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# A Role for Caveolin and the Urokinase Receptor in Integrin-mediated Adhesion and Signaling

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**Abstract.** The assembly of signaling molecules surrounding the integrin family of adhesion receptors remains poorly understood. Recently, the membrane protein caveolin was found in complexes with  $\beta 1$  integrins. Caveolin binds cholesterol and several signaling molecules potentially linked to integrin function, e.g., Src family kinases, although caveolin has not been directly implicated in integrin-dependent adhesion. Here we report that depletion of caveolin by antisense methodology in kidney 293 cells disrupts the association of Src kinases with  $\beta 1$  integrins resulting in loss of focal adhesion sites, ligand-induced focal adhesion kinase (FAK) phosphorylation, and adhesion. The nonintegrin urokinase receptor (uPAR) associates with and stabilizes  $\beta 1$  integrin/caveolin complexes. Depletion of caveolin in uPAR-expressing 293 cells also disrupts uPAR/integrin

complexes and uPAR-dependent adhesion. Further,  $\beta 1$  integrin/caveolin complexes could be disassociated by uPAR-binding peptides in both uPAR-transfected 293 cells and human vascular smooth muscle cells. Disruption of complexes by peptides in intact smooth muscle cells blocks the association of Src family kinases with  $\beta 1$  integrins and markedly impairs their migration on fibronectin. We conclude that ligand-induced signaling necessary for normal  $\beta 1$  integrin function requires caveolin and is regulated by uPAR. Caveolin and uPAR may operate within adhesion sites to organize kinase-rich lipid domains in proximity to integrins, promoting efficient signal transduction.

**Key words:** urokinase receptor • caveolin • integrins • adhesion • cell signaling

**I**NTEGRINS are heterodimeric adhesion receptors implicated in many biological processes characterized by cell movement, including inflammation, tissue remodeling, growth, and tumorigenesis (Hynes, 1992; Ruoslahti and Reed, 1994; Schwartz, 1997). Integrin-dependent cellular adhesiveness depends not only on specific  $\alpha/\beta$  integrin chains expressed by cells but also on ligand-induced integrin redistribution and signal transduction leading to cytoskeletal reorganization (Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Parsons, 1996). Signaling through the integrin family of adhesion receptors requires their association with cytoplasmic elements capable of signal transduction because integrins lack intrinsic signaling capacity. In part, these dynamic aspects of integrin function are regulated by the physical association of integrins with nonintegrin membrane proteins. These include the tetraspan family of membrane proteins, integrin-associated protein, and CD98 (Lindberg et al., 1993; Berditchevski et al., 1996; Fenczik et al., 1997). In addition

we have previously reported that  $\beta 1$  integrin function is regulated by its association with the GPI-linked nonintegrin receptor, the urokinase receptor (uPAR)<sup>1</sup> (Wei et al., 1996). When human embryonic kidney cells are engineered by transfection to express uPAR, uPAR forms complexes with  $\beta 1$  integrins and promotes adhesion of these cells to vitronectin via a vitronectin binding site on uPAR. Incorporation of  $\alpha 5/\beta 1$  integrins into complexes with uPAR also inhibits the ability of these integrins to bind fibronectin. Disruption of the uPAR/integrin complexes by uPAR-binding peptides blocks adhesion to vitronectin and restores the normal adhesiveness of 293 cells for fibronectin. Thus, the ligand specificity and adhesive function of this class of integrins is altered by the presence of uPAR.

A second nonintegrin membrane protein, caveolin, was also found to coimmunoprecipitate with  $\beta 1$  integrins and uPAR in uPAR-transfected 293 cells (Wei et al., 1996). Independently, Wary et al. (1996) observed caveolin to coprecipitate with  $\beta 1$  integrins in detergent-lysed A431 cells, which express high levels of uPAR and caveolin. Caveolin,

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1. *Abbreviations used in this paper:* FAK, focal adhesion kinase; MAPK, mitogen-activated protein kinase; SMC, smooth muscle cells; uPAR, urokinase receptor.

discovered as an Src kinase substrate, is an ~21-kD membrane protein which binds cholesterol and a number of signaling molecules potentially linked with integrin function: Src family kinases, heterotrimeric G proteins, and H-ras (Li et al., 1995, 1996a). Caveolin has an intrinsic propensity to oligomerize and it is currently thought caveolin oligomers contribute to formation of cholesterol-sphingolipid-rich microdomains within membranes and the distinct membrane structures termed caveolae (Parton, 1996; Harder and Simons, 1997). Caveolae are enriched in a number of nonreceptor signaling proteins and growth factor receptor tyrosine kinases, but are not known to contain integrins (Lisanti et al., 1994; Mineo et al., 1996). However, while this work was in progress it was reported that a fraction of cellular caveolin associates with  $\beta 1$  integrins and promotes Fyn-dependent Shc phosphorylation in response to integrin ligation, leading to mitogen-activated protein kinase (MAPK) activation and cellular growth (Wary et al., 1998). In this report we provide direct evidence that caveolin is also important to  $\beta 1$  integrin-dependent fibronectin adhesion and focal adhesion kinase (FAK) activation and propose that this operates, at least in part, by virtue of the ability of caveolin to regulate Src family kinase activity surrounding integrins. Thus, caveolin appears to be a general regulator of  $\beta 1$  integrin function.

## Materials and Methods

### Generation and Transfection of Caveolin cDNA and Antisense Constructs

Human caveolin-1 cDNA was generated by reverse transcription and polymerase chain reaction, subcloned into TA cloning vector (Invitrogen), verified by nucleotide sequencing (Sequenase; United States Biochemical), digested with BamHI and XhoI, and then subcloned into the inducible expression vector pML1 (Lukashev et al., 1994) in an inverted order. Cells were transfected by electroporation, clones selected in hygromycin, and levels of caveolin-1 assessed by immunoblotting after overnight culture with  $3 \mu\text{M}$  CdCl<sub>2</sub>. Caveolin cDNA was also subcloned into the CMV promoter-driven expression vector, pCEP9 (Invitrogen), and clones selected by growth in G418. Transfected cells were then screened for protein expression via immunoblot with antibody specific for caveolin-1 (Transduction Labs).

### Adhesion and Binding Assays

Cells ( $5 \times 10^4$ ) were seeded in fibronectin ( $5 \mu\text{g}/\text{ml}$ ; Sigma Chemical Co.) or vitronectin ( $1 \mu\text{g}/\text{ml}$ ; Becton Dickinson) coated 96-well tissue culture plates, incubated at  $37^\circ\text{C}$  for 1 h, and adherent cells quantified as described previously (Wei et al., 1996). Before the adhesion assay, antisense caveolin 293 or uPAR/293 cells were induced with  $3 \mu\text{M}$  CdCl<sub>2</sub> at  $37^\circ\text{C}$  for 8 h.

Fibronectin binding was assessed on cells seeded in polylysine-coated 96-well tissue culture plates, cultured overnight, induced with  $3 \mu\text{M}$  CdCl<sub>2</sub> at  $37^\circ\text{C}$  for 8 h, and then washed with binding buffer (DMEM, 1 mg/ml BSA). Half of the cells were treated with  $\beta 1$  integrin activating antibody TS2/16 ( $3 \mu\text{g}/\text{ml}$ ; Endogen) at  $4^\circ\text{C}$  for 30 min, and then all cells were incubated with buffer containing <sup>125</sup>I-fibronectin (1, 5, and 10 nM) at  $4^\circ\text{C}$  for 1.5 h. After washing, cells were lysed in 0.2% SDS, 0.2% Triton X-100, 10% glycerol in PBS for 30 min, and radioactivity was measured. Nonspecific binding was determined by inclusion of 0.5 mM RGDS (American Peptide Co.) in the reaction mixture.

### Flow Cytometry

Cells transfected with caveolin antisense constructs were induced with  $3 \mu\text{M}$  CdCl<sub>2</sub> at  $37^\circ\text{C}$  for 8–18 h. Cells were then detached and incubated with PBS containing 0.1% BSA and primary antibodies to uPAR (R2) or inte-

grins  $\beta 1$  (JB1A) (Wilkins et al., 1996),  $\alpha 5\beta 1$  (Chemicon), and  $\alpha v$  (L230) (American Type Culture Collection) on ice for 30 min. After washing, cells were incubated with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co.) and analyzed on a flow cytometer (FACScan<sup>®</sup>; Becton Dickinson).

### Immunofluorescence and Confocal Fluorescence Microscopy

To assess the distribution of caveolin and  $\beta 1$  integrins, human saphenous vein smooth muscle cells (SMC) and 293 or uPAR/293 cells coexpressing caveolin were cultured overnight on glass coverslips in 10% FBS. After washing, cells were incubated for 30 min with JB1A and/or K20 (Immunotec) antibodies to  $\beta 1$  or isotype controls at  $4^\circ\text{C}$  and fixed for 20 min in 3.7% paraformaldehyde. Fixed cells were permeabilized and incubated with rabbit polyclonal antibody to caveolin (Transduction Labs) for 30 min at room temperature, then incubated with FITC- or Rhodamine red-conjugated secondary antibodies and coverslips mounted in Prolong (Molecular Probes). Fluorescence staining was analyzed by confocal laser (model MRC1024; Bio-Rad Laboratories) attached to a Zeiss microscope (model Axiovert S100) using separate filters for each fluorochrome. Optical planes were imported into Adobe Photoshop and processed. To visualize integrin and caveolin clustering, SMC in suspension were incubated with FITC-K20  $\beta 1$  antibodies without or with goat anti-mouse secondary antibodies for 1 h at  $37^\circ\text{C}$ , immobilized on  $50 \mu\text{g}/\text{ml}$  polylysine-coated coverslips for 10 min, and then fixed and permeabilized as above. Cells were stained for caveolin and analyzed by confocal laser microscopy.

### Immunoprecipitation and Blotting

Control or antisense caveolin clones were cultured for 8 h in serum-free DMEM containing  $3 \mu\text{M}$  CdCl<sub>2</sub> on either fibronectin ( $5 \mu\text{g}/\text{ml}$ ), vitronectin ( $1 \mu\text{g}/\text{ml}$ ), or polylysine ( $50 \mu\text{g}/\text{ml}$ ) and lysed on ice for 30 min in buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonylfluoride, and leupeptin ( $10 \mu\text{g}/\text{ml}$ ). After preclearing with protein A-agarose, lysates were incubated with antibodies to  $\beta 1$  integrin (JB1A) or cortactin (Upstate Biotechnology Inc.) at  $4^\circ\text{C}$  overnight. In some experiments, the lysates were made  $10 \mu\text{M}$  in the caveolin-related peptide, cav-1 (DGIWKASFT-TFTVTKYWFYR), or the control cav-x peptide (WGIDKAFFTTSTV-TYKWFYR), in 0.25% DMSO (Michel et al., 1997). The cav-x peptide has an identical amino acid composition and 60% identical sequence to the cav-1 peptide. The immunoprecipitates were blotted with caveolin antibody (Transduction Labs), stripped, and rebotted for Src family kinases (Santa Cruz Biotech), cortactin, or 4G10 (Upstate Biotechnology Inc.). To analyze FAK and EGF receptor activation, clones were induced with CdCl<sub>2</sub> and plated on fibronectin- or polylysine-coated 100-mm dishes or their  $\beta 1$  integrins clustered with antibodies. Cells were lysed or stimulated with EGF ( $10 \text{ ng}/\text{ml}$ ; Upstate Biotechnology Inc.) for 5 min before lysing in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% deoxycholate, 0.1% SDS, 1% Triton X-100) supplemented with protease inhibitors. Lysates were immunoblotted for ErbB-2, -3, and -4 (Santa Cruz Biotech) and phosphotyrosine (Upstate Biotechnology Inc.).  $\beta 1$  immunoprecipitates from m1–m5 clones were immunoblotted with uPAR antibody 399R (American Diagnostica).

### Kinase Assay

To measure total enzymatic activity of  $\beta 1$ -associated Src kinases, induced cells were lysed in Triton X-100 buffer and immunoprecipitated with  $\beta 1$  antibody (JB1A). The immunocomplexes were suspended in  $24 \mu\text{l}$  of reaction solution containing 50 mM Pipes, pH 7.0, 10 mM MnCl<sub>2</sub>, 5  $\mu\text{g}$  of acid-denatured enolase, 10  $\mu\text{M}$  ATP, 10  $\mu\text{Ci}$  of [ $\gamma$ -<sup>32</sup>P]ATP, and reacted at room temperature for 10 min. The kinase reaction was stopped by sample buffer, and samples were resolved on 10% SDS-PAGE and visualized by autoradiography.

For determination of basal activity of Src, induced cells were lysed in RIPA buffer and ~500  $\mu\text{g}$  of lysate immunoprecipitated with Src mAb (Calbiochem) and procedures described above followed.

### Phospho-MAPK Assay

SMC were plated onto fibronectin- ( $5 \mu\text{g}/\text{ml}$ ) or polylysine-coated ( $50 \mu\text{g}/\text{ml}$ ) dishes at  $37^\circ\text{C}$  for 20 min in serum-free DMEM/BSA with or without peptide 25 or 36 ( $75 \mu\text{M}$ ). Cells were lysed in RIPA buffer and immuno-

blotted with phospho-specific MAPK antibody which detects p42 and p44 MAPK catalytically activated by phosphorylation at Tyr204/Thr202 (New England Biolabs). The membrane was stripped and re probed for total MAPK protein as control.

### Migration Assay

Passage 1–3 SMC ( $10^5$ ) were seeded into Transwell (Costar; Becton Dickinson) inserts containing 8- $\mu$ m polycarbonate filters precoated on the bottom with fibronectin, and then cultured overnight in serum-free DMEM containing BSA (5 mg/ml) with or without peptide 25 or 36 (50–100  $\mu$ M). Cells on both sides of the filter were detached by trypsin-EDTA and counted. All assays were performed in triplicate and the data expressed as percent inhibition by the peptides.

## Results

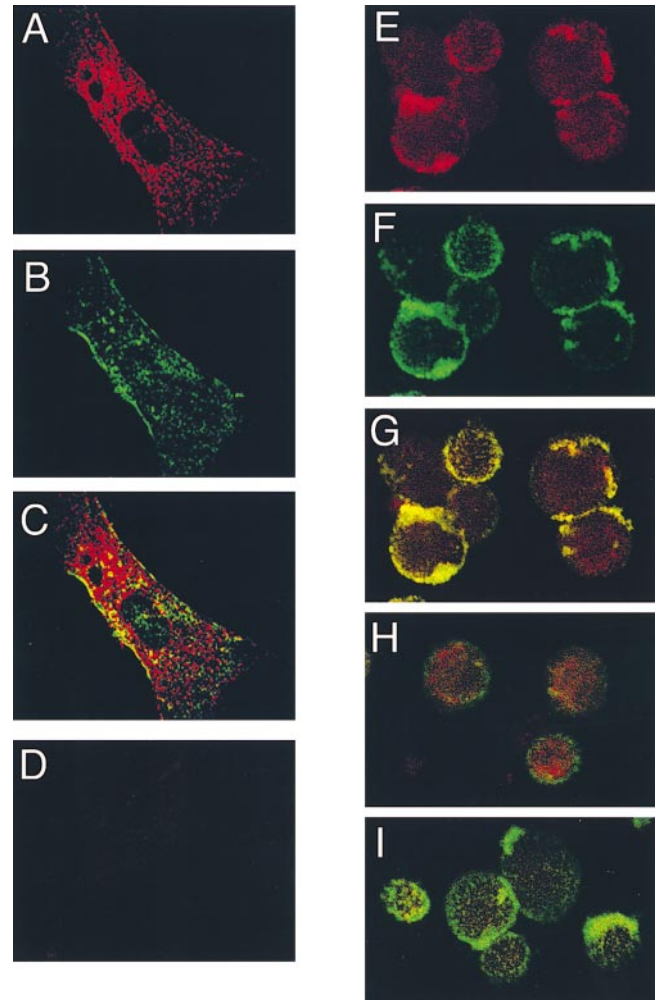
### $\beta$ 1 Integrins and Caveolin Partially Colocalize

Because caveolin oligomers are relatively insoluble in non-ionic detergents, the reported association between  $\beta$ 1 integrins and caveolin could possibly be an artifact of cellular disruption by detergents. Therefore, the distribution of  $\beta$ 1 integrins and caveolin in cells in situ was assessed by immunostaining. Primary cultures of human vascular SMC (Fig. 1, A and B) were examined because these cells express relatively high amounts of  $\beta$ 1 integrins (red) and caveolin-1 (green). Confocal microscopic analysis indicated these two proteins had marked spotty or array-like colocalizations over the cell body of SMC grown on glass coverslips (Fig. 1 C). Substitution of isotype Ig controls for primary  $\beta$ 1 and caveolin antibodies resulted in much weaker or no staining (Fig. 1 D) verifying the specificity of the immunostaining. Because  $\beta$ 1 integrins and caveolin-1 only partially colocalize, we determined if  $\beta$ 1 integrins and caveolin would cocluster. Clustering of SMC  $\beta$ 1 integrins in suspension at 37°C resulted in the emergence of bright fluorescent signals of integrins (green) (Fig. 1 F) and caveolin (red) (Fig. 1 E) indicating clusters. Superimposition of the signals (Fig. 1 G) confirmed coclustering. No caveolin clustering was observed when the integrins were nonclustered (Fig. 1 H) or nonimmune Ig was substituted for the primary caveolin antibodies (Fig. 1 I). These images indicate that fractions of  $\beta$ 1 integrins and caveolin colocalize and are physically linked in cells before cellular disruption with detergents and are consistent with the hypothesis that caveolin has a role in integrin function.

### Caveolin Depletion in 293 Cells Blocks $\beta$ 1 Integrin Function

To explore more directly the importance of caveolin to  $\beta$ 1 integrin function, parental 293 cells were transfected with antisense full-length mRNA for caveolin-1 under the influence of an inducible murine metallothionein promoter. Several clones with inducible suppression of caveolin protein expression were identified and examined. After 12–18 h of induction in the presence of 3  $\mu$ M cadmium, two clones with normal caveolin levels, 6 and 9 (Fig. 2 A), displayed strong adhesion to fibronectin (Fig. 2 B). By contrast, two representative antisense clones, 31 and 32, which had 15–50% the level of caveolin in parental 293 cells (Fig. 2 A), displayed markedly impaired adhesion to fibronectin (Fig. 2 B). Although 293 cells have only weak vitronectin adhesion, mediated by  $\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 1, this adhesion was also

abrogated in caveolin-deficient cells (not shown). As is also indicated in Fig. 2 A, there was substantially less caveolin in these clones even without induction, indicating leakage of antisense expression, which has been reported for this expression vector in previous studies (Lukashev et al., 1994). Adhesion assays mirrored the extent of caveolin depletion: although they were capable of spreading on fibronectin, clones 31 and 32 were less adhesive even in the absence of induction (Fig. 2 B). Nonetheless, clones 31 and 32 grew nearly as well as clones 6 and 9 over many months



**Figure 1.** Caveolin and  $\beta$ 1 integrins colocalize and cocluster. Early passage human SMC (A–C) were cultured in 10% FBS, stained with JB1A/K20 mAb to localize  $\beta$ 1 integrins (A–C), and then fixed/permeabilized and stained with polyclonal caveolin antibodies. Secondary antibodies were then used to visualize optical planes of the cells by confocal microscopy: integrins (red), caveolin (green), and sites of colocalization (yellow). Substitution of isotype mouse Ig and rabbit IgG for primary antibodies resulted in much less or no immunostaining of SMC (D). SMC were also examined for integrin-induced coclustering with caveolin (E–G). SMC were stained in suspension with FITC-K20 antibodies to  $\beta$ 1 integrins followed by clustering with secondary antibodies and then stained for caveolin. In this case integrin (green) and caveolin (red) fluorescent signals emerge and colocalize (E–G) with clustering in comparison with that of nonclustered cells (H) or clustered cells stained without primary caveolin antibodies (I).

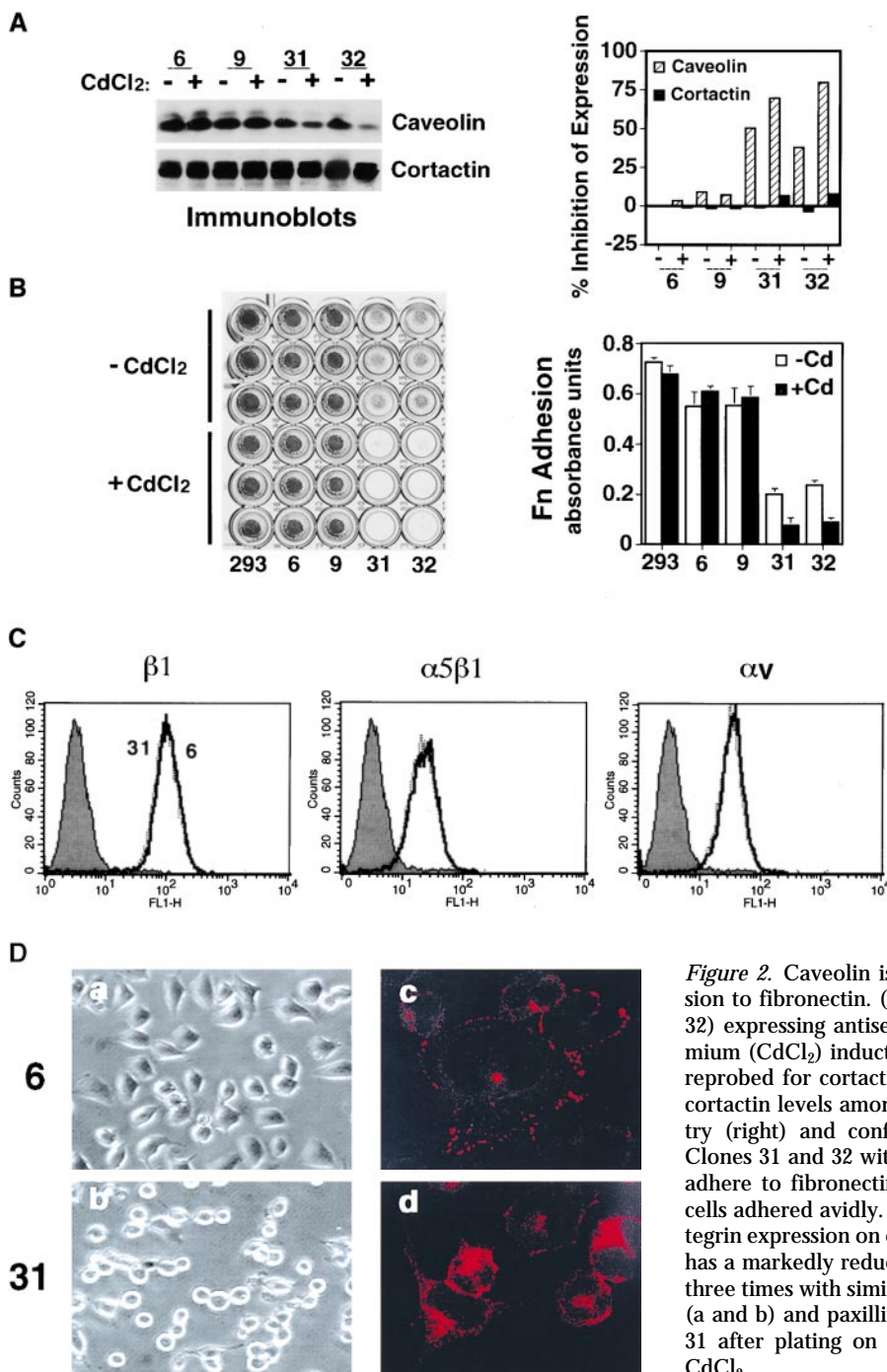
of passage. Flow cytometric analyses revealed no differences in surface level of  $\beta 1$  or  $\alpha v$  or  $\alpha 5\beta 1$  heterodimers among these clones (Fig. 2 C), indicating that depletion of caveolin expression affected the function rather than expression of  $\beta 1$  or its dimerization with  $\alpha 5$  or other integrins.

Caveolin antisense clones also exhibited time-dependent changes in cell shape with decreasing caveolin expression. Clones 6 and 31 were allowed to spread onto fibronectin for 1 h at 37°C, and then antisense caveolin mRNA induced. Within 8 h, clone 31 cells, but not clone 6 cells, became less spread. Within 18 h, clone 31 cells became round (Fig. 2 D) and numerous cells actually detached from the matrix. The shape changes paralleled loss

of focal adhesion sites as indicated by loss of focal paxillin accumulation on ventral surfaces (Fig. 2 D). In spite of the marked changes in adhesiveness and shape, most cells were found to respread and adhere better within hours of removing cadmium.

### Caveolin Depletion in 293 Cells Does Not Impair Integrin Ligand Binding or Lateral Movement but Blocks Signaling through $\beta 1$ Integrins

We next considered mechanisms by which caveolin could influence integrin function. Because integrin expression per se was not changed in caveolin-depleted cells (Fig. 2



**Figure 2.** Caveolin is required for  $\beta 1$  integrin-dependent adhesion to fibronectin. (A) Lysates of 293 cells (clones 6, 9, 31, and 32) expressing antisense caveolin before (-) and after (+) cadmium (CdCl<sub>2</sub>) induction were immunoblotted for caveolin-1 and reprobed for cortactin as a control (left). Relative caveolin and cortactin levels among the clones were quantified by densitometry (right) and confirmed in three separate experiments. (B) Clones 31 and 32 with marked depletion of caveolin (A) did not adhere to fibronectin, whereas clones 6 and 9 and control 293 cells adhered avidly. (C) FACS<sup>®</sup> analysis of  $\beta 1$ ,  $\alpha v$ , and  $\alpha 5\beta 1$  integrin expression on control clone 6, and antisense clone 31 which has a markedly reduced caveolin level. B and C were performed three times with similar results. (D) Photographs of phase images (a and b) and paxillin immunostaining (c and d) of clones 6 and 31 after plating on fibronectin and subsequent induction with CdCl<sub>2</sub>.

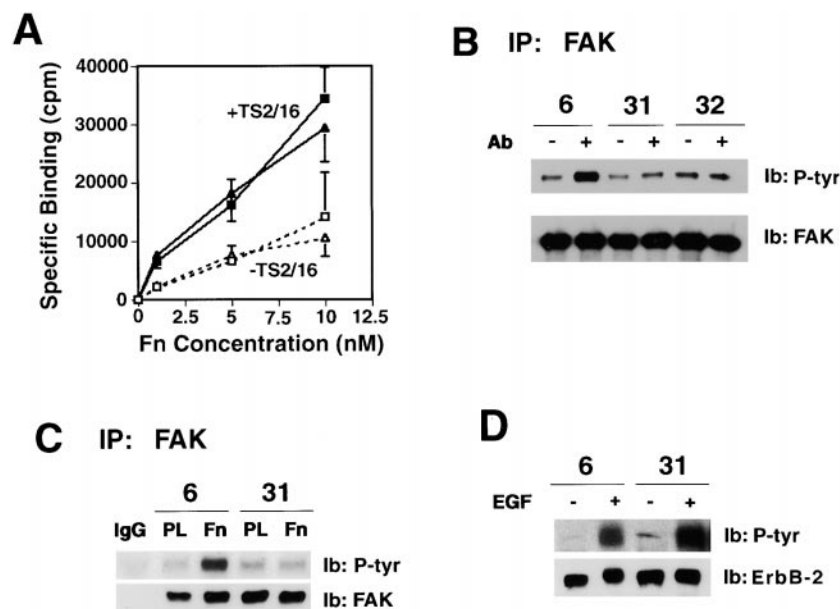
C), the capacity of the various clones to bind  $^{125}\text{I}$ -fibronectin in the absence and presence of a stimulating antibody (TS2/16) known to promote fibronectin binding by the major fibronectin receptor,  $\alpha 5\beta 1$ , was determined (Masumoto and Hemler, 1993). After induction, clones 6 and 31 were found to bind fibronectin equally at  $4^\circ\text{C}$  and exposure of the cells to TS2/16 enhanced fibronectin binding to a similar degree (Fig. 3 A), indicating that the affinity states of  $\alpha 5\beta 1$  in control and caveolin-depleted clones are similar. Further, when surface  $\beta 1$  integrins were cross-linked by antibodies, the  $\beta 1$  integrins of normal (clone 6) and caveolin-depleted cells (clones 31 and 32) clustered equally well as judged by confocal microscopic analysis (not shown). In spite of the capacity of  $\beta 1$  integrins on caveolin-depleted clones to engage ligand, the changes in cell shape and adhesiveness provoked by caveolin depletion (Fig. 2) could not be reversed by the stimulatory  $\beta 1$  integrin antibody (TS2/16). These data imply that a postreceptor binding event(s) important to adhesion, rather than binding per se, is most likely impaired in caveolin-depleted cells.

Src family tyrosine kinases are required for fibronectin receptor  $\alpha 5\beta 1$  and vitronectin receptor  $\alpha v\beta 5$ -mediated cell adhesion (Bergman et al., 1995; Kaplan et al., 1995). Moreover, tyrosine phosphorylation of FAK and other signaling molecules important to cytoskeletal reorganization and cell spreading after ligand engagement of  $\beta 1$  integrins may depend on interactions between FAK and Src family kinases (Parsons, 1996; Schlaepfer and Hunter, 1997). Because caveolin has been shown previously to interact with c-Src (Li et al., 1996 a,b), it is possible that integrin-mediated tyrosine phosphorylation depends on caveolin. To test this hypothesis, we initially treated 293 cells with the tyrosine kinase inhibitor genestein ( $100\ \mu\text{M}$ ) and noted that 293 cells failed to attach and spread on fibronectin (data not shown). When  $\beta 1$  integrins were clus-

tered with antibodies or 293 cells were allowed to adhere to fibronectin, increased FAK phosphorylation was apparent (Fig. 3, B and C). In contrast, 293 cells with suppressed caveolin expression failed to exhibit FAK phosphorylation whether in response to antibody-induced clustering or ligand binding. This defect was not a general defect in tyrosine phosphorylation as autophosphorylation of ErbB-2, an EGF receptor-like tyrosine kinase, after binding of EGF, was not different between control and caveolin-deficient clones (Fig. 3 D).

The  $\beta 1$  integrins were next examined for interaction with nonreceptor tyrosine kinases by coprecipitation. Caveolin depletion of 293 cells (clones 31 and 32) resulted in loss of caveolin and Src family kinases in the  $\beta 1$  immunoprecipitates (Fig. 4 A). The antibody used in this experiment was a pan-Src antibody recognizing multiple members of the *src* gene family. In additional experiments using specific antibodies we found c-Src, Fyn, and Yes in the  $\beta 1$  immunoprecipitates, all of which were almost completely lost in the  $\beta 1$  immunoprecipitates of caveolin-depleted cells (not shown). A major substrate for Src kinase, cortactin, implicated in adhesion and migration (Vuori and Ruoslahti, 1995), was also markedly depleted in these precipitates. In situ kinase assays of  $\beta 1$  immunoprecipitates confirmed markedly reduced activity in cells (clones 31 and 32) depleted of caveolin (Fig. 4 B).

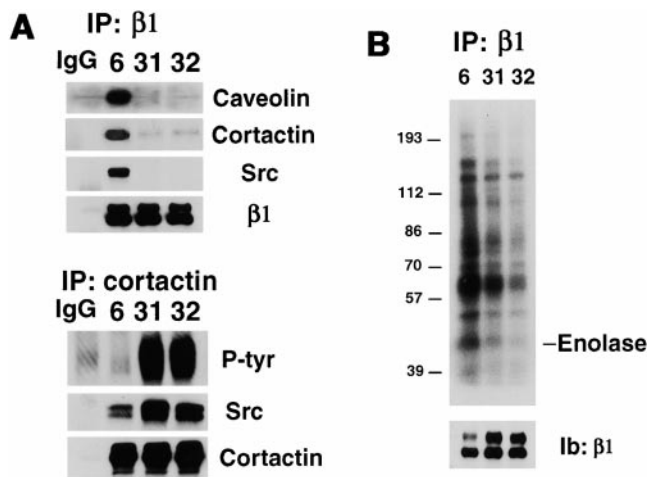
Although little or no Src family kinase or cortactin associates with  $\beta 1$  integrins in caveolin-depleted cells (Fig. 4 A), this was not because tyrosine kinases could not be activated in these cells. Rather, kinase assays of immunoprecipitated c-Src confirmed a two- to fourfold increase in total c-Src activity in the caveolin-deficient clones (not shown). Previously, caveolin levels have been linked inversely to cellular Src kinase activity (Li et al., 1996a). Enhanced total kinase activity correlated with a striking hyperphosphorylation of cortactin in the caveolin-depleted



**Figure 3.** Caveolin depletion does not block integrin clustering but abrogates integrin signaling through FAK. (A) Fibronectin binding to clones 6 ( $\Delta$ ,  $\blacktriangle$ ) and 31 ( $\square$ ,  $\blacksquare$ ) in the absence and presence of the stimulatory TS2/16 antibody. Data are shown as specific binding (mean  $\pm$  SD of triplicate determinations) as a function of  $^{125}\text{I}$ -fibronectin concentration. Similar results were seen in three separate experiments. (B) Clones 6 and 31 after induction and clustering were immunoprecipitated with FAK antibody. The immune complexes were analyzed for tyrosine phosphorylation by antiphosphotyrosine antibody (4G10) (top). The same membrane was stripped and blotted for FAK (bottom). (C) Clones 6 and 31 were induced and allowed to adhere on either polylysine (PL) or fibronectin (Fn) for 40 min before being analyzed for FAK phosphorylation. (D) Clones 6 and 31 were induced and allowed to adhere on fibronectin. Cells were stimulated with EGF for 5 min and lysed and immunoblotted for tyrosine-phosphorylated ErbB-2 (top). The membrane was

then blotted for total ErbB-2. EGF binding to ErbB-2/ErbB-3 heterodimers in epithelial cells was reported recently (Pinkas-Kramarski et al., 1998). All of the experiments involving cell lysates (Figs. 3–6) were performed three to four times with similar results.



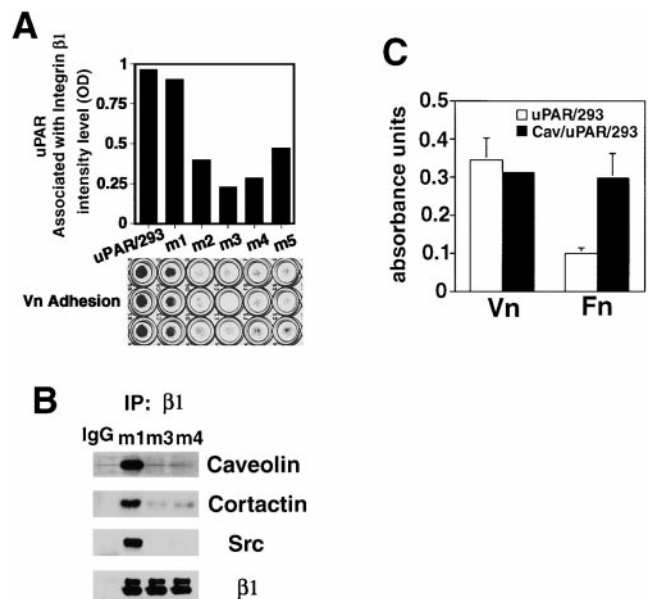


**Figure 4.** Caveolin depletion leads to loss of integrin-associated Src family kinases. (A) The top panel shows immunoprecipitates of  $\beta 1$  integrins of antisense caveolin 293 cells immunoblotted sequentially for caveolin, cortactin, Src family kinases, and  $\beta 1$  integrin. The bottom panel shows immunoprecipitates of cortactin in Cd-induced 293 cells sequentially immunoblotted for phosphotyrosine, Src family kinases (pan-Src Ab), and cortactin. (B)  $\beta 1$  integrin immunoprecipitates were assayed in situ for total Src family tyrosine kinase activity (top). The bottom panel shows the relative amounts of integrin precipitated in each sample.

clones (31 and 32) compared with the control clone 6 (Fig. 4 A). Immunoblots of the cortactin immunoprecipitates revealed increased Src family kinases associated with tyrosine-phosphorylated cortactin, possibly through Src SH2 domain (Thomas and Brugge, 1997). These data indicate that caveolin-deficient 293 cells have dysregulated Src family kinase activity and little or no tyrosine kinase activity associated with their  $\beta 1$  integrins.

#### **Caveolin Depletion Disrupts uPAR/Integrin Complexes and Abrogates uPAR-mediated Adhesion to Vitronectin**

uPAR forms complexes with  $\beta 1$  and  $\beta 2$  integrins and such complexes are important to uPAR-dependent adhesion to matrix vitronectin via a vitronectin binding site on uPAR (Wei et al., 1996). Caveolin is also found in uPAR/ $\beta 1$  integrin complexes. To explore the functional importance of caveolin to these complexes, we created stable cotransfectants of 293 cells expressing both uPAR and the antisense full-length mRNA for caveolin-1. Several clones with inducible suppression of caveolin expression were again identified. Control clones expressing uPAR but without reduction in caveolin levels adhered avidly to vitronectin (Fig. 5 A). Clones with <50% the normal level of caveolin protein (clones m2–m5) adhered weakly or not at all to vitronectin (Fig. 5 A) even though uPAR and  $\beta 1$  integrin surface expression was not changed as judged by FACS<sup>®</sup> analysis (data not shown). Immunoprecipitation of  $\beta 1$  integrins coprecipitated uPAR in proportion to caveolin levels and the ability of the cells to adhere to vitronectin (Fig. 5 A), indicating that caveolin is required for stable uPAR/integrin complexes and vitronectin adhesion. As with nontransfected 293 cells (Fig. 4), depletion of caveolin in



**Figure 5.** Caveolin is required for uPAR/ $\beta 1$  integrin complexes and uPAR-dependent adhesion to vitronectin. (A) The bottom panel shows uPAR/293 cells expressing varying levels of antisense caveolin (clones m1–m5) tested for adhesion to vitronectin as described in Materials and Methods. The corresponding decrease in caveolin levels for each of the clones, as judged by densitometry of immunoblots, was as follows: m1, 0% decrease; m2, 83%; m3, 89%; m4, 96%; and m5, 73%. The top panel shows  $\beta 1$  integrins of uPAR/293 cells cotransfected with antisense caveolin (clones m1–m5) immunoprecipitated and immunoblotted for relative amounts of uPAR as quantified by densitometry. (B) Immunoprecipitates of  $\beta 1$  integrins sequentially immunoblotted for caveolin, Src, and cortactin as in Fig. 4. (C) uPAR-expressing or uPAR and caveolin-coexpressing 293 cells were detached and allowed to adhere on vitronectin (Vn) fibronectin (Fn)-coated 96-well plates. After washing, the percentage of adherent cells was quantitated by spectrophotometric absorbance of stained cells. Adhesion assays were performed three times with identical results.

uPAR/293 cells resulted in almost complete loss of Src family kinases from the  $\beta 1$  integrin immunoprecipitates (Fig. 5 B). As expected, the caveolin-depleted uPAR/293 cells also failed to adhere to fibronectin (not shown).

In prior work, we observed that incorporation of  $\alpha 5/\beta 1$  integrins into complexes with uPAR, while stabilizing caveolin/integrin interactions, inhibited the ability of these integrins to bind fibronectin. Indeed, uPAR-transfected 293 cells had impaired fibronectin adhesion (Wei et al., 1996). In light of the critical role for caveolin in 293 cell adhesion to fibronectin (Fig. 2) and the fact that 293 cells have relatively low levels of caveolin, we considered the possibility that uPAR expression also inhibited  $\beta 1$  integrin function indirectly by sequestering caveolin into uPAR/integrin complexes. Therefore, we transfected uPAR/293 cells with wild-type caveolin-1 and assessed the capacity of these transfectants to adhere to vitronectin and fibronectin. Concurrent caveolin overexpression in uPAR/293 cells had no effect on uPAR-dependent adhesion to vitronectin but completely restored the capacity of these cells to adhere to fibronectin (Fig. 5 C). These data suggest competi-

tion for limited caveolin between unbound  $\beta 1$  integrin and uPAR/ $\beta 1$  integrin complexes may largely explain the impaired fibronectin adhesion of uPAR-transfected 293 cells.

### Disruption of $\beta 1$ Integrin/Caveolin Complexes with Caveolin and uPAR-Binding Peptides

To explore further the functional connection between caveolin and uPAR in assembly of integrin-associated signaling molecules, lysates of uPAR/293 cells were immunoprecipitated for  $\beta 1$  integrins in the presence of either a peptide (peptide 25) (Wei et al., 1996) which disrupts uPAR/integrin associations or a peptide derived from the "scaffolding domain" of caveolin-1 (cav-1). In experiments using purified proteins, cav-1 disrupts self-oligomerization of caveolin and blocks direct interactions between caveolin and other proteins, including c-Src (Li et al., 1996a; Michel et al., 1997). Both peptide 25 and cav-1 blocked coprecipitation of caveolin with  $\beta 1$  integrins in lysates prepared from either uPAR/293 cells or primary human vascular SMC (Fig. 6 A). Both peptides, but not inactive control peptides, also blocked coprecipitation of Src family kinases and cortactin with  $\beta 1$  integrins (Fig. 6 A), verifying that the stable association of these cytoplasmic proteins with integrins is dependent upon the membrane

proteins caveolin and uPAR in these cells. Neither control peptide (peptide 36 and cav-x) had any effect on caveolin, cortactin, or Src coprecipitation with  $\beta 1$  integrins when compared with a no peptide control (Fig. 6 A). Further, peptide 25 had no effect on coprecipitation of caveolin (or Src kinases) with  $\beta 1$  integrins in nontransfected 293 cells (not shown), confirming the specificity of this peptide for uPAR.

Vascular SMC are known to express uPAR and  $\beta 1$  integrins (Clyman et al., 1992; Okada et al., 1995) and we confirmed uPAR expression by immunostaining. Clustering of  $\beta 1$  integrins resulted in uPAR coclustering, though no uPAR was detectable in these  $\beta 1$  immunoprecipitates by immunoblotting (data not shown). Although complex formation of  $\beta 1$  integrins with uPAR could be expected to inhibit fibronectin adhesion (Wei et al., 1996), SMC attach and spread well on fibronectin, mostly via  $\alpha 5\beta 1$ . As discussed above, this may be explained by the relatively high levels of caveolin in SMC as compared with 293 cells. Nonetheless, peptide 25 but not a control peptide, was found to disrupt caveolin and Src kinase associations with  $\beta 1$  integrins almost completely in lysates of SMC (Fig. 6 A). Therefore, we used this peptide to explore the role of caveolin in the integrin function of intact SMC. Incubation of SMC adherent to fibronectin with peptide 25 before ly-

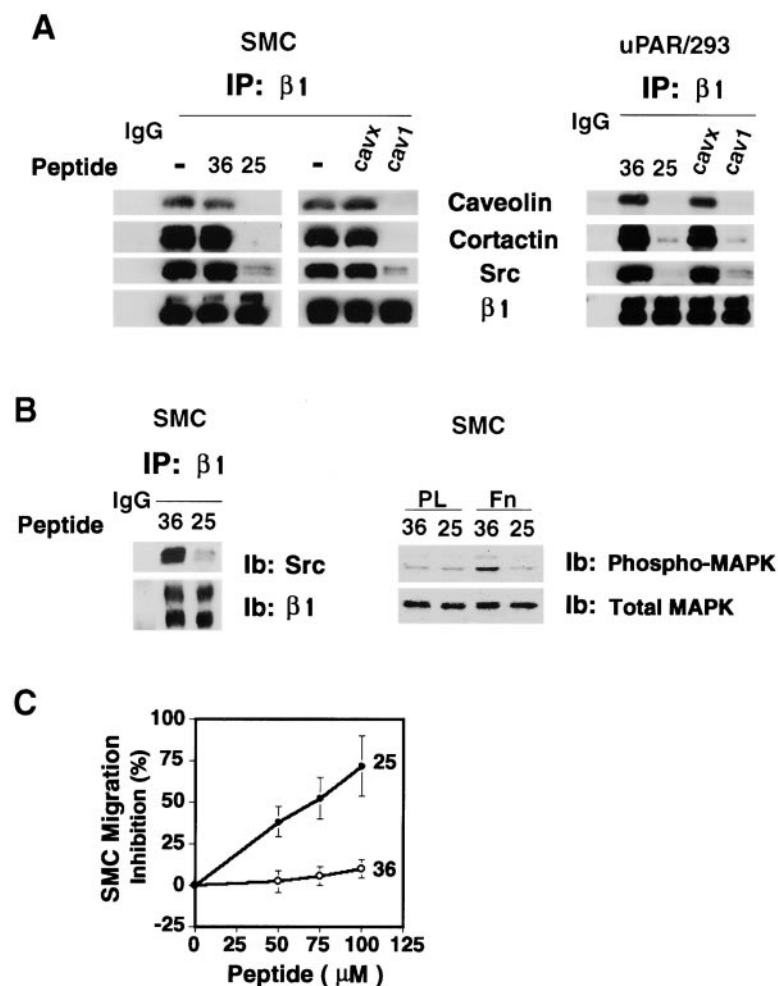


Figure 6. Disruption of  $\beta 1$  integrin/uPAR/caveolin complexes in SMC leads to loss of integrin-associated Src kinases. (A)  $\beta 1$  integrins of uPAR/293 cells and SMC were immunoprecipitated in the presence of 100  $\mu M$  of either peptide 25 or 36, 10  $\mu M$  cav-1, or the control cav-x peptide, then sequentially immunoblotted for caveolin, cortactin, Src family kinases, and  $\beta 1$  integrins. (B) Intact SMC were incubated with 75  $\mu M$  peptide 25 or control 36 and then immunoprecipitated with  $\beta 1$  integrin antibodies and the precipitates immunoblotted sequentially for Src kinases and  $\beta 1$  integrins. SMC were also pretreated with peptides as above and plated onto polylysine (PL) or fibronectin (Fn) for 30 min at 37°C. Lysates were immunoblotted for total MAPK and phosphorylated MAPK as an indicator of the active kinase. (C) SMC were placed in the upper chambers of Transwell inserts undercoated with fibronectin in the presence of peptide 25 or 36 at concentrations indicated in the figure. SMC on the filter bottom were counted after 24 h. The assay was done three times and the data are expressed as mean ( $\pm$  SD) percent inhibition by either peptide.



sis resulted in loss of Src kinases (as well as caveolin) in the  $\beta 1$  immunoprecipitates (Fig. 6 B). Consistent with the loss of signaling molecules, integrin-dependent MAPK activation was also blocked by peptide 25 (Fig. 6 B). It should be noted that the pathway of integrin and uPAR-dependent MAPK activation in these cells is not yet defined. Although MAPK is reproducibly phosphorylated within 30 min of fibronectin attachment, we can detect no Shc or FAK phosphorylation (not shown). The inhibitory effects of the peptide on association of Src family kinases with integrins was correlated with loss of integrin function. Although neither peptide blocked adhesion, peptide 25 delayed SMC spreading on fibronectin and blocked migration of human SMC across microbore filters to fibronectin-coated surfaces (Fig. 6 C), consistent with its effect on integrin signaling.

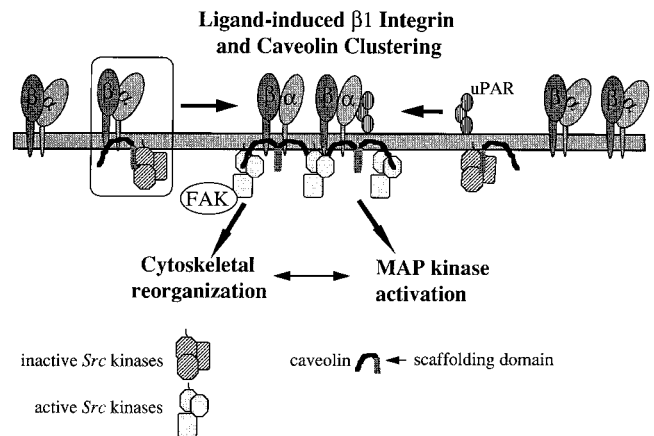
## Discussion

Expression of the capacity of integrins to mediate cellular adhesion has several distinct facets: (a) the conformational state of integrins, affecting the affinity of ligand binding; (b) ligand-induced integrin clustering, enhancing the strength of attachment and promoting interactions between kinases and their substrates important to signal transduction; and (c) signaling itself, activating a cascade of events leading to organization of the cytoskeleton and cell spreading (Schwartz et al., 1995; Burridge and Chrzanowska-Wodnicka, 1996; Parsons, 1996). Numerous *in vitro* studies and recent evaluations of mice with targeted deletions in non-receptor tyrosine kinases implicate FAK and Src family kinases as key mediators of integrin signaling (Furata et al., 1995; Ilic et al., 1997; Meng and Lowell, 1998). Thus, the molecular events leading to assembly and activation of these kinases surrounding integrins are important determinants of integrin function. This conclusion has focused much attention on nonintegrin membrane proteins which might regulate this process and our data identify one such protein as caveolin. While caveolin does not affect integrin expression or the intrinsic capacity of  $\beta 1$  integrins to bind fibronectin (Figs. 2 and 3), caveolin is required for the normal assembly of adhesion plaques which develop in response to ligand engagement and integrin clustering. In the absence of sufficient  $\beta 1$  integrin-associated caveolin, there is loss of integrin-associated Src kinase activity, little or no FAK activation after ligand binding, and impaired association of integrins with structural proteins such as tyrosine-phosphorylated cortactin important to  $\beta 1$  integrin-dependent adhesion (Fig. 4). As a result, the characteristic accumulation of enzymes and structural proteins which comprise integrin-dependent adhesion sites fails to develop (Fig. 2).

Our results confirm and extend the recent findings of Wary and colleagues (1998) that caveolin expression is required for the association of Fyn kinase with  $\beta 1$  integrins and ligand-dependent Shc phosphorylation. These investigators began with cells (Fisher rat thyroid cells) expressing little or no caveolin or  $\alpha 5$  integrin and transfected both proteins. They found that physical association of the Src family kinase Fyn with  $\alpha 5/\beta 1$  required concurrent coexpression of caveolin-1. Fyn but not Src was required for  $\alpha 5/\beta 1$ -dependent Shc phosphorylation. Beginning with cells

having functional  $\beta 1$  integrins and expressing caveolin-1, we suppressed caveolin expression and found  $\beta 1$  integrin association with several Src family kinases to be completely dependent on caveolin. In both cases, deficient  $\beta 1$  integrin-associated Src family kinase activity led to correspondingly marked changes in integrin function. Together, these findings indicate caveolin is a general regulator of  $\beta 1$  integrin function.

How does caveolin promote integrin signaling? Although our understanding is incomplete, we favor a model (Fig. 7) in which caveolin functions primarily to sequester Src family kinases in an inactive configuration at sites proximate to integrins, promoting their presentation to integrins and activation during ligand-induced integrin clustering. Caveolin has been shown previously to interact predominantly with the inactive form of Src and to reduce Src activity when overexpressed in 293 cells, perhaps by sequestering Src kinases from activating phosphatases and substrates (Li et al., 1996b; Rodgers and Rose, 1996). Consistent with this model, caveolin-deficient 293 cells were found to have two- to fourfold higher levels of basal c-Src kinase activity (not shown) and to exhibit striking hyperphosphorylation of a major Src substrate, cortactin (Fig. 4). If one function of caveolin is to suppress basal Src ki-



**Figure 7.** Model for regulation of  $\beta 1$  integrin signaling by caveolin. The model indicates that caveolin binds and maintains Src family kinases in an inactive configuration (dark shade) proximate to a fraction of  $\beta 1$  integrins (complex is encircled). Whether these complexes exist in caveolae as signaling units or separately from caveolae is currently unknown (see text). Ligand engagement of integrins and clustering of caveolin along with integrins results in homo-oligomerization of caveolin and release of Src kinases from caveolin. This in turn allows Src kinases to activate (light shade) through a phosphatase and/or through direct binding to substrates at the developing focal contact site. Available evidence suggests that Fyn kinase may primarily function in MAPK activation whereas Src/Yes may primarily function to activate FAK and phosphorylate other cytoskeletal proteins leading to the organization and development of an adhesion plaque. The model also proposes that as integrins activate in response to ligand-induced clustering, uPAR moves into the complexes enriching them with caveolin and its associated signaling molecules. The importance of uPAR, when present, to the complexes is illustrated by the ability of uPAR-binding peptides to disrupt signaling through  $\beta 1$  integrins in SMC (Fig. 6).

nase activity, the model in Fig. 7 also offers a possible molecular explanation of how caveolin contributes to Src family kinase activation as a consequence of integrin clustering. Caveolin interactions with Src family kinases are thought to occur through the same membrane proximal sites on caveolin as those involved in formation of caveolin homo-oligomers (Okamoto et al., 1998). Because coclustering of caveolin with integrins (Fig. 1) could be expected to favor caveolin homo-oligomer formation, integrin clustering is likely to modify the physical interaction of caveolin with Src kinases. This may lead to outright release of these kinases from caveolin (as shown in Fig. 7) and/or an altered, more accessible position of these kinases in the cluster favoring their activation through contact with substrate or through a phosphatase. In either case, these studies directly implicate caveolin function in the membrane proximate events of ligand-induced,  $\beta 1$  integrin-dependent tyrosine kinase activation. As caveolin levels are reportedly low in a number of transformed cells (Koleske et al., 1995; Okamoto et al., 1998), our data raise the possibility that low caveolin levels contribute to the defective fibronectin adhesion and matrix assembly seen in many cancer cells and thought to be important to their metastatic potential (Hynes, 1992; Schwartz, 1997).

A further implication of the studies reported here and the recent studies of Wary and colleagues (1998) is that Fyn and Src may have overlapping but distinct roles in  $\beta 1$  integrin signaling: Fyn being primarily required for Shc phosphorylation and activation of an MAPK-dependent growth pathway and Src being primarily required for FAK and cytoskeletal phosphorylation and assembly of focal adhesion sites. This concept is supported by results of prior studies examining mice deficient in the negative regulator of Src family kinase activity, Csk (Thomas et al., 1995). Fibroblasts from Csk<sup>-</sup> embryos exhibit hyperphosphorylation of adhesion sites and striking hyperphosphorylation of cortactin, reminiscent of that seen in caveolin-deficient cells (Fig. 4). The hyperphosphorylation of adhesion sites and cortactin was largely corrected by crossing the Csk<sup>-</sup> mice with Src<sup>-</sup> mice but not by crossing with Fyn<sup>-</sup> mice, again implicating Src as primarily being involved in regulation of integrin-dependent adhesion. Clearly this is an oversimplification since we also observe Yes as well as Fyn and Src in  $\beta 1$  integrin/caveolin complexes and even kinase-inactive Src can promote focal contact assembly in Src<sup>-</sup> cells (Kaplan et al., 1995). Still, these recent studies support the possibility that different members of the *src* gene family have favored roles in integrin signaling even in cells expressing multiple family members.

The structural basis for the association between caveolin and  $\beta 1$  integrins is uncertain. First, most if not all cellular caveolin is reportedly found in purified caveolae (Lisanti et al., 1994; Schnitzer et al., 1995). Although integrins have not been observed in purified caveolae, when we subject 293 cells to mechanical lysis, sonicate, and centrifuge the postnuclear supernatant through a sucrose gradient, caveolin in 293 cells appears at the 5/35% sucrose interface, typical for that reported for caveolae preparations in 293 and other cells (Song et al., 1996; Ikezu et al., 1998). Almost all of the  $\beta 1$  integrins appear in the fractions containing caveolin (Yang, X., unpublished observation). These findings are consistent with the scenario that  $\beta 1$  integrin/

caveolin/Src family kinase complexes (encircled in Fig. 7) exist as signaling units in caveolae and separate from caveolae in response to ligand-induced clustering and cytoskeletal reorganization. However, the exact composition of caveolae remains controversial and appears strongly dependent on the method of purification (Lisanti et al., 1994; Schnitzer et al., 1995; Mineo et al., 1996; Stan et al., 1998). An alternative possibility is that some fraction of caveolin traffics outside caveolae, as has been argued by Wary and colleagues (1998). This will be an important issue for future experiments. In either case, the fraction of  $\beta 1$  integrins which colocalize and cocluster with caveolin (Fig. 1) appears to be critical to  $\beta 1$  integrin signaling.

Whether  $\beta 1$  integrins directly bind caveolin is also uncertain. Wary and colleagues (1998) reported that the  $\alpha 5$  transmembrane domain was required for coprecipitation of  $\alpha 5/\beta 1$  and caveolin. However, no direct binding has been demonstrated. Most if not all other proteins that are reported to bind caveolin bind sequences of caveolin that are membrane proximal but in the cytoplasmic compartment (Okamoto et al., 1998). Indeed, in SMC we observed a peptide comprised of a membrane proximal region of caveolin (amino acids 82–101) blocking coprecipitation of caveolin with  $\beta 1$  integrins (Fig. 5). Thus, it is uncertain whether  $\beta 1$  integrins and caveolin directly bind or associate indirectly through a common affinity for certain membrane lipids or a third protein such as uPAR which may directly interact both with integrins and cholesterol-rich lipid domains enriched in caveolin. The physical basis for the association of  $\beta 1$  integrins and caveolin also requires further investigation.

The marked inhibition of  $\beta 1$  integrin function by uPAR-binding peptides in human SMC (Fig. 6) is remarkable. Although we could detect uPAR in SMC by immunostaining and could cocluster uPAR along with  $\beta 1$  integrins, no uPAR was detected in the  $\beta 1$  immunoprecipitations of SMC. This suggests the association of uPAR with  $\beta 1$  integrins and caveolin in these cells is relatively weak. There also appears to be a stoichiometric excess of  $\beta 1$  integrins compared with uPAR in SMC. Thus, it is surprising that the coprecipitation of most caveolin- and integrin-associated Src kinases with  $\beta 1$  integrins would be blocked by peptide 25, raising the possibility of an *in vitro* artifact. This is an unlikely explanation for the findings because the uPAR-binding peptide (peptide 25), but not two control peptides (peptide 36 and a scrambled version of peptide 25), also had clear biochemical and functional effects on intact SMC. Prior treatment of SMC with the active peptide blocked Src kinase association with  $\beta 1$  integrins and inhibited both signaling and migration of the cells on fibronectin (Fig. 6). These results indicate that, although not intrinsically required for the function of integrin/caveolin (Fig. 2), the presence of uPAR organizes caveolin and its associated signaling molecules in an interdependent manner such that integrins, caveolin, and uPAR form a unit promoting integrin function. Perhaps this is possible because only a fraction of  $\beta 1$  integrins associates with caveolin (Fig. 1) and uPAR associates mainly with activated integrins (Wei et al., 1996). As indicated in Fig. 7, integrin activation initiated by ligand-induced integrin clustering may promote formation of these signaling complexes. Similar mechanisms may account for observations that uPAR

is required for normal function of the  $\beta 2$  integrins, CD11/CD18 (Mac-1; Simon et al., 1996; Sitrin et al., 1996; May et al., 1998), and for migration on vitronectin mediated by  $\alpha v\beta 5$  (Yebara et al., 1996), suggesting a promoting effect of uPAR on integrin function may be a widespread phenomenon.

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