purified by affinity chromatography with gelatin-agarose and heparin-agarose (46). The 70-kDa amino-terminal fragment of fibronectin was generated by limited digestion of intact fibronectin with cathepsin D followed by gelatin affinity chromatography as described previously (45). Purified plasma fibronectin (400 μg) and the 70-kDa fragment of fibronectin (100 μg) were iodinated with 1 mCi of Na125I (PerkinElmer Life Sciences) as described previously (47). Iodinated proteins were mixed with bovine albumin, 1 mg/ml, dialyzed against phosphate-buffered saline, and frozen at −80 °C until used.

**Matrix Incorporation Assays**—The 125I-fibronectin assembly into detergent-insoluble matrix was determined as previously described (47). Cultures were incubated with 125I-fibronectin (1 μg/ml, 1 × 106 cpm/ml) in DMEM at 37 °C in the presence of either P25 or S25. Incubation times and peptide doses and/or inhibitors are as designated in the figure legends. After incubation, cells were rinsed three times in PBS, and the detergent-insoluble extracellular matrix was isolated by extraction of cell layers in 1% deoxycholate. Deoxycholate extractions were done in a 20 mM Tris (pH 8.8) buffer containing 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 2 mM N-ethylmaleimide, and 2 mM iodoacetic acid. Cell layers were scraped into 1% deoxycholate, and deoxycholate-insoluble matrix was recovered by centrifugation at 18,000 rpm for 30 min. Radioactivity associated with the pellet was measured by gamma scintillation.

**70-kDa Fragment Binding Assays**—Cell layers were preincubated with peptides and inhibitors as specified in the figure legends prior to the addition of the 125I-labeled 70-kDa fragment (100 ng/ml) in serum-free medium. Following incubation with the 70-kDa fibronectin fragment, cell layers were washed three times with PBS, solubilized in 1 N NaOH, and cell-associated radioactivity was determined by gamma scintillation. Nonspecific binding was determined in the presence of excess (50 μg/ml) unlabeled protein.

**Receptor Activation Assays**—A1-F cells were grown to confluence in 48-well plates and treated with peptides and/or inhibitors as designated in the figure legends. To detect activated β1 integrins, cells were incubated with 100 ng/ml monoclonal antibody, HUTS-4 or 9EG7, for 1 h. To detect activated EGFR, cells were incubated with 400 ng/ml monoclonal antibody 74 for 1 h. Cells were then washed three times with PBS, fixed with 3% paraformaldehyde, and blocked with 2% bovine serum albumin for 1 h. Bound antibody was detected by incubating cells for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG (for HUTS-4 or monoclonal antibody 74) or horseradish peroxidase-conjugated goat anti-rat IgG (9EG7). Cell layers were washed five times with PBS and incubated with substrate (0.1M citrate buffer, 0.5 mg/ml o-phenylenediamine, 1 μg/ml 30% hydrogen peroxide, pH 5). The color was allowed to develop, and the reaction was stopped with the addition of 2 N sulfuric acid prior to measuring the optical density at A490. Measurements were corrected for light scattering by subtracting the optical density obtained at A650.

**Immunofluorescence and Microscopy**—A1-F cells were seeded onto glass coverslips, coated with 10 μg/ml fibronectin, and allowed to adhere and spread for 2 h in serum-free medium. Cells were then treated with 50 μM P25, S25, or 100 ng/ml EGF for 1 h, washed with PBS, fixed for 15 min in 3% paraformaldehyde, permeabilized in 0.3% Triton, and blocked for 30 min in 2% bovine serum albumin. To visualize activated EGFR, cells were stained with monoclonal antibody 74, followed by goat anti-mouse conjugated with secondary antibody Alexa Fluor 594. Cells were then costained for β1 integrin with a fluorescein isothiocyanate-conjugated antibody directed against β1 integrin. Fluoro-
RESULTS

We have previously shown that incubation of monolayers of human dermal fibroblasts with the uPAR ligand, P25, results in a substantial increase in the rate of fibronectin matrix assembly (43). Recent studies have demonstrated that the EGFR may participate in the transduction of uPAR-dependent signaling pathways (41, 42). To investigate a role for EGFR in regulation of matrix assembly by uPAR, the EGFR kinase inhibitor, AG1478, was used to inhibit EGFR activity. Monolayers of human foreskin fibroblasts were incubated with increasing doses of AG1478 for 1 h before treatment with the uPAR ligand, P25, or the scrambled control peptide, S25. After 1 h of incubation with the peptide, matrix assembly site expression was measured using the 70-kDa amino-terminal fragment of fibronectin. Treatment with P25 caused an 8- to 9-fold increase in the binding of the 125I-70-kDa amino-terminal fragment of fibronectin to fibroblast monolayers (Fig. 1A). The control peptide, S25 had no effect on 70-kDa fragment binding. In the presence of increasing concentrations of the EGFR kinase inhibitor, AG1478, there was a dose-dependent decrease in P25-mediated 70-kDa fragment binding. The binding of fibronectin to matrix assembly sites is followed by intermolecular homophilic binding events, which render the fibronectin detergent-insoluble (19). To determine if EGFR is required for the P25-mediated detergent-insoluble matrix formation, cells were preincubated with increasing doses of AG1478. After a 1-h treatment with AG1478, cells were incubated with 125I-fibronectin for 6 h in the presence of 50 μM P25 or S25. Detergent-insoluble extracellular matrix was then isolated by deoxycholate extraction. Fig. 1B shows a 35-fold increase in detergent-insoluble matrix in the presence of P25 and a dose-dependent decrease in P25-enhanced matrix assembly in the presence of AG1478. The scrambled peptide, S25, had no effect on the levels of fibronectin matrix. Similar results were seen using PD168393, another inhibitor of EGFR kinase activity. Treatment of cells with PD168393 prior to addition of P25 resulted in a dose-dependent decrease in the P25-mediated increase in both 70-kDa fragment binding and fibronectin matrix incorporation (Fig. 1, C and D). These results suggest that EGFR activity is required for the P25-mediated increase in fibronectin matrix assembly. In the absence of P25, neither AG1478 nor PD168393 had any effect on incorporation of fibronectin into matrix suggesting that EGFR does not participate in the regulation of basal levels of fibronectin matrix assembly.

In response to growth factor treatment, EGFR undergoes a series of tyrosine-phosphorylation events that lead to its activation. To evaluate whether P25 was causing activation of EGFR, an ELISA assay was performed on P25-treated fibroblast monolayers using a monoclonal antibody (monoclonal antibody 74) that recognizes the activated form of EGFR, the growth factor ligand of EGFR, was used as a positive control for EGFR activation. Our earlier studies have shown that the P25-mediated increase in the expression of 70-kDa binding sites and in the assembly of fibronectin into matrix is regulated by the β1 integrin receptor for fibronectin (43). Recent studies have suggested that uPAR, β1, and EGFR may form physical as well as functional complexes (41). To determine whether P25 was affecting the formation of β1-EGFR complexes, β1 and EGFR were localized by indirect immu-
mRNA fluorescence. Following a 1-h treatment with P25, cells were fixed, permeabilized, and stained with antibodies to the β1 integrin (Fig. 2B, panels A–C) and to the activated EGFR (Fig. 2B, panels D–F). In the presence of control peptide S25, β1 staining was localized to adhesion sites (Fig. 2B, panel A), and there was little staining of activated EGFR (Fig. 2B, panel D). In the presence of P25, active EGFR was localized in adhesion sites (Fig. 2B, panels C and F). Activation of EGFR by its ligand, EGF, resulted in increased staining of activated EGFR, however, EGFR localization remained diffuse over the cell surface (Fig. 2B, panel E). Merged images are shown in Fig. 2B (panels G–I), and colocalization of integrin and EGFR is shown in yellow. Colocalization of activated EGFR with β1 integrin is observed after P25 (panel I) treatment, but no colocalization is seen in the S25- or EGF-treated cells. Treatment of the cells with AG1478 prevents the P25-mediated colocalization of the EGFR and β1 integrin (data not shown) suggesting that the kinase activity of the EGFR is required for its interaction with the β1 integrin. These results indicate that activation of EGFR by P25 but not by EGF, results in the redistribution of EGFR into β1-containing focal adhesions. β1-EGFR complex formation was also assessed by coimmunoprecipitation. Lysates from cells treated with S25, P25, or EGF were immunoprecipitated with an EGFR antibody and analyzed for β1 integrin by Western blotting. As shown in Fig. 2C, the cells treated with P25 show an enhanced association of β1 integrin with EGFR. These data support the morphological observation of colocalization observed by immunofluorescence and confirms that P25 treatment caused the enhanced formation of EGFR-β1 integrin complexes.

Our earlier studies showed that the P25 enhancement of matrix assembly was associated with an increase in the level of activated β1 found on the cell surface (43). As matrix assembly is regulated by the α5β1 integrin, we also tested P25 for its effect on the activation state of the α5 subunit using the SNAK-A51 antibody. As shown in Fig. 3A treatment of cells with P25 resulted in a 2- to 3-fold increase in the

FIGURE 2. P25 treatment causes activation of EGFR and colocalization of activated EGFR with β1. A, confluent monolayers were incubated in DMEM containing increasing doses of P25, 100 μM S25, or 100 ng/ml EGF for 1 h. Cells were then treated with 50 μM S25 (A, D, and G), 100 ng/ml EGF (B, E, and H), or 50 μM P25 (C, F, and I) for 1 h. Cells were fixed, permeabilized, and immunostained for activated EGFR (D–F) and the β1 integrin (A–C). Panels G–I show merged images. Boxed areas in C, F, and I are shown as magnified images (c, f, and i). C, confluent fibroblast monolayers were treated with 50 μM P25 or S25 or 100 ng/ml EGF. EGFR was immunoprecipitated from cell lysates using a mouse anti-EGFR antibody (3 μg), and the resulting immunoprecipitates were analyzed for the β1 integrin (top panel). Blot was then stripped and reprobed with an antibody against total EGFR (bottom panel).

FIGURE 3. P25-mediated β1 integrin activation requires EGFR. A, confluent fibroblast monolayers were incubated with 50 μM P25, S25, or 2 mM Mn2+ for 1 h. Activation of the α5 subunit was measured by ELISA using the SNAK-A51 antibody specific for activated α5. B, confluent fibroblast monolayers were incubated in DMEM containing increasing concentrations of AG1478 for 1 h. Cells were then treated with either 50 μM P25 or S25 for 1 h, and integrin activation was measured by ELISA using the HUTS-4 antibody specific for activated β1 integrin. *, significantly different than P25 treatment alone, t test, p < 0.05.
binding of SNAK-A51 antibody, consistent with P25 inducing an increase in the activation of the α5β1 integrin. To determine whether EGFR activity may be required for the P25-mediated increase in β1 integrin activation, cells were treated with AGI478 before the addition of P25. A monoclonal antibody, HUTS-4, that recognizes the active conformation of the β1 integrin (48), was used to measure the level of activated β1 integrins on the cell surface. Fig. 3B shows that treatment of cells with increasing amounts of AGI478 caused a dose-dependent decrease in the number of β1 integrins activated in response to P25. AGI478 treatment had no effect on the level of integrin activation in untreated cells, suggesting that basal levels of integrin activation are not under EGFR regulation.

To determine whether "classic" activation of the EGFR with its cognate ligand, EGF, would produce similar effects on matrix assembly as the P25-dependent activation of the EGFR, fibroblast monolayers were treated with 100 ng/ml EGF, and β1 integrin activation and 125I-70-kDa binding were measured (Fig. 4, A and B). We found that EGF treatment did not cause an increase in either β1 integrin activation or 70-kDa fragment binding. EGF, but not P25, caused an increase in the phosphorylation of ERK mitogen-activated protein kinase indicating that EGF was active in these cells (data not shown). Activation of EGFR by EGF results in the phosphorylation of EGFR on specific tyrosine residues. To further delineate the differences between P25- and EGF-induced EGFR activation EGFR was immunoprecipitated, and the levels of tyrosine phosphorylation were analyzed (Fig. 5A). Examination of the total levels of EGFR tyrosine phosphorylation revealed that cells treated with P25 show less EGFR phosphorylation than EGF-treated cells. Immunoprecipitates of cells treated with S25 show little detectable EGFR phosphorylation. The phosphorylation of the EGFR was then examined in more detail by Western blotting using antibodies that recognize specific phosphorylation sites. The EGFR contains a number of autophosphorylation sites, including Tyr-992 and Tyr-1068, that are known to become phosphorylated upon receptor activation with EGF. Western blot analysis shows that EGFR treatment resulted in the phosphorylation of the receptor at tyrosines 845, 992, and 1068, whereas P25 treatment caused phosphorylation detectable at only tyrosine 845, suggesting that P25-mediated activation of EGFR was due to Src kinase activity (Fig. 5B).

To evaluate the role of Src kinases in the P25-mediated effects on fibronectin matrix assembly, cells were treated with increasing concentrations of PP2, a chemical inhibitor of Src family kinases prior to treatment with P25. Between the doses of 1 and 20 μM, PP2 treatment caused dose-dependent decreases in both the P25-mediated increases in 125I-70-kDa binding (Fig. 6A) and detergent-insoluble matrix formation (Fig. 6B). PP2 (20 μM) treatment partially inhibited basal levels of matrix assembly causing a 30–40% decrease in both 125I-70-kDa binding as well as 125I-fibronectin incorporation into detergent-insoluble matrix. In addition, PP2 inhibited the P25-mediated activation of β1 integrins, as measured by HUTS-4 binding while having no effect on the basal level of integrin activation (Fig. 6C). To determine whether Src family kinases were being activated in response to P25 treatment, lysates from cells treated with P25 or S25 for increasing periods of time were examined for Src phosphorylation at tyrosine 418. As shown in Fig. 7, Src phosphorylation can be seen as early as 10 min and is increased by as much as 4-fold in response to P25. To evaluate further the role of the Src family kinases in P25-induced matrix assembly, fibroblasts deficient in Src, Yes, and Fyn (SYF cells) were used. Monolayers of control MEFs showed approximately an 8-fold increase in fibronectin incorporation upon P25 treatment (Fig. 8A). The effect of P25 on fibronectin matrix incorporation was lost in the null SYF cells but could be rescued by re-expression of Src (SYFwtSrc). A similar effect was seen when cells were examined...
for β1 integrin activation using 9EG7, a monoclonal antibody that recognizes activated β1 integrins. Fig. 8B shows enhanced β1 integrin activation in the MEFs but not in SYF cells upon P25 treatment. The increase in integrin activation was restored in the SYF cells that re-express Src. Mn²⁺, a known external activator of integrins, was used as a positive control on all three cell lines. These data indicate that the Src family kinases are essential to P25-induced FN matrix assembly and β1 integrin activation.

Recently, it has been shown that Src is involved in the propagation of uPAR-dependent signals to the EGF receptor (42). To test the role of Src family kinases on activation of the EGFR by P25, cells were treated with increasing doses of PP2, and activation of EGFR was assessed using an antibody that recognizes the active conformation of the EGFR. As shown in Fig. 9A, PP2 effectively inhibited the activation of EGFR by P25. PP2 also prevented the P25-induced redistribution of EGFR to β1 containing adhesion sites (data not shown). Finally, to confirm that the P25-mediated phosphorylation of EGFR at Tyr-845 was Src-dependent, cells were pretreated with PP2 prior to treatment with P25. As shown in Fig. 9B, PP2 treatment abolished the phosphorylation of EGFR at Tyr-845 in response to P25 treatment. We further confirmed the dependence of the P25-induced EGFR Tyr-845 phosphorylation on Src by using the SYF cells. As shown in Fig. 9C, phosphorylation of the EGFR at Tyr-845 in response to P25 is observed in the control MEFs, lost in the SYF null cells, but rescued upon re-expression of Src. These data establish a role for Src family kinases in the P25-mediated activation of EGFR. Taken together these data indicate that Src and EGFR function as sequential upstream activators of the β1 integrin.
Finaly, to confirm that the P25 effects on fibronectin matrix assembly and the associated signaling events were dependent on uPAR, experiments were performed using uPAR null fibroblasts (Fig. 10). Monolayers of uPAR-positive mouse embryo fibroblasts show a dose-dependent increase in fibronectin incorporation upon P25 treatment (Fig. 10A). The effect of P25 on fibronectin matrix incorporation is lost in the uPAR null cells but could be rescued by re-expression of human uPAR. There was no difference in the basal incorporation of fibronectin into matrix between the uPAR+/+ and uPAR−/− cells under control conditions, suggesting that uPAR does not affect the basal rate of fibronectin matrix assembly. These data indicate that uPAR is required for the effects of P25 on matrix assembly. A similar effect is seen when cells were examined for β1 integrin activation using 9EG7. Fig. 10B shows enhanced β1 integrin activation in the uPAR+/+ upon P25 treatment. The uPAR null cells, however, show no increase in β1 activation. The increase in integrin activation was restored in the uPAR null cells that express human uPAR. To confirm that the effects of P25 on matrix assembly in uPAR expressing cells were dependent on EGFR and Src kinases, AG1478 and PP2 were used to inhibit EGFR and Src, respectively, and fibronectin incorporation was measured. As shown in Fig. 10C, the effect of P25 on fibronectin matrix incorporation in the uPAR+/+ cells and the uPAR null cells transfected with human uPAR was inhibited by both AG1478 and PP2 confirming a role for both the EGFR and Src family kinases in P25-mediated fibronectin matrix assembly. Western blot analysis indicated that the P25-induced phosphorylation of EGFR was dependent on the presence of uPAR as P25 treatment-induced phosphorylation of EGFR at Tyr-845 in the wt uPAR+/+ cells but not in the uPAR−/− cells (Fig. 10D). Phosphorylation of EGFR by P25 was restored when uPAR−/− cells were transfected with human uPAR. Taken together, these data indicate that uPAR regulates fibronectin matrix assembly through the sequential activation of Src family kinases, EGFR, and the β1 integrin.
DISCUSSION

Our previous studies indicate that uPAR regulates fibronectin matrix assembly in monolayers of human dermal fibroblasts by modulating the activation state of the α5β1 integrin (43). In the present study, we now define the molecular mechanisms mediating the effect of uPAR on fibronectin matrix formation. Treatment of cell layers with the uPAR ligand, P25, results in the activation of Src and EGFR. Sequential activation of these kinases is required for both uPAR-dependent integrin activation and matrix assembly in human dermal fibroblasts. Integrin activation by uPAR is caused by the Src-dependent transactivation of the EGFR resulting in the colocalization of activated EGFR with α5β1 integrin in cell adhesion sites. P25 treatment results in a pattern of tyrosine phosphorylation on the EGFR, which is distinct from that caused by EGFR. Activation of EGFR by EGF has no effect on either integrin activation or matrix assembly. These studies reveal a novel pathway for the regulation of fibronectin matrix assembly involving a Src-dependent, ligand-independent transactivation of EGFR.

Treatment of cells with the uPAR ligand, P25, increases both the expression of activation-dependent epitopes as well as the phosphorylation of EGFR. Activation of EGFR is accompanied by complexing of EGFR with β1 integrins in cell adhesion sites and by an activation of the α5β1 integrin. The effects of P25 on integrin activation and matrix assembly are inhibited by the EGFR kinase inhibitors, tyrphostin AG1478 and PD168393, indicating that EGFR kinase activity is required for integrin activation. Earlier studies have shown that integrin-dependent adhesion results in the complexing of β1 integrins with EGFR and in the ligand-independent trans-activation of EGFR and subsequent activation of ERK (40, 41). Our studies are the first to document the reciprocal interaction in which EGFR lies upstream of integrin activation. Our results show that, in adherent dermal fibroblasts, P25/uPAR-dependent transactivation of EGFR results in the phosphorylation of the EGFR at Tyr-845, a known substrate for Src kinases. P25 treatment does not result in phosphorylation of EGFR at Tyr-1068, a site that is phosphorylated in response to both EGF and integrin ligation (49). In addition, P25/uPAR-dependent phosphorylation of EGFR at Tyr-845 is not accompanied by the activation of ERK. This is consistent with earlier studies showing that the Src phosphorylation of EGFR at Tyr-845 is not required for ERK activation by EGFR (50) and suggests that uPAR activates EGFR through phosphorylation at a distinct subset of sites, which results in the activation of the α5β1 integrin while having no effect on phospho-ERK levels. Other studies have shown that uPAR signaling is linked to EGFR transactivation and subsequent ERK activation in epithelial carcinoma cells in which uPAR has been overexpressed (41) and in breast cancer cells stimulated with uPA (42, 51). However, the specific sites phosphorylated on EGFR in response to uPAR-mediated transactivation were not identified in these studies. Interestingly, in breast cancer cells stimulated with uPA, transactivation of EGFR is associated with an increase in cellular invasion, whereas EGF-mediated activation of EGFR is associated with a proliferative response (42). Taken together with our results, these data are consistent with a model in which uPAR regulates specific cellular outcomes through modulating the sites of EGFR phosphorylation resulting in the bifurcation of the EGFR signaling pathway.

The binding of P25 to uPAR results in the rapid activation of Src kinase, which is required for the activation of EGFR and β1 integrin as well as for the P25-mediated increase in fibronectin matrix assembly. Phosphorylation of EGFR at Tyr-845 is blocked with PP2 consistent with Src kinase activity lying upstream of EGFR activation. Experiments using cells null for the Src family kinases confirm a role for Src upstream of both EGFR and integrin activation. The specific mechanism by which P25 activates Src kinase in our cells is not known. Activation of Src by uPAR occurs following uPA ligation (52) or uPAR overexpression (53), but these mechanisms of Src activation are also not understood. uPAR is linked to the plasma membrane through a glycosylphosphatidylinositol anchor, and it is thought to require a coreceptor to mediate intracellular effects. Potential coreceptors for uPAR-mediated signaling include G-protein-linked receptors, growth factor receptors, and integrins. P25 is known to bind to uPAR and disrupt uPAR-β1 integrin complexes (36, 38). In some cell types, disruption of this complex is associated with a loss of integrin function, including decreased adhesion and fibronectin matrix assembly (31, 54). In other cell types, P25-mediated disruption of β1/uPAR complexes is associated with an increase in β1 integrin function (33, 38, 43). The molecular basis of the diverse effects of P25/uPAR on integrin function is not understood, but likely results from the assembly of cell type-specific uPAR containing multiprotein complexes that function as transmembrane adaptors to mediate uPAR signaling (51, 55). The specific nature of these complexes in our cells is not yet known. Although we were able to detect β1-EGFR complexes both morphologically and biochemically, we were unable to detect uPAR-β1 complexes. Therefore, our data are consistent with a “functional” rather than a “physical” association of uPAR and β1. Our inability to detect physical β1-uPAR complexes is consistent with earlier reports that indicate that uPAR-integrin complexes are typically found under conditions where uPAR is overexpressed but are not detected in cells expressing only endogenous levels of uPAR (reviewed in Ref. 56). Our inability to detect these complexes may reflect the fact that only a small proportion of total uPAR is in association with integrins as has been suggested by others (30, 36) or that in human dermal fibroblasts P25 effects uPAR association with other as yet unidentified transmembrane proteins such as G-protein-linked receptors (57), caveolin (58), or low density lipoprotein-related protein (59). G-protein-linked receptors as well as caveolin and low density lipoprotein-related protein have been linked to the regulation of α5β1 function and fibronectin matrix assembly (30, 60–65). Further studies are needed to define the specific roles of these uPAR coreceptors in the regulation of matrix assembly.

Studies using uPAR null cells confirm that the effects of P25 on integrin activation and matrix assembly are dependent on uPAR, consistent with earlier studies showing that the effects of P25 on matrix assembly are inhibited by uPAR antibodies (43). The effects of P25 on integrin activation and matrix assembly seen in our studies are reminiscent of previous studies that show that overexpressing uPAR stimulates α5β1 integrin activation, fibronectin adhesion, and fibronectin matrix assembly (31, 41, 66). It may be that, under our experimental conditions, where uPAR is expressed at physiological levels, the binding of P25 to uPAR drives uPAR into the same molecular associations seen when uPAR is overexpressed at high levels. We were unable to mimic the effect of P25 on matrix assembly using biological uPAR ligands such as uPA or vitronectin. 4 This is not particularly surprising, because the P25 peptide binds uPAR in a site distinct from both the vitronectin and uPA binding sites (38). The most likely biological counterpart for the P25 peptide is an integrin α subunit (30). Therefore, the binding of P25 to uPAR may place uPAR in a conformation similar to that seen when integrin binds to uPAR. uPAR-integrin or uPAR-P25 may then associate with other as yet unidentified molecules to affect intracellular signaling pathways (e.g. Src kinase). The ability of P25 to mimic the uPAR-integrin complex might explain why in our studies we can affect integrin function in the absence of uPAR-integrin complexes. The effects of integrin or P25 binding on uPAR conformational state and the associa-

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tion of uPAR-P25 or uPAR-integrin with potential coreceptors is an important avenue for further investigation.

Previous studies have used the P25 peptide as well as other "non-biological" uPAR ligands to modulate uPAR function. Such ligands, including the P25 peptide, block growth and invasion of tumors in vivo, however, there is very little known about the mechanism underlying their ability to inhibit tumor progression (33, 67, 68). As discussed by Bu et al. (69), recent data suggest that the ability of non-biological uPAR ligands to block tumor growth may not be due to blockade of uPA binding, as originally thought, but to direct effects of these ligands on uPAR signaling. Clearly, the potential usefulness of uPAR-binding peptides as cancer therapeutic agents dictates the need for further studies to delineate the molecular mechanism of uPAR-based signaling initiated by these ligands.

A role for Src family kinases in fibronectin matrix assembly has been shown previously by Wierzbicka-Patynowski and Schwarzauer (1) who demonstrated decreased levels of matrix assembly in Src-deficient fibroblasts. In our studies, PP2 inhibited both basal levels of matrix assembly as well as the P25-uPAR induction of matrix assembly. This finding is consistent with a role for Src kinases in the regulation of both basal and uPAR-regulated matrix assembly pathways and suggests that Src kinases may lie both upstream and downstream of integrin activation to regulate the extent of fibronectin deposition into the matrix. In contrast, the EGFR inhibitor, AG1478, had no effect on basal levels of matrix assembly but significantly inhibited the assembly of fibronectin in response to P25, indicating a role for EGFR upstream of integrin activation. Activation of EGFR by EGF had no effect on matrix assembly, suggesting that matrix assembly may be regulated through graded signaling pathways of EGFR. These studies are the first to document a role for EGFR in fibronectin matrix assembly and suggest that both uPAR and EGFR may represent novel targets for the regulation of fibronectin matrix deposition under conditions where dysregulated fibronectin matrix deposition may contribute to pathological conditions such as tumor survival and tissue fibrosis.

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