A dual role for caveolin-1 in the regulation of fibronectin matrix assembly by uPAR

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Summary

The relationship between the plasminogen activator system and integrin function is well documented but incompletely understood. The mechanism of uPAR-mediated signaling across the membrane and the molecular basis of uPAR-dependent activation of integrins remain important issues. The present study was undertaken to identify the molecular intermediates involved in the uPAR signaling pathway controlling α5β1-integrin activation and fibronectin polymerization. Disruption of lipid rafts with MβCD or depletion of caveolin-1 by siRNA led to the inhibition of uPAR-dependent integrin activation and stimulation of fibronectin polymerization in human dermal fibroblasts. The data indicate a dual role for caveolin-1 in the uPAR signaling pathway, leading to integrin activation. Caveolin-1 functions initially as a membrane adaptor or scaffold to mediate uPAR-dependent activation of Src and EGFR. Subsequently, in its phosphorylated form, caveolin-1 acts as an accessory molecule to direct trafficking of activated EGFR to focal adhesions. These studies provide a novel paradigm for the regulation of crosstalk among integrins, growth-factor receptors and uPAR.

Key words: Fibronectin, Extracellular matrix, uPAR, Integrin, Caveolin-1

Introduction

Fibronectin is a high-molecular-weight dimeric glycoprotein that is secreted by cells as a soluble dimer and is subsequently polymerized into the insoluble fibrils found as a component of the extracellular matrix of most tissues. Through its interaction with cell-surface integrin receptors, the fibronectin matrix intersects with intracellular signaling cascades that are important in a number of cellular events, including adhesion, migration, differentiation and survival. Assembly of a fibrillar fibronectin network is a complex and tightly regulated process that requires the coordinate regulation of integrin-receptor function, cytoskeletal organization and intermolecular homophilic binding events between assembling fibronectin protomers (reviewed by Mao and Schwarzbauer, 2005). Ongoing polymerization of the fibronectin matrix is important in regulating cell-cycle progression and is essential to the deposition of other matrix components such as collagen, thrombospondin and fibrinogen (Pereira et al., 2002; Sechler and Schwarzbauer, 1998; Sottile et al., 1998; Velling et al., 2002). In addition, fibronectin polymerization is required for the stability of the existing matrix as well as for the remodeling of cell-matrix adhesion sites and the regulation of integrin-based signaling (Sottile and Hocking, 2002). There is now accumulating evidence that matrix fibronectin influences the fate of metastatic cells, both by promoting acquired drug resistance (Hazlehurst and Dalton, 2001; Yu et al., 2002) and by providing niches for the growth of metastatic cells within target organs (Kaplan et al., 2006). Therefore, fibronectin polymerization plays a central role in mediating the reciprocal signaling pathways regulating normal and abnormal cell function.

The assembly of exogenous fibronectin into extracellular matrix occurs in a step-wise process in which fibronectin, via its N-terminal matrix-assembly domain, binds to cell layers. This matrix-assembly domain is contained with the 70-kDa N-terminal fragment of fibronectin and can be used to detect the presence of matrix-assembly sites on the cell surface (Christopher et al., 1997; McKeown-Longo and Mosher, 1985). Matrix-assembly sites can be rapidly up- and down-regulated in response to a variety of stimuli, but the molecular composition of the site is not well understood (Tomasini-Johansson et al., 2006). The binding of the N-terminus of fibronectin to cell layers is both reversible and detergent soluble. Surface-bound fibronectin then undergoes a process of fibrillogenesis in which a series of homophilic binding events results in the formation of detergent-insoluble fibronectin multimers. In most instances, fibronectin matrix assembly, including the expression of assembly sites, is regulated by the receptor for fibronectin, α5β1 integrin (Fogerty et al., 1990; Hocking et al., 1994; Hocking et al., 1996). α5β1 is thought to exist in multiple activation states, which impact its ability to support fibronectin fibrillogenesis (Garcia et al., 2002; Sechler et al., 1997; Wu et al., 1995); however, the biological pathways that regulate the activation state of α5β1 are not well understood.

The receptor for urokinase-type plasminogen activator (uPAR) is a GPI-linked protein that functions both as a facilitator of plasminogen activation and a regulator of the functional activity of several integrin receptors, including α5β1 (reviewed by Ragno, 2006). uPAR has been localized to caveolae – specialized raft structures that are defined by the presence of the cholesterol-binding protein caveolin and are involved in vesicular transport, cellular cholesterol homeostasis and signal transduction. A common denominator of many models of uPAR regulation of α5β1-integrin function has been that uPAR forms complexes in cis with integrins. The consensus has been that the regulation of integrin function, including that of α5β1, by uPAR is a result of direct association (i.e. physical binding) of uPAR to the integrin α subunit, which not only modulates integrin function but provides a co-receptor (i.e.

transmembrane protein) that mediates urokinase-type plasminogen activator (uPA)- and uPAR-initiated signaling (Aguirre-Ghiso et al., 2001; Degryse et al., 2005; Liu et al., 2002; Wei et al., 1996; Wei et al., 2001; Wei et al., 2005; Wei et al., 2007). Cis-forming integrin-uPAR complexes have been identified under conditions of uPAR overexpression but have not been detected under conditions of endogenous uPAR expression levels (Chaurasia et al., 2006; Simon et al., 2000; Wei et al., 2001).

Several peptides have been identified that bind to uPAR and share homology with various integrin α subunits (Simon et al., 2000; Wei et al., 2001). These integrin mimetics disrupt the integrin-uPAR complexes, which form under conditions of high uPAR expression and have therefore been used as antagonists of uPAR signaling (Aguirre-Ghiso et al., 2001; Simon et al., 2000; van der Pluijm et al., 2001; Wei et al., 1996; Wei et al., 2001). Using cell lines expressing endogenous (moderate) levels of uPAR, we previously demonstrated that one of these peptides, P25, binds to uPAR and functions as an agonist to activate a signaling pathway that stimulates fibronectin matrix assembly. We found that P25 binding to uPAR on either fibroblast cells or osteosarcoma cells under conditions in which uPAR is not complexed with integrins leads to a 35-fold increase in the rate of fibronectin matrix assembly (Monaghan et al., 2004; Monaghan-Benson and McKeown-Longo, 2006; Vial et al., 2006). uPAR stimulation of matrix assembly occurs through a novel signaling pathway involving sequential activation of Src kinase, epidermal growth factor receptor (EGFR) and the α5β1 integrin (Monaghan et al., 2004; Monaghan-Benson and McKeown-Longo, 2006; Vial et al., 2006). Although our earlier studies demonstrated that the P25 peptide can exhibit agonist activity to initiate uPAR signaling, it has not been clear how, in the absence of uPAR-integrin complexes, uPAR-generated signals can be transmitted across the membrane. In the present study we identify caveolin-1 as a scaffold or adaptor molecule required for the activation of Src and EGFR by uPAR. We also find that the activation of α5β1 by uPAR requires the phospho-caveolin-dependent trafficking of activated EGFR to focal adhesions. These studies describe a novel pathway for the regulation of α5β1-integrin function by uPAR and identify a dual role for caveolin-1 in this pathway.

Results

The initial step in the assembly of exogenous fibronectin into matrix requires the interaction of the N-terminus of fibronectin with specific sites on the cell surface termed matrix-assembly sites. Expression of matrix-assembly sites is typically monitored in binding assays using the 70-kDa N-terminal fragment of fibronectin (McKeown-Longo and Mosher, 1985; Tomasini-Johansson et al., 2006). Following its binding to matrix-assembly sites, fibronectin undergoes a series of intermolecular homophilic binding events that render it detergent-insoluble as it assembles into fibrils that are characteristic of the extracellular matrix (reviewed by Wierzbicka-Patynowski and Schwarzbauer, 2003). In previous studies we have shown that uPAR ligation with P25 causes a marked increase in both the expression of matrix-assembly sites as well as the incorporation of fibronectin into an insoluble matrix (Monaghan et al., 2004; Vial et al., 2006). uPAR is known to associate with specialized lipid-raft structures, termed caveolae, which serve as signaling platforms to link extracellular GPI-linked proteins with intracellular molecules. To investigate the role of caveolae in the regulation of fibronectin matrix assembly by P25, the cholesterol-depleting agent methyl-β-cyclodextrin (MβCD) was used to disrupt raft structures. Monolayers of human foreskin fibroblasts were incubated with increasing doses of MβCD for 1 hour before treatment with P25 or the scrambled control peptide, S25. Fig. 1A (white bars) shows a 35-fold increase in detergent-insoluble matrix in the presence of P25. The effect of P25 on matrix assembly was lost when cells were pre-treated with MβCD. The effect of MβCD on matrix assembly was partially reversible because treatment of MβCD-treated cells with cholesterol to restore caveolae integrity rescued the ability of P25 to increase fibronectin matrix assembly (Fig. 1A, black bars). Treatment with either cholesterol or MβCD alone had no effect on matrix assembly, suggesting that caveolae do not participate in the basal regulation of fibronectin matrix deposition (data not shown).

As an alternative approach, we also used RNA interference (RNAi) to knockdown caveolin-1 expression in human foreskin fibroblasts (Fig. 1B). Caveolin-1 protein was decreased by approximately 70% in cells transfected with siRNA against caveolin-1 when compared with cells transfected with a non-targeting siRNA (Fig. 1C). As shown in Fig. 1D, treatment of cells with P25 resulted in a sixfold increase in expression of matrix-assembly sites as compared with untreated cells or cells treated with the control peptide, S25. Cells transfected with caveolin-1 siRNA showed a severe attenuation in the enhancement of 70-kDa binding sites seen in response to uPAR ligation with P25 (Fig. 1D). A similar effect was seen when looking at fibronectin binding (Fig. 1E). The caveolin-1-knockdown cells showed an abrogated ability to increase fibronectin binding in response to P25 treatment when compared with cells transfected with a non-targeting siRNA. Caveolin-1 knockdown had no effect on the basal level of 70-kDa fragment or fibronectin binding (data not shown), indicating that caveolin-1 is required for the P25-dependent effects on matrix assembly but that caveolin-1 does not participate in the regulation of basal fibronectin matrix assembly.

Our earlier studies showed that the enhancement of matrix assembly by P25 was associated with an increase in the amount of activated β1 integrin found on the cell surface (Monaghan et al., 2004). To determine whether the effects of P25 on β1-integrin activation require caveolin-1, RNA interference was used to knockdown caveolin-1. As measured by ELISA using an antibody (HUTS4) that recognizes the active form of the integrin, cells transfected with caveolin-1 siRNA showed severe attenuation in the P25 enhancement of β1-integrin activation, as compared with the cells transfected with a non-targeting siRNA (Fig. 2A). There was no difference in the total level of β1-integrin expression between the caveolin-1-siRNA- and non-targeting-siRNA-transfected cells, suggesting that the caveolin-1 knockdown had no effect on the level of β1 integrin expressed at the cell surface (Fig. 2B).

Previously, we have shown that P25-dependent activation of the β1 integrin requires the ligand-independent transactivation of the EGFR. Transactivation of the EGFR by P25 is mediated by the Src-dependent phosphorylation of EGFR at Tyr845 (Monaghan-Benson and McKeown-Longo, 2006). To test the role of caveolae on activation of the EGFR by P25, MβCD was used to disrupt caveolae structures and activation of EGFR was assessed using an antibody that recognizes the active conformation of the EGFR. As shown in Fig. 3A, MβCD effectively inhibited the activation of EGFR by the uPAR ligand, P25. As shown in Fig. 3B, MβCD treatment also inhibited the phosphorylation of EGFR at Tyr845 in response to P25 treatment, consistent with caveolae being required for EGFR activation by P25. To determine whether phosphorylation of the EGFR at Tyr845 following P25 stimulation was caveolin-dependent,

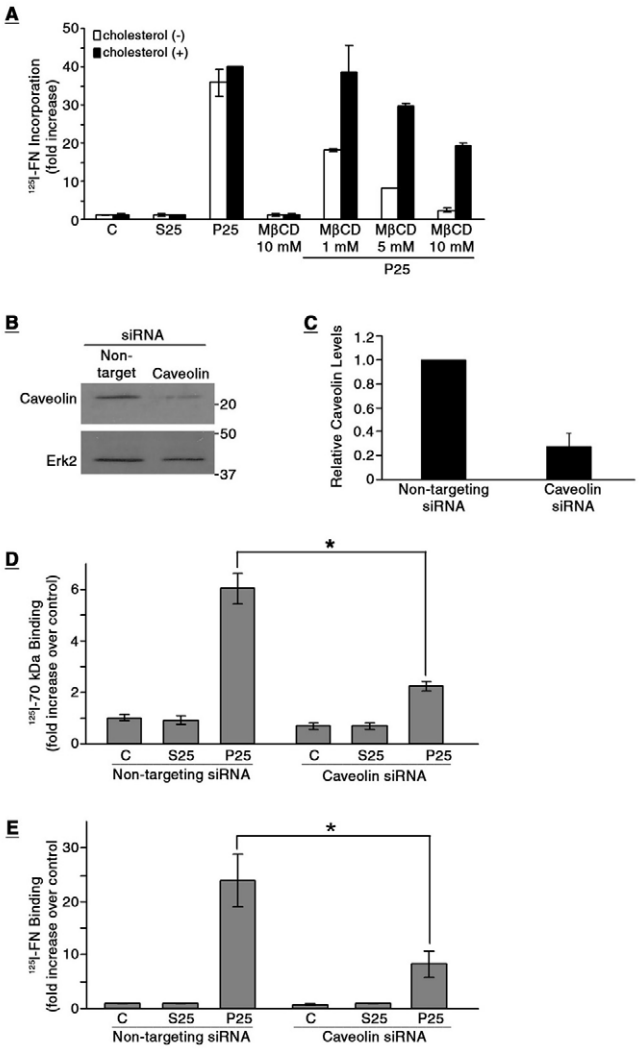

Fig. 1. Cholesterol depletion and caveolin knockdown inhibit the effects of uPAR stimulation on matrix assembly. (A) Monolayers of A1-F cells were treated with increasing doses of MβCD for 30 minutes to deplete cholesterol. After washing, cholesterol was reintroduced into some of the cells (+) by incubating cholesterol-depleted cells with cholesterol (1 mM):MβCD (10 mM). Cells were then incubated with either 50 μM of the uPAR ligand, P25, or the control peptide, S25. Cell layers receiving no peptide served as additional controls (labeled as 'C'). ^{125}I-fibronectin (^{125}I-FN) was added to the medium for 6 hours. Cell layers were extracted in 1% deoxycholate, and soluble and insoluble material was separated by centrifugation. The amount of ^{125}I-FN incorporated into the detergent-insoluble matrix was determined by γ scintillation. Representative data is shown; *n*=3. The error bars represent the s.e.m. of triplicates. All data were normalized against the control value, which was set at 1. (B) Cells were transfected using oligofectamine with siRNA directed against caveolin-1 or a control non-targeting siRNA for 72 hours. Cells were lysed in gel sample buffer under reducing conditions and lysates were electrophoresed, immunoblotted and analyzed for the expression of caveolin. Blots were then stripped and re-probed for total Erk2 as a loading control. Representative data is shown; *n*=4. Caveolin knockdown was evident at 72 hours and persisted through to 96 hours. (C) The blots shown in B were scanned and the data was normalized to Erk2. Values from control siRNA cells were set at 1. Error bars represent s.e.m.; *n*=4. (D) Cells shown in B were treated with 50 μM of P25 or the control peptide, S25, and the ^{125}I–70-kDa-fibronectin-fragment was added to the medium and incubated with the cells for 1 hour. Cells were then rinsed in PBS and solubilized in 1 N NaOH. The amount of ^{125}I–70-kDa that was associated with the cell layers was determined by γ scintillation. (E) Cells shown in B were incubated with P25 or S25 peptides in the presence of ^{125}I-FN for 6 hours. Cells were then rinsed in PBS and solubilized in 1 N NaOH. The amount of ^{125}I-FN that was associated with the cell layers was determined by γ scintillation. Data in D and E are presented as fold increase over control, in which cells incubated in the absence of any peptide (labeled as 'C') serve as control. Bars represent the standard error of triplicate samples. Asterisk (*) indicates that P25 stimulation of matrix assembly in siRNA-knockdown cells is statistically different from control cells receiving non-targeting siRNA (*t*-test, $P<0.05$).

cells were transfected with caveolin-1 siRNA or a non-targeting control siRNA prior to treatment with P25. As shown in Fig. 3C, caveolin-1 knockdown abolished the P25-dependent phosphorylation of the EGFR at Tyr845 as compared with the phosphorylation seen in the control knockdown cells. These data show that caveolin-1 is required for the P25-dependent phosphorylation of the EGFR at Tyr845. Western blot analysis indicated that the P25-dependent phosphorylation of Src at Tyr418 was also dependent on the presence of caveolin-1, because P25 induced the phosphorylation of Src in control cells treated with non-targeting siRNA but not in caveolin-1-knockdown cells (Fig. 3D). These data indicate that the P25-dependent activation of Src and EGFR requires caveolin-1.

Earlier studies have shown that caveolin can both regulate Src activity as well as serve as a substrate for active Src (Lee et al., 2000; Li et al., 1996). To determine whether caveolin-1 was being phosphorylated by Src in response to P25, lysates from cells treated with P25, or the control peptide, S25, were examined for caveolin-1 phosphorylation at Tyr14. As shown in Fig. 4A, caveolin-1 phosphorylation at Tyr14 was seen in response to P25 treatment. To confirm that the effect of P25 on caveolin-1 phosphorylation was dependent on uPAR, uPAR expression was knocked-down through RNAi (Fig. 4B). As seen by scanning of western blots (Fig. 4C), uPAR knockdown with siRNA resulted in a decrease in uPAR protein levels of approximately 90%. As shown in Fig. 4D, uPAR knockdown attenuated the phosphorylation of caveolin-1 at Tyr14 in response to P25 treatment as compared with the phosphorylation seen in the control knockdown cells. Because previous studies have demonstrated that the Src-dependent phosphorylation of caveolin-1 at Tyr14 can occur in response to EGF treatment (Orlichenko et al., 2006), we evaluated whether the EGFR or Src family kinases were required for the P25-dependent phosphorylation of caveolin-1. EGFR and Src kinase activities were inhibited using AG1478 and PP2, respectively. As shown in Fig. 4E, the enhancement of Tyr14 phosphorylation on caveolin -1seen in response to P25 was inhibited by PP2 but not by AG1478, indicating that the phosphorylation of caveolin-1 in response to P25 is dependent on the Src family kinases. Our studies indicate that P25-dependent activation of Src requires caveolin-1 and are consistent with earlier studies showing that uPAR as well as Src are localized to caveolae (Li et al., 1996; Stahl and Mueller, 1995). To determine whether uPAR and Src are associated with caveolae in dermal fibroblasts, uPAR was immunoprecipitated from fibroblast lysates before and after treatment with P25. As shown in Fig. 4F, western blot analysis of uPAR immunoprecipitates indicated that uPAR was associated with both caveolin-1 and Src before and after treatment with P25. These results indicate that uPAR-caveolin-Src complexes are found within the cell, and are consistent with caveolin-1 functioning as a transmembrane scaffold that facilitates signaling between uPAR and Src. The data shown in Figs 3 and 4 indicate that caveolin-1 is required for the activation of Src and EGFR by P25, and that activated Src phosphorylates caveolin-1 at residue Tyr14. Taken

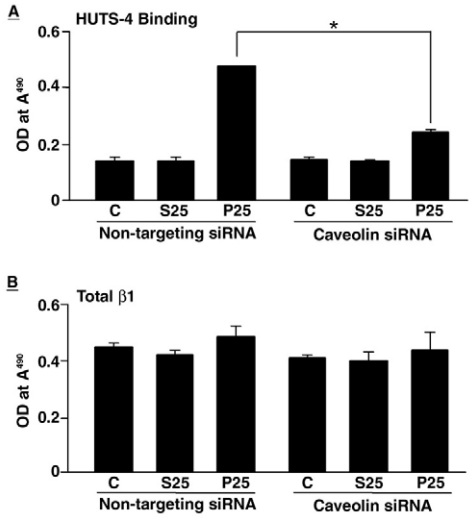

Fig. 2. Caveolin knockdown inhibits the effects of uPAR stimulation on β1-integrin activation. Cells were transfected with caveolin siRNA or control non-targeting siRNA for 72 hours. Cells were treated with 50 μM of the uPAR ligand, P25, or the control peptide, S25, for 1 hour. (A) Cells were fixed and incubated with HUTS-4, a monoclonal antibody against the activated β1 integrin, for an additional 1 hour. Bound antibody was detected by ELISA. (B) Cells were fixed and incubated with P5D2, a monoclonal antibody against total β1, for an additional 1 hour. Bound antibody was detected by ELISA. Bars represent the standard error of triplicate samples. Asterisk (*) indicates siRNA knockdown is statistically different from control cells receiving non-targeting siRNA (*t*-test, $P<0.05$).

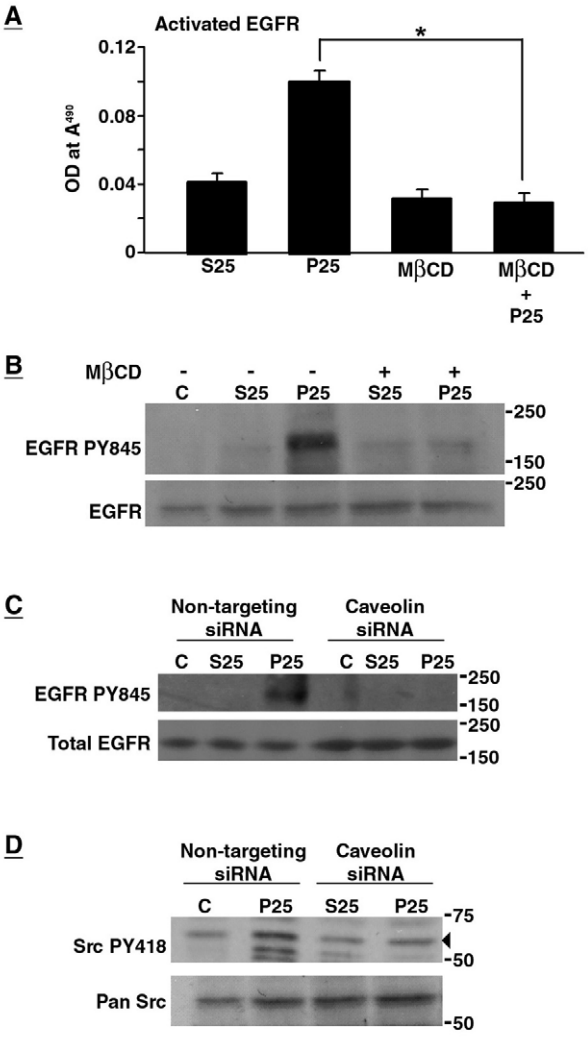

Fig. 3. Cholesterol depletion and caveolin knockdown inhibit uPAR-mediated EGFR phosphorylation. (A,B) Confluent fibroblast monolayers were incubated with MβCD (10 mM) for 30 minutes. Cells were washed and incubated with 50 μM of the uPAR ligand, P25, or the control peptide, S25. (A) Cells were fixed and incubated with mAb74, a monoclonal antibody directed against an activated conformation of EGFR, for 1 hour. Bound antibody was detected by ELISA. $n=3$; error bars indicate s.e.m. Asterisk (*) indicates data that is significantly different from P25 treatment alone (*t*-test, $P<0.05$). (B) Cell layers were lysed in gel sample buffer under reducing conditions. Lysates were electrophoresed and immunoblotted using an antibody against the EGFR phosphorylated at Tyr845 (EGFR PY845). The blot was then stripped and re-probed with a total EGFR antibody to assure equal loading. (C,D) Cells were transfected with caveolin siRNA or a non-targeting siRNA for 72 hours. Cells were then incubated with 50 μM S25 or P25 for 1 hour. Cell layers were lysed in sample buffer. (C) Lysates were electrophoresed and immunoblotted using an antibody against EGFR PY845. The blot was then stripped and re-probed with a total EGFR antibody to assure equal loading. (D) Lysates were electrophoresed and immunoblotted in parallel using an antibody against Src family kinases phosphorylated at Tyr418 (Src PY418) or a pan Src antibody to detect total Src. The arrowhead indicates Src.

together, these results suggest that caveolin-1 functions both upstream and downstream of Src in this uPAR-dependent signaling pathway.

Recent studies have suggested that phosphorylated caveolin localizes to focal adhesions (del Pozo et al., 2005; Gaus et al., 2006). To determine whether P25 treatment was affecting the localization of phosphorylated caveolin to focal adhesions, β1 integrin and caveolin phosphorylated on Tyr14 (caveolin PY14) were localized by indirect immunofluorescence. Following a 1-hour treatment with P25, cells were fixed, permeabilized and dual-stained with antibodies to caveolin PY14 and to the β1 integrin (Fig. 5A). In the presence of the control peptide S25, β1 staining was localized to adhesion sites and there was some staining of phosphorylated caveolin also at adhesion sites. Treatment with P25 caused an increase in the staining of phospho-caveolin in adhesion sites, suggesting that P25 treatment results in an enhanced localization of caveolin PY14 to areas of cell-matrix contact. uPAR was not seen in adhesion sites, but remained diffuse over the cell surface (data not shown). The enhanced localization of caveolin PY14 to the focal adhesions was also demonstrated by isolating substrate-attached material (SAM), which is enriched in focal-contact-associated proteins (Cathcart and Culp, 1979). Western blot analysis indicated that SAMs prepared from P25-treated cells were greatly enriched in caveolin PY14 as compared with control cells. We previously reported that the EGFR localizes to adhesion sites after P25 treatment (Monaghan-Benson and McKeown-Longo, 2006). Consistent with this earlier report, Fig. 5B shows that SAMs prepared from cells treated with P25 also exhibited increased levels of EGFR when compared with SAMs prepared from S25-treated cells. These data indicate that uPAR stimulation with P25 results in the redistribution of caveolin PY14 and EGFR to adhesion sites.

The observation that both the EGFR and caveolin PY14 localize to adhesion sites suggests that their redistribution following P25 treatment might be interdependent. Experiments were designed to determine whether the EGFR was required for caveolin PY14 to localize to adhesion sites and conversely whether caveolin-1 was required for the EGFR to localize to adhesion sites. To address these

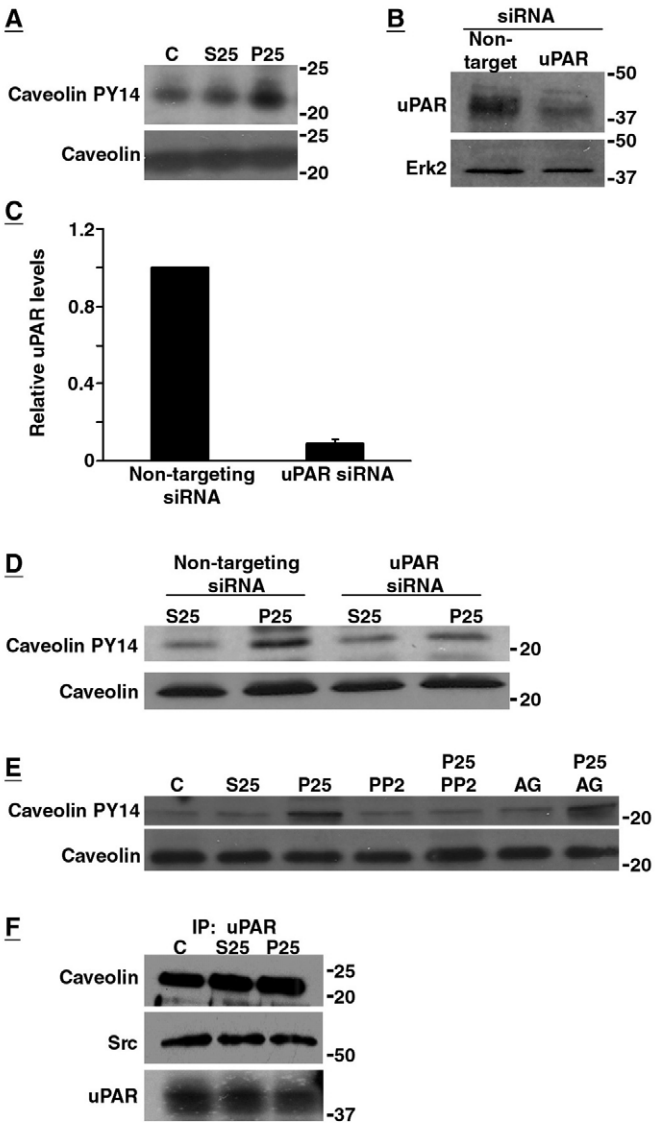

Fig. 4. uPAR stimulation results in Src-dependent phosphorylation of caveolin at Tyr14. (A) Confluent fibroblast monolayers were treated with either 50 µM of the uPAR ligand, P25, or the control peptide, S25, for 1 hour. Cell layers were lysed in sample buffer. Lysates were electrophoresed, immunoblotted and analyzed using an antibody that recognizes caveolin phosphorylated at Tyr14 (caveolin PY14). Blots were then stripped and re-probed with a total caveolin antibody to verify equal loading. (B) Cells were transfected with *uPAR* siRNA or a non-targeting siRNA for 72 hours. Cell layers were lysed in unreduced sample buffer. Lysates were electrophoresed and immunoblotted using an antibody against uPAR or Erk2. (C) Gels were scanned and uPAR values were normalized to Erk2. Values from control siRNA cells were set at 1. Bars reflect the range of knockdown (n=2). (D) Cells treated with either non-targeting siRNA or *uPAR* siRNA were incubated with 50 µM P25 or S25 for 1 hour. Cell layers were lysed in sample buffer and lysates were analyzed by western blot for caveolin PY14. Blots were stripped and re-probed for total caveolin to verify equal loading. (E) Fibroblast monolayers were pre-treated with 10 µM PP2 or 5 µM AG1478 (AG) for 1 hour before treatment with 50 µM P25 or S25. Cell lysates were electrophoresed and immunoblotted using an antibody against caveolin PY14. Blots were then stripped and re-probed for total caveolin. (F) Cells were treated with P25 or S25 as described in A and then extracted in RIPA buffer. uPAR was immunoprecipitated from lysates using anti-uPAR antibody 3937. Immune complexes were precipitated with protein A/G agarose beads, solubilized in unreduced sample buffer and electrophoresed into gels. Components of the complexes were analyzed by western blotting.

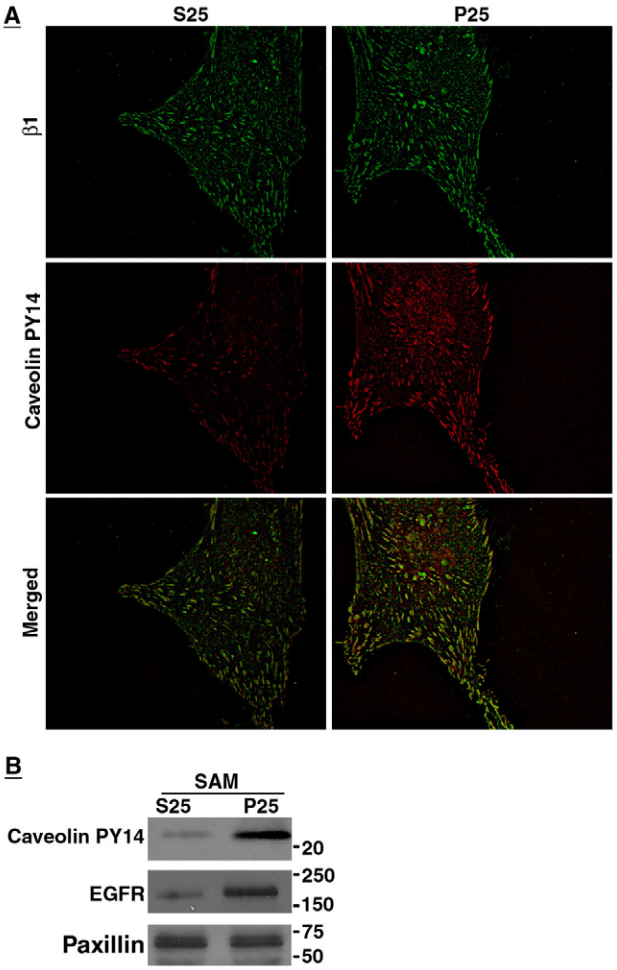

Fig. 5. Treatment of cells with uPAR ligand causes enhanced localization of caveolin PY14 to areas of cell-matrix contact. (A) Fibroblasts were seeded onto glass coverslips coated with 10 µg/ml fibronectin for 2 hours. Cells were then treated with 50 µM P25 or the control peptide, S25, for 1 hour. Cells were fixed, permeabilized and immunostained for caveolin PY14 and the β1 integrin. (B) Confluent monolayers of A1-F cells were incubated with 50 µM S25 or P25 for 1 hour. SAM was isolated by removing cells from the substrate with EGTA and was solubilized in sample buffer under reducing conditions. SAMs were electrophoresed and western blotted. Blots were cut and stained for caveolin PY14, EGFR and paxillin.

issues, EGFR was knocked down in cells using siRNA (Fig. 6A). Scanning of western blots indicated a nearly 60% decrease in EGFR protein (Fig. 6B). SAMs prepared from EGFR-knockdown cells show that caveolin PY14 exhibited enhanced localization to the adhesion site in response to P25 treatment, even in the absence of the EGFR (Fig. 6C). To evaluate whether caveolin-1 was required for the movement of EGFR to focal adhesions, caveolin-1 knockdown was used (Fig. 2). As shown in Fig. 6D, SAMs prepared from caveolin-1-siRNA-treated cells no longer show enhanced localization of the EGFR to adhesion sites after P25 treatment.

To determine whether the phosphorylation of caveolin-1 at Tyr14 is required for P25-dependent effects on fibronectin matrix assembly, mouse embryo fibroblasts (MEFs) that were null for caveolin-1 were infected with adenovirus containing either wild-type caveolin-1 or caveolin-1 in which Tyr14 was mutated to a phenylalanine (Y14F). Fig. 7A shows expression of FLAG-tagged

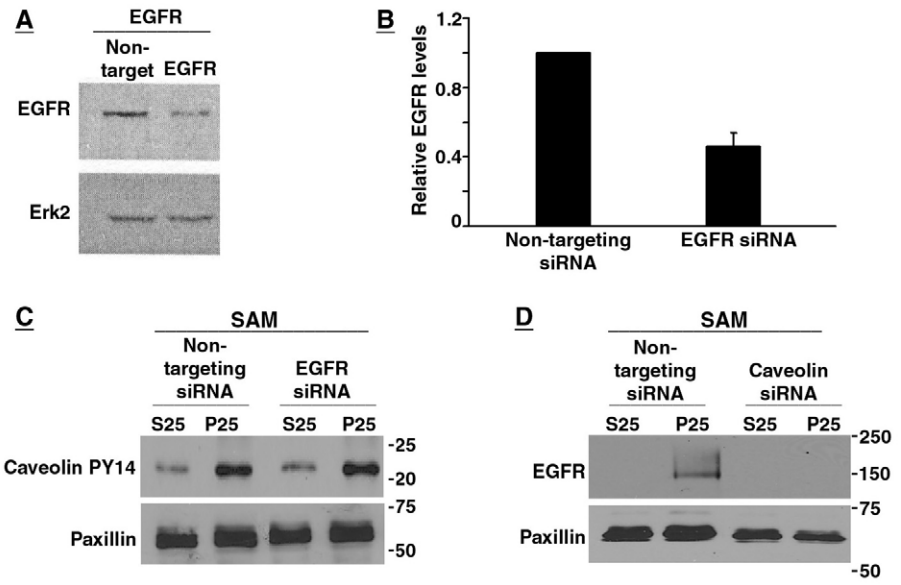

Fig. 6. Caveolin is required for uPAR-dependent EGFR translocation to the focal adhesion. Cells were transfected with *EGFR* siRNA (A-C) or caveolin siRNA (D, and see Fig. 2) as described in the Materials and Methods. (A) Cells treated with *EGFR* siRNA or non-targeting siRNA were lysed in sample buffer under reducing conditions, electrophoresed and analyzed by western blot for EGFR. Blots were stripped and re-probed for Erk2 as a loading control. (B) Blots shown in A were scanned and data was normalized against Erk2. Values from cells receiving control siRNA were set at 1. Bars reflect the range of knockdown (n=2). (C) Cells treated with control siRNA or *EGFR* siRNA were incubated with 50 μM P25 or S25 for 1 hour. SAMs were isolated by removing cells from the substrate with EGTA treatment and solubilized in sample buffer under reducing conditions. SAMs were electrophoresed, immunoblotted and analyzed for caveolin phosphorylated at Tyr14. Blots were stripped and re-probed for paxillin. (D) SAMs were prepared from cells treated with control siRNA or caveolin siRNA and electrophoresed, immunoblotted and analyzed for EGFR. Blots were stripped and re-probed for paxillin.

wild-type and Y14F-mutant caveolin protein in MEF cells. In control monolayers of wild-type MEFs expressing endogenous caveolin, treatment of cells with the uPAR ligand, P25, caused an increase in the accumulation of fibronectin in the cell layer (Fig. 7B). As expected, the caveolin-1-null cells did not respond to P25. The effects of P25 on fibronectin binding were restored upon infection of caveolin-1-null cells with virus containing wild-type caveolin-1. However, infection of cells with virus containing caveolin Y14F only modestly increased fibronectin binding, suggesting that the phosphorylation of caveolin-1 at Y14 is a crucial step in the regulation of fibronectin matrix assembly by P25. A similar effect was seen when cells were examined for β1-integrin activation using 9EG7, a monoclonal antibody that recognizes activated β1 integrins. Fig. 7C shows enhanced β1-integrin activation in the control MEFs but not in caveolin-1-null MEF cells upon stimulation with P25. The increase in integrin activation was restored in the null cells when wild-type caveolin-1 was expressed, but not when caveolin Y14F was expressed. These data indicate that phosphorylation of caveolin-1 at Tyr14 is essential to P25-dependent stimulation of β1-integrin activation and fibronectin matrix assembly.

To determine whether the phosphorylation of caveolin-1 is required for the P25-dependent phosphorylation of the EGFR at Tyr845, the caveolin-1-null cells expressing either wild-type caveolin-1 or the Y14F caveolin-1 mutant were evaluated. As shown in Fig. 8A and 8B, phosphorylation of the EGFR at Tyr845 in response to P25 was observed in the control MEFs ($cav^{+/+}$) and was lost in the caveolin-1-null cells ($cav^{-/-}$) but rescued upon re-expression of either wild-type caveolin-1 or caveolin Y14F. These data suggest that caveolin-1 but not phospho-caveolin-1 is required for the P25-dependent phosphorylation of the EGFR. To determine whether the phosphorylation of caveolin-1 at Tyr14 was required for the P25-dependent translocation of the EGFR to adhesion sites, SAMs were collected from MEF cells expressing wild-type or mutant caveolin (Fig. 8C,D). We observed that, in control MEF cells, P25 treatment resulted in enhanced levels of EGFR in adhesion sites. There was no enhancement in the localization of the EGFR to adhesion sites in the caveolin-null cells. Re-expression of wild-type caveolin in the null cells rescued the effect of P25 on EGFR trafficking. By contrast, expression of mutant caveolin (Y14F) did not rescue the effect of P25 on EGFR localization, suggesting that phosphorylation of caveolin-1 at Tyr14 is required for the localization of the EGFR to adhesion sites in P25-treated cells. Taken together these data suggest that caveolin-1 is required for the P25-dependent phosphorylation of EGFR and that phosphorylation of caveolin-1 at Tyr14 is required for the translocation of EGFR to focal-adhesion sites. To further examine the relationship between caveolin-1 and the EGFR, co-immunoprecipitation analysis was performed. As demonstrated in Fig. 9A, caveolin-1 and the EGFR strongly co-immunoprecipitate under control and S25-treated conditions. However, upon treatment with P25, there was a significant reduction in the amount of caveolin-1 that immunoprecipitated with the EGFR, suggesting that P25 treatment dissociates the EGFR from caveolin-1. This would be consistent with EGFR leaving caveolae after treatment with P25. By contrast, the association of EGFR with phospho-caveolin-1 was increased following P25 treatment (Fig. 9B), consistent with EGFR and phospho-caveolin-1 translocating to focal adhesions. Taken together with the results of our previous studies (Monaghan-Benson and McKeown-Longo, 2006), the data presented in this manuscript are consistent with the hypothesis that ligation of uPAR by P25 mediates the transactivation of EGFR, resulting in the loss of EGFR from caveolae and in the redistribution of EGFR and phospho-caveolin-1 to adhesion sites. As we have previously shown (Monaghan-Benson and McKeown-Longo, 2006), this redistribution of EGFR results in the formation of EGFR-integrin complexes and is associated with an increase in the activation of the α5β1 integrin.

Discussion

Our earlier studies indicate that uPAR regulates fibronectin matrix assembly in human dermal fibroblasts through a mechanism involving the sequential activation of the Src family kinases, the EGFR and the α5β1 integrin (Monaghan-Benson and McKeown-Longo, 2006). In the current manuscript, we now show that caveolin-1 plays an important role in this pathway, functioning as both a scaffolding protein to mediate uPAR-dependent activation of Src kinase and as an accessory molecule to mediate trafficking

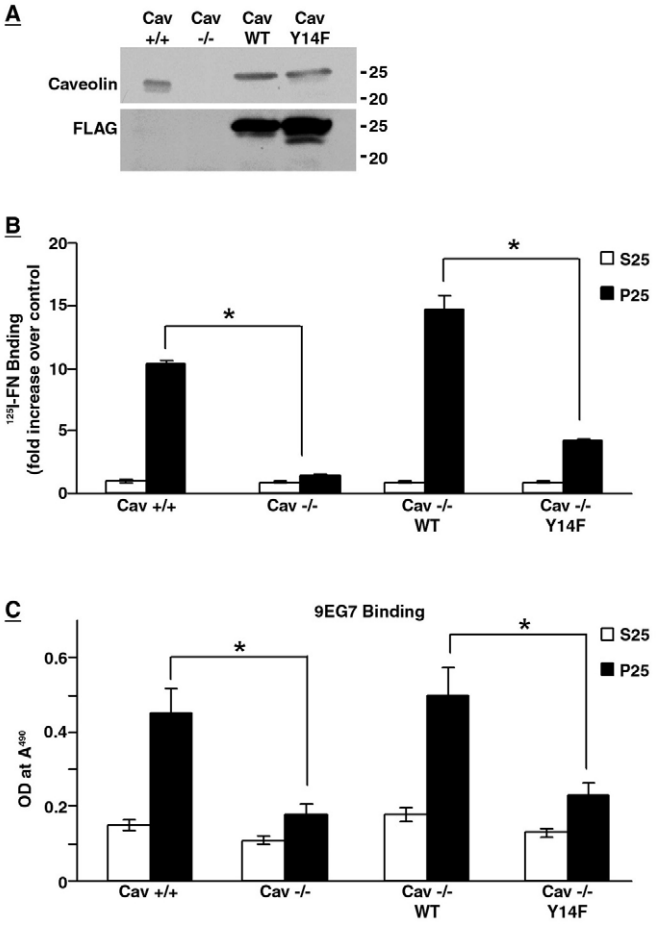

Fig. 7. Mutation of Tyr14 on caveolin inhibits the effects of P25 on fibronectin matrix assembly and β1-integrin activation. (A) MEF caveolin$^{+/+}$ (cav$^{+/+}$) and MEF cav$^{-/-}$ cells infected with adenovirus containing wild-type (WT) or mutant (Y14F) FLAG-tagged caveolins were lysed and analyzed for caveolin expression by western blot. Blots were stripped and re-probed for FLAG to visualize ectopic expression of caveolins. (B) Monolayers of normal MEFs (cav$^{+/+}$), caveolin-null cells (cav$^{-/-}$) and caveolin-null cells infected with wild-type caveolin virus (WT) or caveolin Y14F mutant virus (Y14F) for 24 hours were incubated with 50 μM of either S25 or P25 in the presence of ^{125}I-fibronectin (^{125}I-FN) for 6 hours. Cell layers were rinsed in PBS and solubilized in 1 N NaOH. Radioactivity was measured by γ scintillation. (C) Cells were fixed and incubated with 9EG7, a monoclonal antibody against the activated β1 integrin, for an additional 1 hour. Bound antibody was detected by ELISA. The data are presented as fold increase over control, where the S25 value serves as the control for each experiment. Error bars indicate s.e.m. of triplicate samples. Asterisk (*) indicates statistical difference in effects of P25 on cells expressing wild-type caveolin versus cells expressing either no caveolin or mutant caveolin (*t*-test, $P<0.05$).

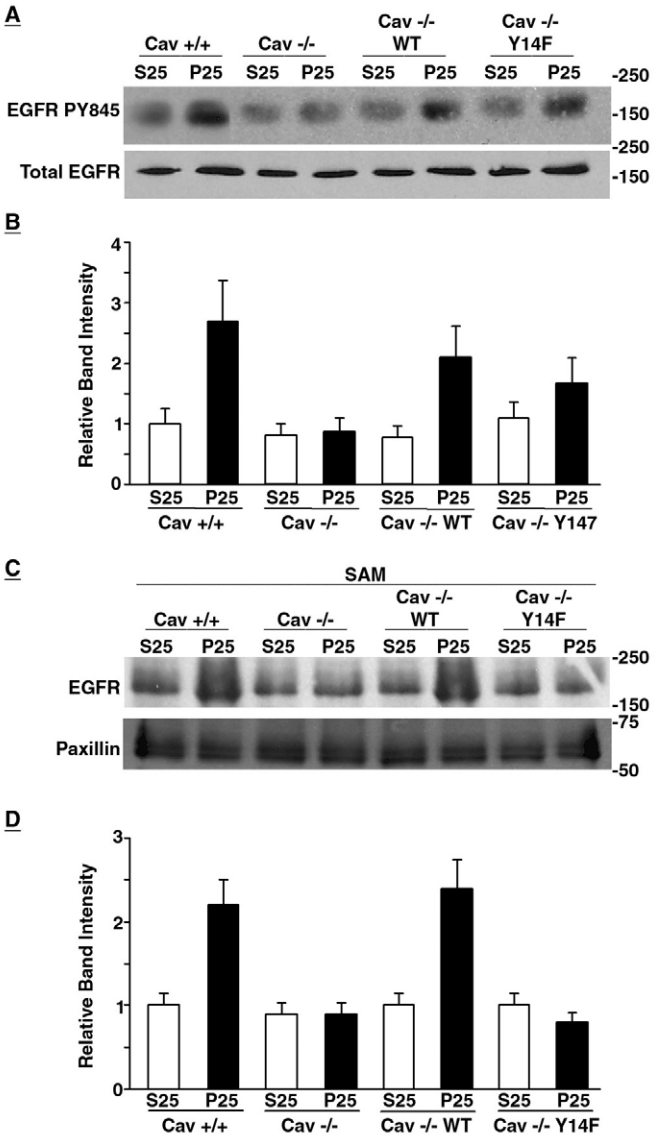

Fig. 8. Phosphorylation of caveolin is required for EGFR translocation but is dispensable for EGFR phosphorylation. Monolayers of caveolin-null cells were infected for 24 hours with virus containing wild-type caveolin or the caveolin Y14F mutant. Confluent fibroblast monolayers were treated with 50 μM of either S25 or P25 for 1 hour. (A) Cell layers were lysed in sample buffer. Lysates were electrophoresed, immunoblotted and analyzed for phosphorylated EGFR at Tyr845 (EGFR PY845). Blots were then stripped and re-probed with a total EGFR antibody to verify equal loading. (B) Western blots shown in A were scanned and the S25 value of EGFR PY845 seen in Cav$^{+/+}$ cells was set at 1. The bars show the range over two experiments. (C) Cells were then removed with EGTA treatment and SAM was isolated. Lysates were electrophoresed and immunoblotted for EGFR. Blots were then stripped and re-probed for paxillin. (D) Western blots shown in C were scanned and the EGFR value obtained from Cav$^{+/+}$ cells was set at 1. The bars show the range over two experiments.

of trans-activated EGFR to focal adhesions. Previous studies have demonstrated that uPAR, Src and the EGFR localize to caveolae, suggesting that caveolae might serve as a scaffold to assemble this signaling platform (Carlin et al., 2005; Couet et al., 1997; Li et al., 1996). Consistent with these earlier studies, we show that, in dermal fibroblasts, uPAR, Src and EGFR are all associated with caveolin-1 and that disruption of caveolae integrity, through cholesterol depletion or caveolin-1 knockdown, inhibits the ability of the uPAR ligand, P25, to enhance fibronectin matrix assembly, suggesting that crucial steps of the uPAR signaling pathway leading to enhanced fibronectin assembly occur in caveolae. Our studies indicate that caveolin-1 is required at two distinct steps in this pathway: as a scaffold or adaptor to facilitate the activation of Src and EGFR, and as an accessory protein to direct the trafficking of EGFR to focal contacts.

Activation of Src by uPAR occurs in some cells following binding of its cognate ligand, uPA (Carlin et al., 2005; Konakova et al., 1998; Nguyen et al., 2000), or in a ligand-independent manner following uPAR overexpression (Aguirre-Ghiso et al., 2001; Zhang et al., 2003). Our studies have indicated that the binding of P25,

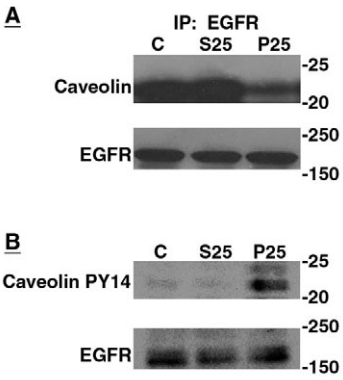

Fig. 9. uPAR ligation results in dissociation of EGFR from caveolin and increased association of EGFR with phospho-caveolin. Fibroblast monolayers were incubated with 50 μM of either P25 or S25 for 1 hour. Cells were lysed in RIPA buffer. EGFR was immunoprecipitated from cell lysates using a mouse anti-EGFR antibody (3 μg) and the resulting immunoprecipitates were solubilized in gel buffer and analyzed by western blot for EGFR and (A) caveolin or (B) phospho-caveolin.

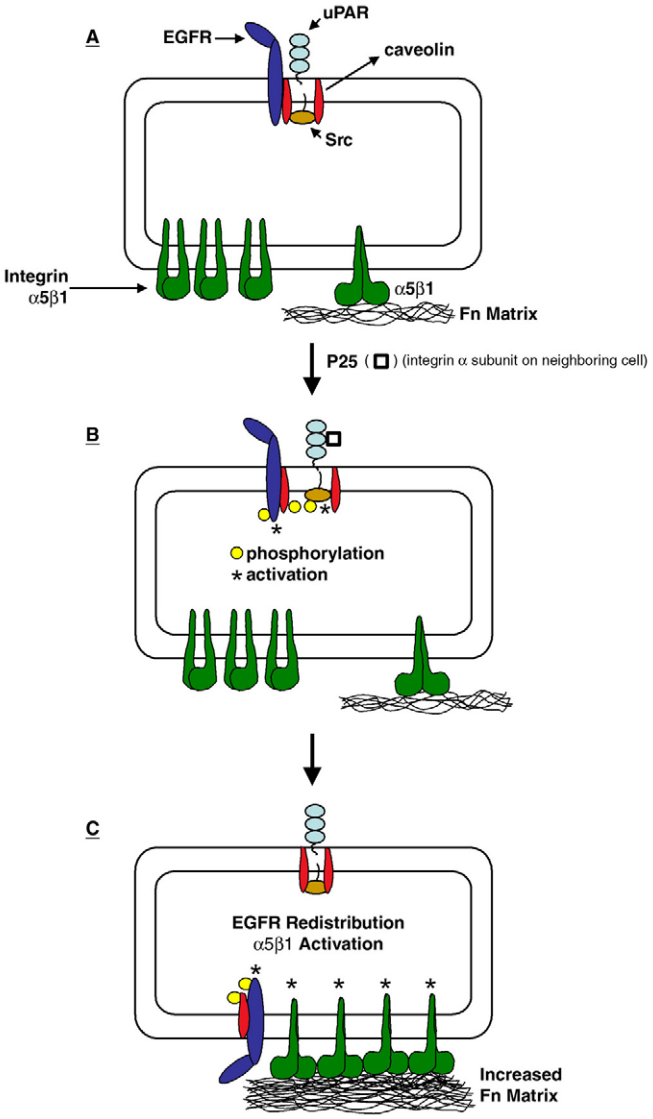

Fig. 10. Model of caveolin function in the regulation of fibronectin matrix assembly by uPAR. This figure shows that, (A) in resting cells, EGFR, uPAR and Src are found in association with caveolin. Both uPAR (via its GPI anchor) and Src (via myristylation) are assumed to partially insert into the lipid bilayer. The binding of an integrin α subunit on a neighboring cell (mimicked by P25; B) to uPAR results in the activation of Src, and to the Src-dependent phosphorylation of EGFR (Y845) and caveolin. Phosphorylation of Src and EGFR are dependent on caveolin, suggesting that caveolin functions as a scaffold to facilitate interactions between uPAR, Src and EGFR. (C) Phospho-caveolin and phospho-EGFR are then trafficked to focal adhesions. The redistribution of phospho-EGFR to focal adhesions is dependent on phospho-caveolin, suggesting that phospho-caveolin functions as an accessory molecule to facilitate proper trafficking of EGFR. Activated EGFR is found in complexes with α5β1 integrin, resulting in an increase in activation state. Increased integrin activation results in an increase in assembly of the fibronectin matrix.

but not uPA, to uPAR results in the rapid activation of Src kinase (Monaghan-Benson and McKeown-Longo, 2006). Our finding that the P25 peptide can function as an agonist to initiate uPAR signaling is in contrast to earlier studies in which integrin mimetic peptides, including P25, have been shown to function as antagonists to inhibit uPAR-dependent signaling (Aguirre-Ghiso et al., 2001; Wei et al., 2001). In these earlier studies, the inhibitory activity of these peptides was shown to result from the ability of the peptides to disrupt uPAR-integrin complexes, which are formed on cells engineered to express high levels of uPAR. The molecular basis for the contradictory effects of the P25 peptide is not known. The dermal fibroblasts used in our study express endogenous (moderate) levels of uPAR and do not contain any detectable uPAR-integrin complexes either before or after P25 treatment (Monaghan et al., 2004; Monaghan-Benson and McKeown-Longo, 2006). The ability of P25 to activate uPAR signaling in dermal fibroblasts suggests that, under conditions in which uPAR signaling is not dependent on uPAR overexpression and/or the formation of uPAR-integrin complexes, the P25 peptide functions as a uPAR agonist. To reconcile these disparate results, we would propose that the P25 peptide can function both as an agonist and as an antagonist of uPAR signaling. Whether one sees an agonist or an antagonist activity would depend on a combination of factors: cell type, uPAR expression levels and the extent of uPAR-integrin 'cis'-forming complexes.

The mechanisms by which uPAR (a GPI-anchored protein that lacks an intracellular domain) activates Src are not well understood. Earlier studies have reported that uPAR-initiated signaling depends on uPAR forming cis-activating complexes with transmembrane proteins such as integrins, G-protein-linked receptors or growth-factor receptors (reviewed by Ragno, 2006). Recent studies have suggested that the GPI anchor itself might be sufficient to initiate signaling (Madsen et al., 2007; Nicholson and Stanners, 2006). Our studies suggest that, in dermal fibroblasts, caveolin-1 is an essential component of the mechanism by which uPAR activates Src. Myristylation of Src promotes its association with the inner leaflet of the plasma membrane (Kaplan et al., 1988), and several studies, including the results presented here, indicate that uPAR–Src–caveolin-1 complexes are present on the cell surface (Cao et al., 2004; Schwab et al., 2001; Stahl and Mueller, 1995; Wei et al., 1999). Caveolin-1 binds Src and regulates its kinase activity (Li et al., 1996), and this interaction controls the ability of Src to engage diverse effector pathways (Mastick and Saltiel, 1997; Wary et al., 1998). Taken together, these data support a role for caveolin-1 as a scaffold or adaptor molecule that promotes uPAR regulation of Src activity.

In addition to binding Src, caveolin-1 can also directly interact with the EGFR and stabilize the receptor kinase in an inactive conformation (Couet et al., 1997). We demonstrate that the EGFR co-immunoprecipitates with caveolin-1 in resting cells and, upon stimulation with P25, EGFR is phosphorylated by Src and dissociates from caveolin-1. These findings are consistent with earlier studies demonstrating that EGFR rapidly moves out of caveolae following activation (Abulrob et al., 2004; Mineo et al., 1999), and suggest that caveolin-1 acts to sequester inactive EGFR and prevent its engagement with downstream signaling pathways. Treatment of cells with P25 disrupted the association of EGFR with caveolin-1, and enhanced the association of EGFR with phospho-caveolin-1 and β1 integrin in focal contacts. These data suggest that, once activated by Src-dependent phosphorylation, the EGFR, together with phospho-caveolin-1, leaves the caveolae and translocates to the focal-adhesion site where EGFR complexes with the α5β1 integrin (see also Monaghan-Benson and McKeown-Longo, 2006). The functional significance of the translocation of EGFR to focal contacts is not yet known. Our previous studies have shown that treatment of cells with inhibitors of EGFR kinase blocks EGFR translocation to focal contacts and also blocks integrin activation (Monaghan-Benson and McKeown-Longo, 2006). The present data indicate that phospho-caveolin-1 is required for both the trafficking of EGFR to focal adhesions as well as for the P25-dependent increase in matrix assembly. Taken together, these data establish a strong correlation between EGFR localization to focal contacts and integrin activation, and suggest that integrin activation in response to uPAR ligation occurs in focal contacts. This concept is supported by our earlier studies showing an increase in active integrins present in focal contacts of P25-stimulated cells (Vial et al., 2006). How the effects of caveolin-1 on membrane order (Gaus et al., 2006) and the complex formation between phosphorylated EGFR and α5β1 integrin contribute to changes in integrin activation remain important avenues for further investigation.

A recent study has suggested that caveolin-1 can function as an accessory protein to regulate trafficking of G-protein-coupled receptors (Syme et al., 2006). Phosphorylated caveolin-1 localizes to focal contacts, where it has been reported to preserve membrane order (del Pozo et al., 2005; Gaus et al., 2006) and to recruit proteins to the focal adhesion (Sanguinetti and Mastick, 2003). Consistent with these observations, we showed that phosphorylation of caveolin-1 on Tyr14 was required for the movement of EGFR to focal-adhesion sites, suggesting that caveolin-1 might be acting as an accessory protein to regulate trafficking of the EGFR. This role for caveolin-1 is supported by a recent study showing that hyperphosphorylation of caveolin-1 in tumors is associated with the transport of active EGFR to perinuclear regions of the cell (Khan et al., 2006). Recent studies have shown that an EGF-like repeat from the matricellular protein, tenascin, binds to and redistributes EGFR to lamellipodia. Similar to our findings using P25, trafficking of activated EGFR to lamellipodia in response to tenascin is not accompanied by the activation of Erk or the internalization of EGFR, events that are typically seen in response to EGF (Iyer et al., 2007; Iyer et al., 2008). A recent study in CHO-K1 cells has now shown that uPAR-mediated Src-dependent transactivation of EGFR (phosphorylation of Tyr845) can occur in response to EGF (Jo et al., 2007). These findings suggest that, in some cells, the uPAR → Src → EGFR Tyr845 phosphorylation can represent an arm of the EGF-EGFR signaling pathway. EGFR signaling is dysregulated in many tumors and EGFR inhibitors are used to treat many of these cancers. Therefore, the pathway regulating uPAR-EGFR crosstalk might be an important therapeutic target. Determining the importance of caveolin-1 in regulating uPAR-dependent bifurcations of the EGFR signaling pathway represents an important avenue for future study.

Fig. 10 presents our working model for the proposed mechanism of uPAR-dependent regulation of matrix assembly. Our model proposes that integrins can act as transcellular ligands for uPAR to activate intracellular signaling pathways regulating the assembly of the fibronectin matrix. Earlier studies have shown that integrin-uPAR interactions can occur in trans between cells (Tarui et al., 2001). On the basis of this model, one can speculate that, in dermal fibroblasts, transcellular ligation of uPAR by integrins initiates uPAR signaling, leading to the activation of Src kinase. Because several integrin α subunits have been reported to bind to uPAR, further studies are needed to identify those integrins that can act transcellularly to initiate uPAR signaling to Src. Such transcellular interactions between integrin and uPAR might be of significance in the tumor microenvironment. Most tumor cells do not assemble extracellular matrix, but are dependent on fibronectin matrix for survival signals and for recruitment of angiogenic vasculature. The binding of tumor-cell integrins to stromal fibroblast uPAR would be expected to result in a rapid upregulation of fibronectin deposition by the fibroblast, thereby providing the tumor cell with the fibronectin matrix necessary to promote tumor progression. Consistent with this model, a recent study has shown that the α6β1 integrin that is present on pancreatic cancer cells binds to uPAR that is present on peritumor fibroblasts to promote activation of MMP2 (He et al., 2007). We have, therefore, proposed that, in our system, the P25 peptide simulates transcellular binding between integrin receptors on one cell and uPAR on a neighboring cell. The binding of P25 to uPAR that is present in caveolae results in the caveolin-1-dependent activation of Src kinase. In this initial step, caveolin-1 functions as a transmembrane adaptor and scaffold to mediate uPAR-dependent Src activation and Src-dependent transactivation of the EGFR. After phosphorylation by Src, phospho-caveolin-1 then functions as an accessory protein to direct the trafficking of phospho-EGFR from caveolae to focal adhesions. Phospho-EGFR then complexes with the α5β1 integrin, increasing the activation state of this protein and stimulating fibronectin fibrillogenesis. Our data indicate that caveolin-1 serves a complex role in the uPAR pathway, regulating fibronectin matrix assembly in fibroblasts. An earlier study has shown that caveolin-1 also controls the endocytosis and turnover of the fibronectin matrix (Sottile and Chandler, 2005), and, together with our study, points to caveolin-1 as an important regulator of fibronectin levels present in the matrix. The present studies are the first to document a role for caveolin-1 in the assembly of the fibronectin matrix, and provide a novel paradigm for the regulation of crosstalk among integrins, growth-factor receptors and uPAR.

Materials and Methods

Reagents

Unless otherwise stated, all chemicals were purchased from Sigma. Peptides P25, sequence AESTYHHLSLGYMYTLN, and S25, sequence NYHYLESSMTAL-YTLGH, were synthesized by Cell Essentials (Boston, MA). The anti-β1 antibody, clone P5D2, and the HUTS-4 antibody against the active conformation of the β1 integrin were purchased from Chemicon (Temecula, CA). Monoclonal antibody 9EG7, which recognizes the activated form of the β1 integrin, was purchased from Pharmingen. Monoclonal antibody 74, against the activated EGFR, the anti-EGFR and anti-caveolin-1 antibodies were from BD Pharmingen. The EGFR phosphotyrosine-specific antibodies were from Cell Signaling. Anti-Src PY418 and pan Src antibodies were obtained from BIOSOURCE (Camarillo, CA). Erk2 was from Santa Cruz Biotechnology (Santa Cruz, CA). PP2, a Src inhibitor, was from BIOMOL (Plymouth Meeting, PA). Anti-uPAR antibody 3937 was from American

Diagnostica. The polyclonal antibody to uPAR was obtained from Andrew Mazar (Attenuon, San Diego, CA). Adenovirus for wild-type caveolin-1 and the caveolin-1 mutant Y14F were prepared as previously described (Sanguinetti and Mastick, 2003). Iodinated human plasma fibronectin and the 70-kDa N-terminal fragment were prepared as described previously (Zheng and McKeown-Longo, 2002). Human foreskin fibroblasts (A1-F) were a gift from Lynn Allen-Hoffmann (University of Wisconsin, Madison, WI). The caveolin-1$^{+/+}$ and caveolin-1$^{-/-}$ MEF cell lines were purchased from ATCC. All cells were grown in Dulbecco's modified eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT).

Matrix-assembly assays

Cultures were incubated with ^{125}I-fibronectin (1 μg/ml; 1×10^6 cpm/ml) or ^{125}I-labeled 70-kDa fragment (100 ng/ml) in serum-free DMEM at 37°C. 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 scraped into 1 N NaOH to determine the total cell-layer-associated radioactivity. In some experiments, the detergent-insoluble extracellular matrix was isolated by extraction of cell layers in 1% deoxycholate (DOC) dissolved 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. DOC-insoluble matrix was obtained following centrifugation at 39,000 ***g*** for 30 minutes.

Receptor-activation assays

Activated integrins and EGFR were assayed by ELISA as described previously (Monaghan-Benson and McKeown-Longo, 2006). Activated levels of β1 integrin were detected by incubating cells with either 100 ng/ml of 9EG7 or 100 ng/ml HUTS-4 antibody. Total β1 was detected using the P5D2 antibody. To detect activated EGFR, cells were incubated with 300 ng/ml monoclonal antibody 74. Bound antibodies were detected using either goat anti-mouse conjugated with HRP (used with P5D2, HUTS-4 and mAb 74) or goat anti-rat conjugated with HRP (used with 9EG7). Freshly prepared substrate [0.1 M citrate buffer, 0.5 mg/ml o-phenylenediamine, 1 μl/ml 30% hydrogen peroxide (pH 5)] was added and the OD was measured at A^{490}. Measurements were corrected for light scattering by subtracting the OD at A^{630}.

Immunofluorescence microscopy

Cells were seeded onto glass coverslips, coated with 10 μg/ml fibronectin and allowed to adhere and spread for 2 hours in serum-free medium. Cells were then treated with 50 μM P25 or S25 for 1 hour, washed with PBS, fixed for 15 minutes in 3% paraformaldehyde, permeabilized in 0.3% Triton and blocked for 30 minutes in 2% bovine serum albumin. To visualize β1, cells were stained with monoclonal antibody AIIB2 (1:300). Caveolin PY14 was visualized using an antibody concentration of 1:300. This antibody was recently reported to cross-react with paxillin (Hill et al., 2007). We also found that, in addition to phospho-caveolin-1, this antibody recognized a 68-kDa band on western blots; however, staining of this 68-kDa protein was unaffected by treatment of cells with P25. Appropriate Alexa-Fluor-594- or -488-conjugated goat anti-mouse, rabbit or rat secondary antibodies were used at a concentration of 1:800. Fluorophores were visualized using an Olympus BX-60 microscope equipped with a cooled charge capture device sensi-camera. Images were acquired using Slidebook software (Intelligent Imaging Innovation, Denver, CO) and processed using Adobe Photoshop.

Immunoblot and immunoprecipitation analysis

For immunoprecipitation, cells were extracted on ice for 30 minutes in RIPA buffer plus protease inhibitors. After pre-clearing with protein A/G agarose beads, lysates were incubated with monoclonal antibodies against EGFR or uPAR for 3 hours at 4°C. Immune complexes were then precipitated for 1 hour at 4°C with protein A/G agarose beads. After washing, the complexes were resuspended in non-reduced sample buffer, boiled for 10 minutes and analyzed by western blotting. For western blot analysis of whole-cell lysates, cells were rinsed in PBS, lysed in sample buffer, electrophoresed into SDS-PAGE gels and transferred to nitrocellulose membranes (Schleicher and Schuell Bioscience). All gels were run under reducing conditions, unless otherwise noted in the figure legend. Bound antibodies were detected by enhanced chemiluminescence (Amersham Biosciences). In some instances, membranes were stripped and re-probed with a second antibody to verify equal loading. For quantification of western blots, intensity values of bands were measured using ImageJ software (NIH).

Cholesterol depletion and addition

Cells were depleted and repleted of cholesterol as previously described (Mandal et al., 2005). Briefly, cells were depleted of cholesterol by incubation with MβCD for 30 minutes. MβCD was then washed out of the wells and experiments were performed. Cholesterol replenishment was done by incubating cells with a 1:10 ratio of cholesterol:MβCD.

siRNA transfection

Cells plated at ~10% confluence and left overnight were transfected with siRNA (Dharmacon, Lafayette, CO) at a concentration of 25 nM using OligofectAMINE (Invitrogen) according to the manufacturer's instructions. A non-targeting siRNA (Dharmacon) was used as a control. Cells were transfected for 4 hours in serum-free medium, following which 1.5 ml of DMEM + 10% FBS was added. Cells were harvested after 72 hours.

Adenoviral infection of caveolin-1$^{-/-}$ cells

Wild-type caveolin and caveolin-1 Y14F recombinant adenoviruses were generated as described previously (Sanguinetti and Mastick, 2003). Caveolin-1-null MEFs were infected with adenovirus for 24 hours in DMEM + 10% FBS. Infection efficiency (>85%) was monitored through the visualization of GFP, which is coexpressed by these recombinants.

Isolation of SAM

SAM was isolated by a modification of the method of Ciambrone and McKeown-Longo (Ciambrone and McKeown-Longo, 1990). Cells were rinsed three times with 4°C DMEM and gently agitated in 10 mM HEPES, 1 mM EGTA at 37°C for 60 minutes to lift the cells. The SAM left on the bottom of each well was solubilized with reducing gel sample buffer and analyzed by western blotting.

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