Focal Adhesions in (Myo)fibroblasts Scaffold Adenylyl Cyclase with Phosphorylated Caveolin*

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Fibroblast-myofibroblast transformation, a critical event for enhanced extracellular matrix deposition, involves formation of an actin stress fiber contractile apparatus that radiates from focal adhesions (FA) in the plasma membrane. Activation of adenylyl cyclase (AC, i.e. increases in cAMP) negatively regulates such transformation. Caveolae and their resident protein caveolins scaffold signaling molecules, including AC isoforms, whereas phosphorylated caveolin-1 (phospho-cav-1) may localize to FA. Here, we used adult rat cardiac fibroblasts to examine distribution and expression of AC, phospho-cav-1, and FA proteins to define mechanisms that link increases in cAMP to caveolin-1 phosphorylation, actin/FA assembly, and fibroblast-myofibroblast transformation. Sucrose density gradient centrifugation, immunoprecipitation, and immunohistochemical analysis revealed that, unlike cav-1, phospho-cav-1 enriches in membrane fractions that express FA proteins and localize at the ends of actin stress fibers. We detected AC in both cav-1 and phospho-cav-1 immunoprecipitates, but FA kinase (FAK), phospho-FAK (FAK Tyr-397), paxillin, and vinculin were detected only in phospho-cav-1 immunoprecipitates. Treatment with the AC activator forskolin or a cAMP analog increased cav-1 phosphorylation but decreased FAK Tyr-397 phosphorylation in a cAMP-dependent protein kinase-dependent manner. These events precede actin cytoskeletal disruption, an effect that was blocked by small interfering RNA knock-down of cav-1. Inhibition of protein tyrosine phosphatase 1B abrogated cAMP-mediated disruption of actin cytoskeleton, cav-1 phosphorylation, and FAK Tyr-397 dephosphorylation. The data thus define a novel organization of signaling molecules that regulate fibroblasts: scaffolding of AC by phospho-cav-1 at FA sites in a caveolae-free microdomain along with components that mediate inhibition of actin/FA assembly and fibroblast-myofibroblast transformation via increases in cAMP.

Fibroblast-myofibroblast transformation is a key event in the deleterious remodeling that results in exaggerated production of connective tissue following injury of the lung, liver, kidneys, skin, and heart (1–4). Relatively little is known regarding the precise cellular mechanisms that lead to and regulate myofibroblast formation, although a primary component is the formation of a contractile apparatus composed of α-smooth muscle actin-containing microfilaments: i.e. stress fibers that anchor and radiate from FA along the plasma membrane (5). Recent evidence suggests that FA serve as organizing centers for regulatory and structural proteins so as to facilitate rapid, precise control of cell proliferation, differentiation, and function (6, 7). Among these are anchoring proteins, such as vinculin, paxillin, talin, and α-actinin, which link the actin cytoskeleton to transmembrane integrin receptors at FA (8). Formation of focal contacts involves tyrosine phosphorylation of the non-receptor protein tyrosine kinase, FAK (9). In response to growth factor stimulation and integrin engagement, FAK is autophosphorylated on Tyr-397 (7), providing a docking site for Src kinase, which phosphorylates additional tyrosine residues in the FAK catalytic domain, resulting in full activation of FAK (10). Tyr-397 phosphorylation helps couple FAK to downstream signaling pathways that regulate cell proliferation, survival, motility, and fibroblast-myofibroblast transformation (6, 11, 12).

Caveolae are cholesterol- and sphingolipid-rich, flask-like invaginations of the plasma membrane that serve as organizing centers for certain transporters, receptors, and post-receptor signaling components, facilitating rapid, coordinated regulation of cell function (13–16). Caveolae contain unique proteins (e.g. caveolin-1, caveolin-2, and caveolin-3 (cav-1, cav-2, and cav-3)), with regions in their primary sequence that scaffold and organize signaling molecules (17). In addition to their role as scaffolding proteins, cav-1 and cav-2 are substrates for phosphorylation by tyrosine kinases such as Src family kinase (18–20), in the case of cav-1 on tyrosine 14 (18, 21, 22). Unphosphorylated cav-1 is distributed randomly within the cell membrane (16) but can undergo stretch-induced translocation to non-caveolar regions, where it associates with β₃-integrins and the Src family proteins, Fyn and Shc (23). In contrast, phosphorylated cav-1 (phospho-cav-1) localizes in close proximity to FA (21, 24). The functional activity of phospho-cav-1 is poorly understood, but its localization near FA and associated microfilaments suggests a role in the regulation of actin and FA dynamics and potentially in the modulation of fibroblast-myofibroblast transformation.

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Adenylyl cyclases (AC) are membrane-bound proteins that catalyze conversion of ATP to cAMP, a ubiquitous second messenger that has numerous effects on cell function and morphology, primarily (albeit not exclusively (25)) through the activation of the cAMP-dependent protein kinase, PKA. One such effect is to blunt FAK phosphorylation and to induce disassembly of actin stress fibers and FA (26). Other studies implicate cAMP-promoted PKA activation in the phosphorylation of cav-1 as a prelude to cell rounding (27). We recently provided evidence...
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that AC activation or cAMP analogs that activate PKA inhibit cardiac myofibroblast formation via effects on α-smooth muscle actin formation (28). To date, however, no evidence has linked AC and phospho-cav-1 in terms of effects on cell morphology or function. Here, we set out to test such a linkage. We find that in adult rat cardiac fibroblasts (CF): 1) phospho-cav-1 colocalizes with AC at FA at plasma membrane sites that are independent of caveolae or lipid rafts; 2) stimulation of AC or incubation with a cAMP analog increases phosphorylation of cav-1 but decreases FAK Tyr-397 phosphorylation in parallel with a disruption of basal and transforming growth factor β-stimulated actin and FA assembly; and 3) protein tyrosine phosphatase (PTP) inhibition abolishes the effects of increased cAMP on cell morphology. The data thus provide unique evidence that phospho-cav-1-mediated scaffolding of AC at FA sites, independent of caveolae, facilitates rapid dysregulation of actin and FA assembly, thereby leading to inhibition of fibroblast-myofibroblast transformation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—a-Smooth muscle actin, vimentin, paxillin, and phospho-FAK (Tyr-397) were purchased from Sigma; total FAK, phospho-cav-1 (Tyr-14), and cav-1 (mouse monoclonal and rabbit polyclonal) antibodies were from BD Transduction Laboratories; phospho-serine, phospho-Src (Tyr-416), and total Src antibodies were from Cell Signaling; PTP1B antibody was from Calbiochem; AC5/6 antibodies were from Santa Cruz Biotechnology; and Alexa Fluor 647 phalloidin probe for F-actin was from Molecular Probes. The Src kinase inhibitor (4-amino-5-(4-chlorophenyl)-7-[(t-butyl)pyrazolo(3,4-d)pyrimidin-5-yl]pyrazole (PP2), cAMP analog (8-CPT-cAMP), PKA inhibitor (Rp-cAMPS, adenosine 3′,5′-cyclic phosphorothiolate-Rp), and PTP1B inhibitor (3-(3,5-dibromo-4-hydroxy-benzoyl)-2-ethyl-benzofuran-6-sulfonicacid-(4-thiazol-2-ylsulfanyl)-phenyl)-amide) were purchased from Sigma.

Isolation and Culture of Adult Rat CF—CF were isolated from adult Sprague-Dawley rats (250–300 g, male) and cultured as described previously (28).

Membrane Fractionation—CF were fractionated using a modification of a detergent-free method (29, 30). CF from two 10-cm plates were washed twice in ice-cold phosphate-buffered saline and scraped in 1 ml of 150 mM NaCl (pH 11.0) containing 1 mM EDTA, protease inhibitor mixture (Sigma), and phosphatase inhibitor mixture (Calbiochem). Cell lysates were sonicated on ice with three cycles of 20-s bursts. Approximately 1 ml of homogenate was mixed with 1 ml of 80% sucrose in 25 mM MES, 150 mM NaCl (pH 6.5) to form 40% sucrose and centrifuged at 175,000 g for 1 h. Protein-agarose pellets were washed using a SW41Ti rotor (Beckman Instruments) for 3 h at 4 °C. Samples were removed in 1-ml aliquots to form 12 fractions.

Immunoprecipitation, Immunohistochemical, and Immunoblot Analysis of CF—Immunoprecipitations were performed using either protein A-agarose or protein G-agarose (Roche Applied Science). CF from a 10-cm plate were washed twice in ice-cold phosphate-buffered saline and scraped in 1 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Igepal) containing protease inhibitor mixture (Sigma) and phosphatase inhibitor mixture (Calbiochem). Lysates were precleared with protein-agarose for 1–3 h at 4 °C, incubated with primary antibody for 1–3 h, immunoprecipitated with protein-agarose overnight at 4 °C, and centrifuged at 13,000 × g for 5 min. Protein-agarose pellets were washed once in lysis buffer followed by subsequent washes in wash buffer 2 (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% Igepal CA-630) and wash buffer 3 (10 mM Tris-HCl, pH 7.5, 0.2% Igepal CA-630). Immunoblot and immunohistochemical (with image deconvolution) analyses were conducted as described (28). Colocalization was assessed by CoLocalizer Pro 1.3 analysis software.

RNA Interference—The expression of PTP1B and cav-1 was suppressed in fibroblasts by using targeted siRNA (Ambion). The specific PTP1B siRNA sequences were as follows: siRNA 1, sense, 5′-GGGAUGGCA AAUCUCUCUtt-3′, antisense, 5′-AAAGAGAUUUGGCUAUUUttCt-3′; siRNA 2, sense, 5′-GCUUGAUAAGGAGGATT-3′, antisense, 5′-UCCUCUAUUUUAACGCGt-3′; siRNA 3, sense, 5′-GCCAGUCAUCCCAGCATt-3′, antisense, 5′-UGCAGGAAAGACUGGCtt-3′. The specific cav-1 siRNA sequences were as follows: siRNA 1, sense, 5′-GGUGAAGUGAAGAGAUGtt-3′, antisense, 5′-CAGGCUUUCUCAUUCCtt-3′, siRNA 2, sense, 5′-GGGAUUGACUGGUGUCAAt-3′, antisense, 5′-UGUGAGGACUAAGUCUUCtt-3′, siRNA 3, sense, 5′-GGGACACACUGUUCGACGtt-3′, antisense, 5′-CGUGCAACUGUGUGUCCtt-3′. Cells were treated with 1.33 μg of siRNA (three separate constructs) using Lipofectamine 2000 (Invitrogen) as the transfection reagent for 24 h. Transfection reagent and negative siRNA (scrambled sequence of similar length, Ambion) served as controls. Cells were co-transfected with Block-it fluorescent oligomer (Invitrogen) to determine siRNA-positive cells. Functional knockdown of gene was assessed by attenuation of effect of forskolin on cell morphology, as determined by immunohistochemistry.

Data Analysis—Statistical comparisons and graphical representation were performed using GraphPad Prism 3.0 (GraphPad Software). Statistical significance was set at p < 0.05.

RESULTS

Phospho-cav-1 Localizes at FA Sites, Independent of Caveolae, in Adult Rat CF—Using immunohistochemistry and immunoblot analysis, we examined the localization of cav-1 and phospho-cav-1 in adult rat CF. Although cav-1 distributes throughout the plasma membrane, phospho-cav-1 localized primarily at FA sites that were independent of caveolae (Fig. 1A), as identified by cav-1 staining. Quantitation revealed that 21.1 ± 4.2% of total cav-1 is phosphorylated in CF. Phospho-cav-1 localized at the end of actin microfilaments in a manner identical to phospho-cav-1 exhibited a high degree of colocalization with tyrosine phosphorylated FAK (FAK Tyr-397) (Fig. 1B, bottom middle panels). Phospho-cav-1 localized primarily at FA sites that were independent of caveolae (Fig. 1B, upper panels). Phospho-cav-1 exhibited a high degree of colocalization with tyrosine phosphorylated FAK (Figs. 1A, bottom left), which is generated by autophosphorylation at FAK following integrin engagement, making FAK Tyr-397 a highly specific FA marker (9). To confirm interaction between phospho-cav-1 and FA proteins, we conducted pull-down assays using cav-1 and phospho-cav-1 antibodies and probed immunoprecipitates for expression of FAK, FAK Tyr-397, paxillin, and vinculin (Fig. 1B). Phospho-cav-1 was detected exclusively in phospho-cav-1 immunoprecipitates with little or no detection in cav-1 immunoprecipitates. These findings imply that phospho-cav-1, unlike cav-1, localizes at FA sites where it interacts with multiple FA proteins.

We used sucrose density gradient fractionation to assess the distribution of cav-1 and phospho-cav-1 in buoyant/lipid rafts (fractions 4–5) versus “heavy” membrane fractions (fractions 10–12). We found (Fig. 1C) that cav-1 and phospho-cav-1 were present in both buoyant and heavy membrane fractions but that expression of phospho-cav-1 was much greater in the heavy fractions, consistent with a non-caveolar distribution. Similar findings were observed using detergent-based cell fractionation methods (data not shown). FAK, FAK Tyr-397, paxillin,
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FIGURE 1. Phospho-caveolin-1 localizes at focal adhesion sites, independent of caveolae, in CF. A, immunohistochemistry was performed using adult rat CF (passage < 3) to localize cav-1, phospho-cav-1, FAK, FAK Tyr-397, paxillin, and F-actin (phalloidin) and nuclear staining of DNA with DAPI (blue). Colocalization is represented in yellow. B, colocalization was verified by immunoblot (IB) analysis of cav-1 and phospho-cav-1 immunoprecipitates (IP) to detect expression of FAK, FAK Tyr-397, paxillin, and cav-1. C, relative distribution of cav-1 and phospho-cav-1 in buoyant/lipid raft (fractions 4–5) versus heavy membrane (fractions 10–12) domains was determined using sucrose gradient fractionation followed by immunoblot analysis and subsequent quantitation of immunoreactive bands. Values represent mean ± S.E. of at least four experiments compared using two-way analysis of variance with post hoc multiple comparison tests. * denotes p < 0.05 between heavy and buoyant fractions. D, localization of FAK, FAK Tyr-397, paxillin, and vinculin was compared using sucrose gradient fractionation followed by immunoblot analysis.

FIGURE 2. A portion of AC localizes with phospho-cav-1 at focal adhesion sites in CF. A, immunohistochemistry was performed using CF co-stained for cav-1 (green) or phospho-cav-1 (green) and AC5/6 (red) and nuclear staining of DNA with DAPI (blue). B, AC 5/6 was detected by immunoblot (IB) in both cav-1 and phospho-cav-1 immunoprecipitates (IP). Cav-1 and phospho-cav-1 were detected in AC 5/6 immunoprecipitates. C, distribution of AC 5/6 in both buoyant/lipid raft (fractions 4–5) and heavy membrane (fractions 10–12) domains was determined using discontinuous sucrose gradient fractionation followed by immunoblot analysis.

and vinculin were only detected in the heavy cellular fractions (Fig. 1D). These findings demonstrate that phospho-cav-1 is localized primarily at FA sites independent of caveolae/buoyant lipid rafts.

AC 5/6 Localizes with cav-1 throughout the Cell Membrane and with Phospho-cav-1 at FA Sites—AC 5/6 is predominantly detected in buoyant/caveolar membrane fractions, but a portion of AC 5/6 localizes in heavy/non-caveolar membrane fractions in cardiac cells (31, 32). Immunohistochemical analysis and quantification of AC 5/6 and phospho-cav-1 staining of CF revealed that, in addition to being distributed with cav-1 throughout the plasma membrane, 17 ± 4% of AC 5/6 colocalizes with phospho-cav-1 and 57 ± 12% of phospho-cav-1 colocalizes with AC 5/6 at FA sites (Fig. 2A). We verified this interaction by co-immunoprecipitation using AC 5/6 and phospho-cav-1 antibodies (Fig. 2B). AC 5/6 enriches in the buoyant/caveolar fraction (the buoyant fraction contains only 5% of the protein found in the heavy fraction), but is also found in heavy fractions with phospho-cav-1 (Fig. 2C). Thus, a portion of AC 5/6 colocalizes with and may be scaffolded by phospho-cav-1 at FA sites.

AC Activation or a cAMP Analog Stimulates cav-1 Phosphorylation in a Src Kinase- and PKA-dependent Manner—The addition of forskolin (10 μM) or 8-CPT-cAMP (100 μM) induced time-dependent increases in cav-1 phosphorylation (Fig. 3A). Pretreatment of CF for 30 min with a Src kinase inhibitor (PP2; 10 μM) or a PKA inhibitor (Rp-cAMPS; 100 μM) abolished the forskolin-stimulated increase in phospho-cav-1 (Fig. 3B), demonstrating that cAMP acts in both a Src kinase- and a PKA-dependent manner to increase phospho-cav-1. Consistent with previous reports (33), forskolin also promoted rapid (5 min) activation of Src (Fig. 3C), as indicated by increased phosphorylation of Src at tyrosine 416 (Tyr-416). The stimulation of phospho-cav-1 by AC/cAMP may be facilitated by their colocalization at FA sites.

cAMP/PKA-mediated cav-1 Phosphorylation Precedes Disruption of Actin Cytoskeleton—Based on AC/cAMP-mediated inhibition of myofibroblast transformation (28), we hypothesized that scaffolding of AC 5/6 by phospho-cav-1 at FA sites may facilitate actin cytoskeleton disassembly. Using immunohistochemistry, we examined the kinetics of cav-1 phosphorylation and actin reorganization following stimulation of CF with forskolin (10 μM) or H2O2 (5 mM), an agent that promotes cav-1 phosphorylation (21) (Fig. 4A). Within 5 min of forskolin treatment, phospho-cav-1 intensity was dramatically increased and remained elevated for 30 min. By 30 min, the actin cytoskeleton began to break down, undergoing complete disruption after 60 min. H2O2 treatment resulted in a similar, rapid (5 min) increase in cav-1 phosphorylation that was followed by disintegration of the actin cytoskeleton (60 min), further demonstrating the parallel between enhanced cav-1 phosphorylation and disruption of the actin cytoskeleton. Similar to previous findings (34), the disruption of the...
actin cytoskeleton between 30 and 60 min coincided with a reduction in phospho-cav-1 intensity and a redistribution of phospho-cav-1 toward the interior of the cell.

To verify the role of phospho-cav-1 in AC/cAMP-mediated disruption of actin cytoskeleton, we used siRNA to knock-down cav-1 expression in CF (Fig. 4B). Using immunohistochemistry, we observed a decrease in cav-1 staining in siRNA-treated cells that correlated with a reduced ability of forskolin (1 μM) to disrupt the actin cytoskeleton. In contrast, negative (scrambled) siRNA-treated cells exhibited a high degree of cav-1 staining and complete disintegration of the actin cytoskeleton in response to forskolin treatment. A submaximal (1 μM) concentration of forskolin was used to avoid any phospho-cav-1-independent effects on microfilament assembly as a result of cAMP-promoted decrease in Rho-activity (35). Combined, these data demonstrate the involvement of phospho-cav-1 in AC/cAMP-mediated regulation of actin cytoskeletal dynamics.

cAMP/PGA-stimulated cav-1 phosphorylation precedes disruption of the actin cytoskeleton. Immunohistochemistry was performed using adult rat CF grown for 48 h in serum-free media and then stimulated with serum-free media alone (control) or for 5, 15, 30, and 60 min with forskolin (10 μM) or H2O2 (5 mM) (A). B, CF were incubated for 24 h with media alone (control), cav-1 siRNA, or a negative (scrambled siRNA) control and then treated with or without forskolin (1 μM) for 60 min. CF were stained for cav-1 (green), F-actin (phalloidin, red), and DNA (DAPI, blue). Fluorescence intensity at all time points was normalized relative to basal to account for increased cav-1 phosphorylation following stimulation.

FIGURE 3. cAMP/PGA stimulates cav-1 phosphorylation in a Src kinase- and PKA-dependent manner. Adult rat CF were grown for 48 h in serum-free media and then stimulated with serum-free media alone (control (Ctrl)) or for 5, 15, and 30 min with forskolin (10 μM) or 8-CPT-cAMP (100 μM) (A and C) or for 15 min with forskolin (10 μM) alone or in the presence of a Src kinase inhibitor (PP2; 10 μM) or a PKA inhibitor (Rp-cAMPS, 100 μM) (B). Inhibitors were added 30 min prior to stimulation. Expression of phospho-cav-1, total cav-1, Src Tyr-416, and total Src was determined by immunoblotting. Phospho-cav-1 levels were normalized for total cav-1, Src Tyr-416 levels were normalized for total Src, and data are expressed as fold change relative to control. Values represent mean ± S.E. of at least three experiments and were compared using a one-way analysis of variance with post hoc multiple comparison tests. #, denotes p < 0.05 as compared with control; * denotes p < 0.05 as compared with forskolin.

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FIGURE 5. cAMP/PI3K-mediated disruption of actin cytoskeleton and focal adhesion assembly involves decreased FAK activation. CF grown for 48 h in serum-free media were stimulated with media alone (control (Ctrl)) or for 5, 15, and 30 min with forskolin (10 μM) or B-CPT-cAMP (100 μM) (A) or for 15 min with forskolin (10 μM) alone or in the presence of a PAK inhibitor (RPA-CAMP, 100 μM) (B). FAK (Tyr-397) and total FAK were then determined by immunoblotting. Inhibitors were added 30 min prior to stimulation. Data were normalized for total FAK expression and expressed as fold-change relative to control. Values represent mean ± S.E. of at least three experiments and were compared using a one-way analysis of variance with post hoc multiple comparison tests. #, denotes p < 0.05 as compared with control; *, denotes p < 0.05 as compared with forskolin. C, immunohistochemistry was performed using CF grown for 48 h in serum-free media and then stimulated for 60 min with media alone (control), transforming growth factor β (TGFβ) (10 ng/ml) alone, or in the presence of forskolin (10 μM). CF were then co-stained for F-actin (phalloidin, red) and FAK (green) and nuclear staining of DNA (DAPI, blue). α-SMA, α-smooth muscle actin.

lateral assembly of actin stress fibers and FA complexes (Fig. 5C). The inhibitory effects of cAMP/PI3K on fibroblast-myofibroblast transformation may thus be a consequence of dephosphorylation of FAK.

cAMP-mediated cav-1 Phosphorylation and FAK Dephosphorylation Are Abrogated by a Protein Tyrosine Phosphatase 1B (PTP1B) Inhibitor—Because both cav-1 and FAK are phosphorylated on tyrosine residues, we examined the role of PTP activity in cAMP-mediated cav-1 phosphorylation and FAK dephosphorylation by treating CF with forskolin in the presence of a PTP1B inhibitor. Forskolin induced a time-dependent increase in PTP1B activation (Fig. 6A). Inhibition of PTP1B reduced the ability of forskolin to stimulate cav-1 phosphorylation (Fig. 6B) and dephosphorylate FAK Tyr-397 (Fig. 6C). Together, these data demonstrate a role for PTP1B in the cAMP-mediated regulation of cav-1 phosphorylation and FAK Tyr-397 dephosphorylation, effects that appear to be involved in the disruption of cytoskeletal dynamics.

cAMP-mediated Cytoskeleton Disruption Is Dependent upon PTP1B—To examine the role of PTPs in the cAMP-mediated effects on cell morphology, we treated CF with forskolin in the presence of vanadate, a nonspecific PTP inhibitor, or a PTP1B-selective inhibitor (Fig. 7A). PTP inhibition with vanadate dose-dependently abolished the ability of forskolin to disrupt actin and FA assembly. Selective inhibition of PTP1B produced identical effects. Moreover, knock-down of PTP1B using siRNA abolished the ability of forskolin to promote disassembly of actin and FA (Fig. 7B). Together, these results demonstrate that PTP1B is required for cAMP-mediated breakdown of the actin cytoskeleton and FA.

DISCUSSION

First identified in its phosphorylated form in v-Src transformed cells (36), cav-1 has well characterized effects on cell morphology and disease (14, 37) but little is known regarding the biological role of phosphocav-1. Cav-1 is phosphorylated on tyrosine 14 in response to cellular stress, hormone, and growth factor stimulation, and when phosphorylated, serves as a docking site for SH2 domain-containing proteins such as Grb7 (18). The SH2 domain of Grb7 interacts with FAK through tyrosine 397 with subsequent effects on cell migration (38). Based on its apparent localization near FA sites, phospho-cav-1 may be involved in stabilization of the actin cytoskeleton (39). Phospho-cav-1 could therefore provide an anchoring site for proteins that control cell morphology and function via regulation of actin cytoskeleton dynamics.

We hypothesized that localization of phospho-cav-1 near FA sites, combined with the ability of cav-1 to scaffold AC, would provide a favorable organization of structural proteins and catalytic molecules to facilitate regulation of CF morphology and function by increased cAMP. We demonstrate that phospho-cav-1 localizes at FA sites, independent of membrane caveolae, where it interacts with the FA proteins FAK, paxillin, and vinculin. At these sites, phospho-cav-1 scaffolds AC, which, when activated, disrupts actin and FA assembly via dephosphorylation of the FA regulatory molecule FAK, with concomitant stimulation of cav-1 phosphorylation. Thus, cAMP-stimulated cav-1 phospho-

AC/CAMP promotes dephosphorylation of FAK and, in parallel, phosphorylation of cav-1 in CF. The fact that both FAK and cav-1 undergo PKA-dependent changes in tyrosine phosphorylation led us to hypothesize a regulation of protein tyrosine PTP activity by cAMP. Nonspecific PTP inhibitors attenuate prostaglandin-mediated FAK dephosphorylation and actin disruption (26). Phosphotyrosine phosphatase PTP1B exerts similar effects on cav-1 under control stimulation of AC cytoskeleton and FAK phosphorylation (40, 41). PTP1B also colocalizes with cav-1 (42) and can promote the activity of Src kinase via dephosphorylation of c-Src tyrosine 529, the Src autoinhibitory site (43). Active Src is able to promote cav-1 phosphorylation (18, 39). Since cAMP can stimulate PTP1B (44) and Src activity (33), we propose that PTP1B is the downstream mediator for the cAMP/PKA-mediated effects on phospho-cav-1, FAK, and actin cytoskeleton in adult rat CF.

Consistent with this hypothesis, inhibition of PTP1B (45, 46) abolished the ability of cAMP/PKA to disrupt actin cytoskeleton and FA formation, phosphorylate caveolin-1, and dephosphorylate FAK (Fig. 6). The current findings thus demonstrate a novel regulatory pathway whereby scaffolding and activation of AC at FA sites blocks FAK activation, leading to rapid disruption of actin and FA assembly, events that depend upon PTP1B. cAMP/PKA-stimulated phosphorylation of cav-1 appears to potentiate these effects by positive feedback on the increased scaffolding of AC by phospho-cav-1 at FA sites.
Few successful strategies currently exist for inhibiting deleterious ECM production that is associated with fibrotic disorders (Lotersztajn et al. (47); Weber and co-workers (48)). The results here provide new

FIGURE 6. Inhibition of PTP1B dose-dependently blocks the effects of forskolin on phospho-cav-1 and FAK Tyr-397. A, the ability of AC/cAMP to activate PTP1B was examined in CF grown for 48 h in serum-free media and then stimulated with serum-free media alone (control) or with forskolin (10 μM) in the absence or presence of the indicated concentrations of vanadate, a nonspecific PTP inhibitor, or PTP1B inhibitor. CF were then stained for FAK (green), F-actin (phalloidin, red) and DNA (DAPI, blue). B, CF were incubated for 24 h with media alone (control), cav-1 siRNA, or a negative (scrambled siRNA) control and then in the absence or presence of forskolin (1 μM) for 60 min. CF were stained for phospho-cav-1 (green), F-actin (phalloidin, red), and DNA (DAPI, blue).

FIGURE 7. A proposed mechanism for PTP1B-dependent cav-1 phosphorylation and FAK Tyr-397 dephosphorylation during cAMP/PKA-mediated inhibition of fibroblast-myofibroblast transformation. Stimulation of AC increases cAMP production and activates PKA, thereby activating PTP1B and resulting in: 1) dephosphorylation of FAK Tyr-397 and inactivation of FAK and 2) activation of Src kinase (43) and increased phosphorylation of cav-1. These events result in disruption of actin and FA assembly and inhibition of fibroblast-myofibroblast transformation. cAMP/PKA-mediated phosphorylation of cav-1 provides a positive feedback mechanism that increases scaffolding of AC by phospho-cav-1 at FA sites and potentiates cAMP/PKA-mediated inhibition of fibroblast-myofibroblast transformation.
mechanistic information regarding the ability of cAMP/PKA to control downstream catalytic molecules in a manner that inhibits fibroblast-myofibroblast transformation, thereby suggesting potential targets to attenuate maladaptive connective tissue remodeling (organ fibrosis) via cAMP/PKA-mediated regulation of actin and FA regulatory proteins at FA.

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